Kinetic Characterization and Cross-Resistance Patterns of HIV-1 Protease Mutants Selected under Drug Pressure†

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ABSTRACT: Eleven different recombinant, drug-resistant HIV-1 protease (HIV PR) mutants—R8Q, V32I, M46I, V82A, V82F, V82I, I84V, V32I/I84V, M46I/V82F, M46I/I84V, and V32I/K45I/F53L/A71V/I84V/L89M—were generated on the basis of results of *in vitro* selection experiments using the inhibitors A-77003, A-84538, and KNI-272. Kinetic parameters of mutant and wild-type (WT) enzymes were measured along with inhibition constants (K_i) toward the inhibitors A-77003, A-84538, KNI-272, L-735,524, and Ro31-8959. The catalytic efficiency, k_{cat}/K_m, for the mutants decreased relative to WT by a factor of 1.2−14.8 and was mainly due to the elevation of K_m. The effects of specific mutations on K_i values were unique with respect to both inhibitor and mutant enzyme. A new property, termed vitality, defined as the ratio $(K_i k_{cat}/K_m)_{mutant}/(K_i k_{cat}/K_m)_{WT}$ was introduced to compare the selective advantage of different mutants in the presence of a given inhibitor. High vitality values were generally observed with mutations that emerged during *in vitro* selection studies. The kinetic model along with the panel of mutants described here should be useful for evaluating and predicting patterns of resistance for HIV PR inhibitors and may aid in the selection of inhibitor combinations to combat drug resistance.

Human immunodeficiency virus (HIV) encodes a protease (HIV PR) which is responsible for the posttranslational processing of the polyprotein gene products of *gag* and *gag-pol* to yield the structural proteins and enzymes of the viral particle (Debouck, 1992). HIV PR is a member of the aspartic proteinase family and is composed of two noncovalently associated, structurally identical monomers. The active site of the enzyme contains two conserved catalytic aspartyl residues, one from each monomer (Wlodawer & Erickson, 1993).

HIV PR is required for viral infectivity since either mutation of the residues essential for its activity or chemical inhibition of the enzyme leads to the production of immature, noninfectious viral particles (Kaplan et al., 1993; Kohl et al., 1988; McQuade et al., 1990; Seelmeier et al., 1988). Therefore, HIV PR is considered to be an attractive target for the rational design of antiviral drugs for AIDS. A wide variety of potent HIV PR inhibitors have been designed using substrate-based and structure-based approaches [for reviews, see Debouck (1992), Huff (1991), Meek (1992), Tomasselli et al. (1991), and Wlodawer and Erickson (1993)]. The crystal structures of numerous HIV PR/inhibitor complexes have been solved to aid the process of inhibitor design and to provide a structural basis for the development of a new generation of inhibitors [for review, see Wlodawer and Erickson (1993)]. Several inhibitors are currently in clinical trials for patients with AIDS (Ho et al., 1995; Jacobsen et al., 1994; Wei et al., 1995).

Recent studies have shown that HIV infection is characterized by a dynamic viral turnover in the steady state (Ho et al., 1995; Wei et al., 1995). The rapid replication rate coupled with the lengthy duration of infection favors the emergence of resistant mutants to targeted antiviral agents (Coffin, 1995). Consistent with this picture is the fact that drug resistance has severely limited the clinical effectiveness of HIV reverse transcriptase inhibitors (Richman, 1993). Theoretically, HIV PR has several advantages as a target for therapy. The enzyme consists of two identical monomers so that a single mutation at the genetic level generates a double mutant at the structural level. This together with the relatively small size of HIV PR should lead to some restrictions in mutational possibilities. Nevertheless, reports of *in vitro* resistance to a number of HIV PR inhibitors (Anderson et al., 1994; El-Farrash et al., 1994; Ho et al., 1994; Jacobsen et al., 1995; Kaplan et al., 1994; Markowitz et al., 1995; Otto et al., 1993) presage the emergence of mutants in the clinical setting *in vivo* (Condra et al., 1995; Emini et al., 1994; Jacobsen et al., 1994).

