Non-active Site Changes Elicit Broad-based Cross-resistance of the HIV-1 Protease to Inhibitors*

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Three high level, cross-resistant variants of the HIV-1 protease have been analyzed for their ability to bind four protease inhibitors approved by the Food and Drug Administration (saquinavir, ritonavir, indinavir, and nelfinavir) as AIDS therapeutics. The loss in binding energy ($\Delta G_{\text{r}}$) going from the wild-type enzyme to mutant enzymes ranges from 2.5 to 4.4 kcal/mol, 40–65% of which is attributed to amino acid substitutions away from the active site of the protease and not in direct contact with the inhibitor. The data suggest that non-active site changes are collectively a major contributor toward engendering resistance against the protease inhibitor and cannot be ignored when considering cross-resistance issues of drugs against the HIV-1 protease.

The objective of anti-retroviral therapy for human immunodeficiency virus type 1 (HIV-1) infection is complete viral suppression below the limits of detection. This goal has not been routinely achieved until the development of potent inhibitors targeted against the HIV-1 protease, an enzyme essential for viral replication. One of the greatest barriers to achieving long term viral suppression is the emergence of drug-resistant strains of HIV. The appearance of drug-resistant viruses and the evaluation of drug cross-resistance for a given enzymatic target (reverse transcriptase or protease) have been the most serious issues in the treatment of HIV-infected individuals. The correlation of active site mutations in HIV-1 protease to decreases in inhibitor binding has been well documented, whereas the role of non-active site mutations has not been clearly defined (1). To date, several non-active site changes have been described as compensatory mutations that tend to offset the detrimental effects of active site mutations toward enzyme catalysis (2, 3), but the effect of non-active site mutations toward binding has hitherto not been analyzed quantitatively. In this report, our findings show that non-active site amino acid substitutions are major factors leading to the decreases in inhibitor binding to three clinically derived variants of the HIV-1 protease.

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1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1.

Enzyme Preparation—Synthetic oligonucleotide cassettes of 444 base pairs were designed according to the wild-type sequence of pET-3b-HIVPR (4). Point mutations were incorporated into the DNA with a bias toward optimal codon usage in Escherichia coli to yield the amino acid mutations listed in Table I. The oligonucleotides were annealed and ligated into pUC18 or pUC19 by Midland Certified Reagent Co. The primary sequence was verified before subcloning into a pET-3b expression vector into NcoI and Bpu102I sites and reconfirmed by automated double-stranded DNA sequencing. Clones carrying the mutant DNA were transformed and expressed as described previously (3, 5). The cells were lysed in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% Nonidet P-40, 10 mM MgCl₂, and 100 µg/ml DNase I using a microfluidizer processer (Microfluidics International Corp., Newton, MA). The mutant protease was extracted, refolded, and purified over affinity columns as described (3). Protein concentrations were determined by amino acid analysis, and purity was confirmed by SDS-gel electrophoresis.

Kinetic Assays—All enzyme-catalyzed reactions were performed under initial velocity and steady-state conditions. Specifically, conditions for the enzyme catalyzed hydrolysis of the MA/CA cleavage site peptide VSQN-(β-naphthylalanine)-PIV were established with respect to time and enzyme concentration to yield linear initial velocity data. The enzyme concentrations employed in the assay were as follows: wild-type, 5 µM; A-44 and A-44r, 200 µM; V-18, K-60, and K-60r, 10 µM; V-18r, 20 µM (r = active site revertant). Binding constants for each competitive inhibitor were first estimated by determining IC₅₀ values with 12 inhibitor concentrations and solving for an estimated Kᵢ value using the equation $K_i = IC_{50} \times K_M/\left[\text{I} \right] + [\text{I}]$. The $K_i$ value was then reetermined in separate assays using a series of inhibitor concentrations that equaled 0.5, 1, 2, and 3 times the estimated $K_i$ value. Six substrate concentrations ranging from 50 to 600 µM were employed for each inhibitor concentration. The final $K_i$ values were derived from replots of $K_M/\text{V}_{\text{max}}$ versus inhibitor concentration from double-reciprocal plots. The $K_i$ values for each inhibitor with wild-type enzyme and selected others (e.g. the K-60 and saquinavir pair) were determined multiple times to yield an average S.D. of 4.2% ($n = 14$). Other assay conditions were as described previously (3) with the exception that detection of product was monitored with fluorescence (excitation = 270 nm, emission = 330 nm).