In this report we describe kinetic parameters for 11 different drug-resistant HIV PR mutants that reflect many of the active site mutants, and some nonactive site mutants, that have been generated during *in vitro* selection experiments using the inhibitors A-77003 (Kempf et al., 1991), A-84538 (Kempf et al., 1995), and KNI-272 (Kageyama et al., 1993). In an effort to understand the nature of cross-resistance, we compared inhibition constants for mutant and wild-type (WT) enzymes toward a set of inhibitors including A-77003, A-84538, KNI-272, Ro31-8959, and L-735,524 (Figure 1). The latter four drugs are currently in clinical trials. The panel of mutants described here may be useful for the initial

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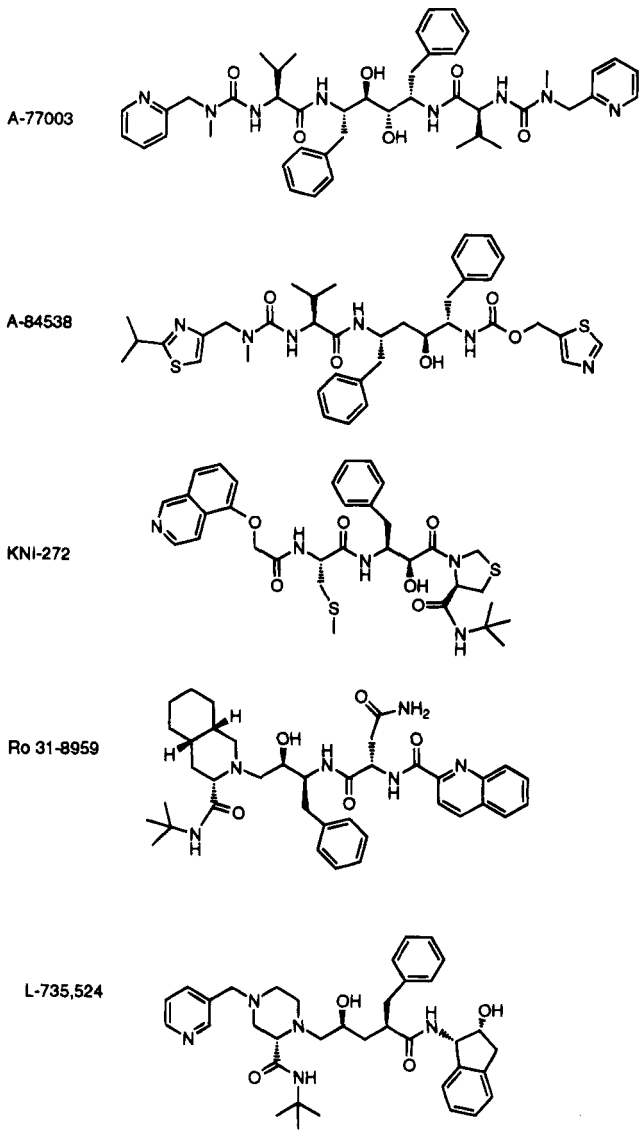

FIGURE 1: Structures of HIV-1 protease inhibitors used in this study. Note that the clinically used designations for Ro31-8959, A-84538, and L-735,524 are Saquinavir, ABT-538 or Ritonavir, and MK-639, respectively.

screening of inhibitors to evaluate possible drug resistance patterns.

MATERIALS AND METHODS

Reagents. HIV PR inhibitors were kindly donated by Abbott Laboratories (A-77003 and A-84538), Dr. D. P. Clough, Roche Research Centre (Ro31-8959), Dr. H. Hayashi, Japan Energy Co. (KNI-272), and Dr. J. P. Vacca, Merck Research Laboratories (L-735,524). A PET11BA vector containing the HIV PR precursor gene (BA) was kindly provided by Dr. Y.-S. Cheng of DuPont-Merck Pharmaceuticals (Cheng et al., 1990). The P7PR gene encoding the full-length WT HIV PR and the V32I and V82I mutant protease-encoding genes (Kaplan et al., 1994) were a gift from Dr. R. Swanstrom, Lineberger Comprehensive Cancer Center, University of North Carolina. PET21 and PET23 plasmid vectors were obtained from Novagene (Madison, WI). HIV PR substrate Lys-Ala-Arg-Val-Tyr-Phe(NO_2)-Glu-Ala-Nle-NH_2 was from Bachem Bioscience, Inc.

Cloning, Expression, and Purification of WT and Mutant HIV PR. The construction and expression of HIV PR mutants were performed using a PET23 or PET21 expression vector as described (Liu, 1994). Unless stated otherwise, all purification steps were done at 4 °C. Wild-type HIV PR and the R8Q and V82A mutants were purified from a PET23 expression system (Liu, 1994). Final purification was achieved by gel filtration using Sephadex G-75 or Superose-12 equilibrated in 0.1% acetic acid, 0.2 M NaCl, and 10% glycerol. At this stage the protease was usually more than 90% pure as judged by SDS−PAGE.

The V32I, M46I, V82I, V82F, I84V, V32I/I84V, M46I/V82F, M46I/I84V, and V32I/K45I/F53L/A71V/I84V/L89M mutants were purified from a PET21 expression system using a modified protocol of K. Appelt (personal communication). Cells were resuspended in 50 mM Tris-HCl buffer, pH 8.0, 25 mM NaCl, and 0.2% β-mercaptoethanol (buffer A), sonicated, and centrifuged. Inclusion bodies were washed first with buffer A, then with buffer A containing respectively 0.1% Triton X-100, 1 M NaCl, and 1 M urea, and finally with buffer A alone. Purified inclusion bodies were solubilized by the addition of buffer A containing 8 M urea at room temperature. The solution was clarified by centrifugation and loaded onto a 2.6 × 9.5 cm Q-Sepharose column. Flow-through fractions were dialyzed against three changes of refolding buffer which consisted of 25 mM sodium phosphate, pH 7.0, 25 mM NaCl, 0.2% β-mercaptoethanol, and 10% glycerol. After refolding, the V32I, V82I, V82F, I84V, and V32I/I84V mutants were more than 90% pure as judged by SDS−PAGE. For unknown reasons, mutants containing the M46I mutation (M46I, M46I/V82F, M46I/I84V), as well as the multiple mutant V32I/K45I/F53L/A71V/I84V/L89M, exhibited very low expression levels. Final purities of the latter mutants ranged from 40% to 60%.

Kinetic Measurements. Protease activity and K_i's were measured essentially as described (Kageyama et al., 1993) using the fluorogenic substrate Lys-Ala-Arg-Val-Tyr-Phe-(NO_2)-Glu-Ala-Nle-NH_2 (Peranteau et al., 1995). Excitation and emission maxima were 277 and 306 nm, respectively.

RESULTS

In vitro drug selection experiments resulted in a variety of mutant viruses with reduced sensitivity to a number of structurally-related C_2 symmetry-based inhibitors, including P-9941 (Otto et al., 1993), A-77003 (Ho et al., 1994; Kaplan et al., 1994), A-84538 (Markowitz et al., 1995), and A-75925 (Mashera et al., 1994), norstatine-containing compounds, such as KNI-272 (Anderson et al., 1994) and RPI-312 (El-Farrash et al., 1994), a nonpeptidyl cyclic urea, XM323 (King et al., 1994), and L-735,524 (Tisdale et al., 1994). In all cases, the reduction in sensitivity was associated with mutations in HIV PR. Among the most frequent active site mutations were R8Q, V32I, V82A, V82I, V82F, and I84V alone or in combination with each other. A number of mutations that lie outside the active site have also been reported. We cloned, expressed, and purified 11 different HIV PR mutants, including the mutants mentioned above and several nonactive site mutants, and compared their kinetic parameters and inhibition constants with those of WT HIV PR using the inhibitors A-77003, A-84538, KNI-272, Ro31-8959, and L-735,524.

Effect of Mutations on the Catalytic Activity of HIV PR. Kinetic parameters for the cleavage of the synthetic fluorogenic substrate are shown in Table 1. The catalytic efficiency (k_{cat}/K_m) for all mutants is decreased compared to WT by a factor of 1.2−14.8. Usually the decrease in

Table 1: Kinetic Constants of WT and Mutant HIV PR[a]

protease	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)
WT	3.7 (1.0)	42.3 (1.0)	11.4 (1.0)
R8Q	15.5 (4.2)	39.8 (0.9)	2.6 (0.2)
V32I	3.7 (1.0)	35.3 (0.8)	9.5 (0.8)
M46I	9.3 (2.5)	47.3 (1.1)	5.2 (0.5)
V82A	7.1 (1.9)	19.6 (0.5)	2.8 (0.3)
V82F	14.8 (4.0)	44.0 (1.0)	3.0 (0.3)
V82I	7.1 (1.9)	24.0 (0.6)	3.4 (0.3)
I84V	17.1 (4.6)	68.0 (1.6)	4.0 (0.4)
M46I/V82F	4.6 (1.3)	31.5 (0.7)	7.0 (0.6)
M46I/I84V	21.2 (5.7)	40.0 (1.0)	1.9 (0.2)
V32I/I84V	34.8 (9.4)	52.0 (1.2)	1.5 (0.1)
V32I/K45I/F53L/A71V/I84V/L89M	20.0 (5.4)	15.3 (0.4)	0.8 (0.07)

[a] In the designation of mutants, the WT amino acid with the residue number is followed by the substituted residue using the single-letter amino acid abbreviations. Ratios of kinetic constants for mutant over WT HIV PR are shown in parentheses.

efficiency was due to an elevation of K_m of up to 9.4-fold. On the other hand, k_{cat} was less affected by the mutations. For 5 of 11 mutants, V82F, R8Q, M46I, M46I/I84V, and V32I/I84V, k_{cat} was essentially unchanged, and for I84V it was about 1.6-fold higher than for WT. For most of the other mutants, k_{cat} was 1.5−3-fold lower compared to WT.

Effect of Mutations on Inhibitor Binding. We compared inhibition constants (K_i) of WT HIV PR and 11 mutants toward five different inhibitors (Figure 1) including two C_2 symmetry-based compounds, A-77003 and A-84538, and three substrate-based inhibitors, KNI-272, L-735,524, and Ro31-8959 (Table 2). These inhibitors were chosen for several reasons. First, all but one are in clinical trials. Second, resistance studies have been performed using all five. Third, the different design principles of symmetry-based (Erickson et al., 1990; Erickson, 1993) and substrate-based inhibitors (Wlodawer & Erickson, 1993) may lead to different resistance properties. For example, symmetry-based inhibitors may be more adversely affected by single HIV PR mutations because of symmetric interactions. On the other hand, one can argue that substrate-based inhibitors should be less susceptible to resistance mutations since these compounds should more closely resemble natural substrates. All the compounds tested are tight-binding inhibitors of the WT enzyme with inhibition constants in the low picomolar range using our assay conditions. While we observed significant increases in K_i values for several inhibitors, the K_i's for all of the mutants remained in the subnanomolar or low nanomolar range. Given the relatively high antiviral IC_{50} values of the inhibitors for the mutants, typically 10−100× greater than their K_i's, our results seem to imply that most of the mutant enzyme activity should be inhibited in tissue culture. This apparent contradiction is due to the fact that there is not a 1:1 correlation between antiviral IC_{50} values and enzyme K_i's. Therefore, in the absence of any information about inhibition constants under *in vivo* conditions, it seems more sensible to compare ratios of K_i's (mutant/WT) rather than their absolute values.

All of the mutants listed were found in viruses with decreased susceptibility to one or more inhibitors during *in vitro* selection studies. Consequently, all of them have elevated K_i values against one inhibitor or another. As was expected, the effect of a mutation on the K_i for a given inhibitor depends on the position of the mutation. For example, for KNI-272 the K_i values were similar for WT and for the V82I and M46I/V82F mutants but were 2.5-fold lower for V82F and 258-fold higher for the multiple mutant, V32I/K45I/F53L/A71V/I84V/L89M. At the same time, the change in K_i for a given mutant depends on the inhibitor used. The mutant/WT K_i ratio for V82F was actually less than 1.0 for KNI-272 (0.4) and Ro31-8959 (0.7) but was around 20 for A-84538 and A-77003 and 84.7 for L-735,-524. Thus, the changes in inhibition constant are unique with respect to both inhibitor and mutant, in agreement with other findings (Lin et al., 1995; Sardana et al., 1994; Vacca et al., 1994). This observation can be rationalized from the different structures of the inhibitors as well as from the specific structural conformational changes in the active site subsites of HIV PR introduced by a given mutation. The V82A/A-77003 mutant complex has been shown to undergo a conformational change that resulted in unanticipated subsite repacking effects (Baldwin et al., 1995). The extent to which such effects are possible with this mutant will probably differ for different inhibitors since residue 82 is in a flexible region of the enzyme (Erickson, 1993).

Vitality of Mutants. The reduction in sensitivity of a given mutant toward a given inhibitor can be described by the ratio $K_{i,mutant}/K_{i,WT}$. However, this ratio reflects only a portion of the selective advantage of the mutant over the wild-type enzyme. Besides having a reduced sensitivity to inhibitor, the mutant enzyme should also maintain a certain minimum level of activity in order for the virus to be functional. The proteolytic efficiency of the enzyme is described by the k_{cat}/K_m ratio. If we assume that the activity of WT HIV PR is optimized for the virus, any reduction of k_{cat}/K_m might lead to a weaker, less functional virus even though we demonstrated above that virus with a substantial k_{cat}/K_m deficit can still grow. Given two different protease mutants which have the same elevated K_i toward a particular inhibitor, the one with the higher k_{cat}/K_m, that is, the more efficient enzyme, is more likely to be selected under the pressure of this inhibitor. In order to be able to compare the selective advantage of different mutants in the presence of a particular inhibitor to WT HIV PR and to each other, we introduce a quantity, vitality, that is described by the equation

$$\text{vitality} = (K_i k_{cat}/K_m)_{mutant}/(K_i k_{cat}/K_m)_{WT}$$

The vitality of different mutants toward various inhibitors is presented in Table 3. The vitality of WT HIV PR is defined as 1.0. The higher the vitality, the greater the chance for the mutant to support virus replication in the presence of a given inhibitor and thus the higher the probability for the mutation to be selected. For example, this analysis indicates that the I84V, V32I/I84V, and V32I/K45I/F53L/A71V/I84V/L89M mutants are the most preferable mutations in the presence of KNI-272.

DISCUSSION

Our kinetic studies indicate that the active site mutations selected under drug pressure affect primarily K_m as opposed to k_{cat}. These observations may be important since, at the relatively high concentration of substrate inside virus particles, the initial velocity of cleavage will be close to V_{max} and independent of K_m. The number of Gag protein equivalents ($Pr55^{gag}$ + $Pr160^{gag\text{-}pol}$) per retroviral particle has been estimated for Moloney leukemia virus at around 2800

Table 2: K_i Ratios for WT and Mutant HIV PR

protease	$K_{i,mutant}/K_{i,WT}$				
	KNI-272	A-77003	A-84538	L735,524	Ro31-8959
WT[a]	1.0 (9.8 pM)	1.0 (8.1 pM)	1.0 (9.3 pM)	1.0 (3.6 pM)	1.0 (7.0 pM)
R8Q	3.2	**165.0** [32][b] [62][c]	23.1	8.2	4.3 [3.7][b]
V32I	**5.3**	**16.4** [7.6][c]	3.4	*8.0* [7.4][d]	1.6 [7.3][e]
M46I	*6.5*	*4.8* [1.4][b]	*1.9*	*4.3*	3.6
V82A	5.2	*14.3*	10.4	*21.6*	3.7
V82F	0.4	20.0	*23.5*	*84.7*	0.7
V82I	1.8	**23.5** [1.0][c]	1.5	6.9 [0.15][d]	2.6 [0.67][e]
I84V	**32.0**	9.1	*6.7*	*10.0* [8.7][d]	*5.8*
M46I/V82F	*0.9*	*7.0*	*2.6*	*6.9*	1.0
M46I/I84V	*29.0*	*19.4*	**9.4**	*21.5*	*4.5*
V32I/I84V	**156.0**	*108.0*	*63.5*	*80.3*	*14.2*
V32I/K45I/F53L/A71V/I84V/L89M	**258.0**	*170.0*[f]	*43.7*	*56.0*	*14.0*

[a] K_i's for the wild-type enzyme are shown in parentheses. Data from other studies is listed in brackets. Values in bold designate mutants that were actually found during *in vitro* selection studies using the inhibitor indicated. Values for mutants that either contain a subset of one or more mutations or were present as a subset of multiple mutants that were found during *in vitro* selection experiments are italicized. [b] Ho et al., 1994. [c] Kaplan et al., 1994. [d] Vacca et al., 1994. [e] Sardana et al., 1994. [f] The virus containing this mutant protease can grow at 30× the IC_{90} concentration of A-77003.

(Karpel et al., 1987). This leads to a concentration of Gag near 10 mM, assuming a particle radius of 50 nm. Under such conditions, k_{cat} should be the main parameter responsible for substrate cleavage efficiency, assuming that $K_m \ll 10$ mM. There is currently no information about K_m values for HIV PR under *in vivo* conditions.

It is not clear how accurately *in vitro* resistance patterns will predict resistance in patients receiving HIV PR inhibitors. At least two of the mutants, V82A and V82F, have been detected in viral isolates obtained from patients treated with L-735,524 (Emini et al., 1994). According to our data, the catalytic efficiencies of both V82A and V82F are about 25% of WT HIV PR, consistent with a recent report of the activity of the V82A mutant toward a related substrate with a Phe residue instead of Tyr at the P1 position (Lin et al., 1995). Only 4 of 11 mutants have lower k_{cat}/K_m ratios—from 7% to 23% of WT. There must exist a threshold of catalytic efficiency below which mutant protease can no longer support virus replication. A recent mutagenesis study of HIV PR indicated that the catalytic activity (k_{cat}) threshold is between 2% and 25% (Rose et al., 1995). Our studies suggest that the minimal k_{cat}/K_m threshold value is less than 7% that of the WT enzyme since the virus that contains the hextuple HIV PR mutant, with an efficiency of about 7%, is still viable (Anderson et al., 1994). We conclude therefore that it is possible that such a level of activity might be enough to make most, if not all, of the mutants described here viable. Validation of this conclusion awaits more *in vivo* studies as well as further investigation of kinetic properties of the mutants using substrates containing the natural cleavage sites of HIV PR from gag and gag-pol polyproteins.

Kinetic parameters for the V32I and V82I mutants were previously reported using the peptide substrate H_2N-Val-Ser-Gln-Asn-(β-naphthylalanyl)-Pro-Val-Ile-Glu-OH (Sardana et al., 1994). The k_{cat} values for both of these mutants were only slightly lower than for WT HIV PR and are consistent with our data. However, due to large differences in K_m (about 12-fold higher for V32I and 3-fold lower than WT for V82I), k_{cat}/K_m ratios were about 6% and 209% of WT values, respectively. Using the same substrate, Vacca et al. (1994) reported a k_{cat}/K_m ratio for the I84V mutant to be only 3% of the WT value. However, we obtained relative catalytic efficiencies for V32I, V82I, and I84V of 83%, 30%, and 35%, respectively. The K_m differences might be due to differences in the substrate used in both studies. Their substrate has the bulky β-naphthylalanine in the P1 position which may result in a different mode of binding in the enzyme active site.

Our K_i data are in general agreement with other published results. For example, 7.4- and 8.7-fold increases in K_i were found for the V32I and I84V mutants, respectively, toward L-735,524 (Vacca et al., 1994). These values approximate the 8- and 10-fold increases shown in Table 2. Similarily, the 7.6-fold increase in the K_i of V32I toward A-77003 (Kaplan et al., 1994) compares well with our value of a 16.4-fold increase. The K_i of R8Q toward Ro31-8959 was reported to be 3.7-fold higher (Ho et al., 1994), which again approximates our finding of a 4.3-fold increase.

For other mutant−drug combinations the correlation is not as good. R8Q with A-77003 exhibited 32-fold (Ho et al., 1994), 62-fold (Kaplan et al., 1994), and 165-fold (Table 2) increases in K_i. However, the overall 5-fold range of differences is not that great. The main discrepancy was found with the V82I mutant and various inhibitors. The K_i for this mutant was reported to actually decrease by 6.9-fold for L-735,524 (Vacca et al., 1994) and decrease 1.5-fold for Ro31-8959 (Sardana et al., 1994) and to remain unchanged for A-77003 (Kaplan et al., 1994). We found decreased sensitivity for all three inhibitors by factors of 6.9, 2.6, and 23.5, respectively. This may be explained by the difference in the experimental conditions used for the K_i determinations. We point out that all the data obtained in the present study are internally consistent with respect to assay conditions and wild-type and mutant enzyme preparation and handling.

Correlation between Vitality of Mutant and Its Appearance during in Vitro Selection. Two HIV PR mutations, V32I and I84V, emerged after passaging HIV_{LA1} with 0.33 μM KNI-272 (Anderson et al., 1994). At higher concentrations of the inhibitor the M46I mutation appeared in combination with V32I, and the triple mutant V32I/M46I/I84V also emerged. Vitality values for V32I and I84V are 4.4 and 11.2, respectively (Table 3). M46I and M46I/I84V, which are subsets of the triple mutant, V32I/M46I/I84V, have values of 3.0 and 4.9. For the dominant double mutation, V32I/I84V, the vitality is much higher—20.3. At 8 μM KNI-272, multiple mutants appeared during the *in vitro* selection (B. Anderson and H. Mitsuya, manuscript in preparation).

Table 3: Vitality Values of WT and Mutant HIV PR[a]

protease	vitality value				
	KNI-272	A-77003	A-84538	L735,524	Ro31-8959
WT	1.0	1.0	1.0	1.0	1.0
R8Q	0.7	**38.0**	5.3	1.9	1.0
V32I	**4.4**	**13.6**	2.8	*6.6*	1.3
M46I	*3.0*	*2.2*	*0.9*	*2.0*	1.7
V82A	1.3	*3.6*	2.6	*5.4*	0.9
V82F	0.1	5.2	*6.1*	*22.0*	0.2
V82I	0.6	**7.0**	0.5	2.1	0.8
I84V	**11.2**	3.2	*2.4*	*3.5*	*2.0*
M46I/V82F	*0.5*	*4.3*	*1.6*	*4.2*	0.6
M46I/I84V	*4.9*	*3.3*	**1.6**	*3.7*	*0.8*
V32I/I84V	**20.3**	*14.4*	*8.3*	*10.4*	*1.9*
V32I/K45I/F53L/A71V/I84V/L89M	**18.1**	*22.1*[b]	*3.0*	*7.3*	*1.0*

[a] Vitality values were calculated as described in the text. Values in bold designate mutants that were actually found during *in vitro* selection studies using the inhibitor indicated. Values for mutants that either contain a subset of one or more mutations or were present as a subset of multiple mutants that were found during *in vitro* selection experiments are italicized. [b] The virus containing this mutant protease can grow at 30× IC_{90} concentration of A-77003.

One of these mutants, V32I/K45I/F53L/A71V/I84V/L89M, has a vitality of 18.1. For comparison, the vitality values of enzymes with subsite mutants that were not found during *in vitro* selection studies with KNI-272—V82F, M46I/V82F, R8Q, V82A and V82I—ranged between 0.1 and 1.3. A structurally similar inhibitor, KNI-227, selects for I84V but rarely for V32I (Anderson et al., 1994). Values for these two mutants are 3.8 and 0.75, respectively, with KNI-227 (data not shown).

The vitality value for the R8Q mutant protease, which is the most severe mutation found in the presence of the C_2 symmetric diol A-77003 (Ho et al., 1994; Kaplan et al., 1994), is the highest at 38.0. Two other mutations—V32I and V82I—which were found as both single and double mutants with A-77003 (Kaplan et al., 1994) also have relatively high vitalities of 13.6 and 7.0, respectively. Virus containing the KNI-272 selected hextuple mutant can also grow in cell culture at 10 μM A-77003 (unpublished data), and this mutant protease has the second highest vitality value, 22.1 with A-77003. M46I is thought to be mainly a compensatory mutation which serves to improve the efficiency of virus replication in the presence of A-77003 (Ho et al., 1994). It was found in one study only as a part of a double mutation, mainly with R8Q. Accordingly, this mutant has a rather low vitality value of 2.2. The same is true for M46I with respect to A-84538 (vitality value 0.9). This mutation was found mainly in combination with V82F and I84V and by itself did not change the sensitivity of virus to the drug (Markowitz et al., 1995). The V82F and I84V mutations, which are present also as a part of multiple mutations primarily with each other and with M46I, were both shown to decrease the sensitivity of virus to A-84538. The vitality values for these mutants are correspondingly higher at 6.1 and 2.4, respectively. However, the more rapid emergence of the lower vitality I84V mutant with A-84538 is contrary to our expectations.

The V32I and V82A mutants that were found during *in vitro* selection studies with L-735,524 (Tisdale et al., 1994) have rather high vitality values, 6.6 and 5.4, respectively. Another mutation, V82F, which together with V82A was reported to be present in *in vivo* isolates from patients treated with this drug (Emini et al., 1994), has a value of 22.0.

None of the mutants used in this study (except I84V) were found in drug selection studies using Ro31-8959, and the vitality values for this inhibitor are very low with all of our mutants (0.18−2.0). The I84V mutant which was detected together with G48V and L90M after passage of an AZT-resistant clone in the presence of Ro31-8959 (Tisdale et al., 1994) has the highest vitality value at 2.0.

Some of the mutants that exhibited relatively high vitality values for a given inhibitor were not found during *in vitro* drug selection studies with the corresponding compound. For instance, the V32I/I84V double mutant was reported only for KNI-272 but has high vitality values for all inhibitors used in this study, except Ro31-8959. However, this mutant has one of the lowest activities compared to WT HIV PR (Table 1). It is possible therefore that higher concentrations of inhibitors would be necessary to select for this mutation.

The magnitude of the vitality value in general correlates with the appearance of mutants during *in vitro* drug resistance selection studies, and it will be interesting to see if this relationship continues to hold as more resistance data become available from clinical studies. The situation presented here may be complicated by the possibility of mutations not only in the protease but also in other regions of the *gag* and *pol* genes (Lamarre et al., 1994). Such mutations for example may affect protease cleavage sites, making the *gag* and *gag-pol* polyproteins better substrates for the mutant protease.

Implications for Inhibitor Design. Comparison of the mean vitality values for each inhibitor summed across the entire panel of mutants (mean of all values for a given column in Table 3) may be one indication of the relative resistance potential for these agents. A-77003 has the highest mean value, 10.6, and is the most symmetric of the compounds. It is also the longest inhibitor in that it occupies six subsitess—S3 to S3′—in the enzyme. Crystallographic studies demonstrate that the P3/P3′ pyridine rings in A-77003 make highly favorable stacking interactions with the charged guanidinium side chains of Arg8/108 (Hosur et al., 1994). Modeling studies indicated that these interactions would be diminished in the R8Q mutant. The other four compounds occupy only five subsites—S3 to S2′—and their aromatic P3 moieties do not make stacking interactions with Arg8 (T. N. Bhat and J. W. Erickson, unpublished data). A-84538, which has a symmetric core (P1−P1′) structure, has the second lowest average value, 3.2. The most "substrate-like" inhibitor, KNI-272, has the second highest value, 5.9, among the P3−P2′-containing compounds. Thus, there does not appear to be any consistent correlation between resistance potential and inhibitor properties such as symmetry or substrate mimicry. Somewhat surprising is that Ro31-8959, with the bulkiest P1′ substituent, remains potent with all the S1′ subsite mutants which also exhibit the lowest overall vitality values. While the major HIV PR mutants that emerge with Ro31-8959, G48V and L90M, were not tested in this study, the consistently low vitality values, to within a factor of 2, for all subsite mutants were unexpected.

It should be noted that the vitality values presented in this study were calculated from k_{cat}/K_m data using a single peptide substrate. While inhibition constants should not depend on the substrate, k_{cat}/K_m ratios do, and therefore vitality values will differ for different substrates. Thus, future studies should focus on a panel of substrates that represent all the natural HIV PR cleavage sites in the *gag* and *gag-pol* polyproteins. Nevertheless, our results show that even with

a single substrate the kinetic model presented in this study may be useful for predicting possible subsite mutations in HIV PR which could be selected in the presence of a given inhibitor. Our panel of mutants may also be used to assess cross-resistance patterns for various inhibitor combinations as an aid to planning clinical studies, as well as for screening for inhibitors targeted to selected HIV PR mutants. Our studies represent an initial step toward the development of a quantitative understanding of the effects of active site mutations on inhibitor binding that is tantamount to the elaboration of structure-based strategies to combat drug resistance.

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