RESULTS AND DISCUSSION

Variants of the HIV-1 Protease—Suboptimal doses of indinavir in early clinical trials were effective in reducing viral loads during the initial weeks of treatment. Subsequently, isolates from patients began to show mutations in the protease gene that correlated directly to in vivo resistance not only to indinavir but also to a diverse panel of HIV-1 protease inhibitors (6). In similar fashions, cross-resistance was also observed for viral isolates from patients treated with other inhibitors (7–11). Under the selective pressure of an inhibitor, it has been shown that more than 20 of the 99 amino acid residues of HIV-1 protease can mutate to yield a replication-competent yet resistant enzyme (12). Most protease sequences from the HIV-1 viruses that achieve a high level of cross-resistance (>99% with >5 different inhibitors) contain a constellation of approximately 8–12 amino acid changes, which include a set of mutations in the active site and another set located in other regions (flaps, hinge, etc.) of the protease. At least a handful of substitutions, in and away from the active site of the protease, is needed to elicit phenotypic resistance against indinavir.² For

2 We note that a previous investigation (6) has shown that a panel of mutational substitutions in the HIV-1 protease is required to elicit phenotypic resistance to the protease inhibitor. Single substitutions of some of the non-active site residues (e.g. Leu-10, Val-32, Met-36, Met-46, Leu-63, Leu-90) exert no effect on viral susceptibility to indinavir. There are other non-active site substitutions (e.g. Lys-20,
indinavir, it is possible for four different active site amino acids to mutate to yield some form of resistant HIV-1 protease. However, in most cases the sequences examined from patient isolates contain only one active site amino acid change. The predominant active site change occurs at residue position 82. It is from this subset of patient isolates (6) that three mutant enzymes have been chosen for this study. The mutation sites of these mutants are shown in Table I. Each isolate contains 9–11 changes from the wild-type sequence (13), contains a single unique active site modification, and is highly cross-resistant. Cross-resistance—Table II lists the $K_i$ values of the four HIV protease inhibitors against the wild-type and three mutant proteases in a low salt buffer. Although each of the four inhibitors exhibits binding to the wild-type protease with equilibrium constants in the sub-nanomolar range ($K_i = 0.06–0.24 \text{ nM}$), the introduction of various sets of mutations (Table I) leads to a 60–1800-fold increase in $K_i$ for the inhibitors. Hence, cross-resistance is demonstrated at the enzyme level as it has been at the virus level (6) with this panel of HIV-1 protease variants.

Effect of Non-active Site Residues on Binding—To assess the collective contribution of non-active site mutations for the variants of the HIV-1 protease, we have reverted the single active site mutation in each variant back to the wild type. The resultant mutants now contain only point mutations away from the active site of the protease. As seen in Table II, there remains a severe loss in binding affinity for each of the “active site revertants” as compared with the wild-type protease.3 The $K_i$ values for the three variants and four inhibitors ($n = 12$) have increased 5–80-fold over the values obtained for the wild-type enzyme. The results clearly reveal that a significant contribution of resistance is due to amino acid changes away from the immediate vicinity of the inhibitor binding pocket as defined in numerous x-ray crystallographic studies.

The quantitative contribution of active site and non-active site mutations toward affinity can be evaluated in terms of the Gibbs free energy of binding, $\Delta G_b$.4 The loss in affinity, $\Delta \Delta G_b$, for the active site revertants ranges from 1 to 2.6 kcal/mol. These values account for 40–65% of the total binding energy of the inhibitor to the protease variants A-44, V-18, and K-60. Therefore, approximately one-half of the loss in affinity of the inhibitors can be attributed to the collective action of the non-active site amino acid substitutions induced by the inhibitor. The results are summarized in Table III as expressed in the ratio of $\Delta \Delta G_{\text{active site revertant}}/\Delta \Delta G_{\text{mutant}}$. It is noteworthy that for the four inhibitors examined, this ratio falls within a narrow range for the contribution of the non-active site residues toward binding. This result is unexpected because these protease variants are derived from patients taking indinavir as their sole protease inhibitor. The only outlier is with saquinavir in the case of the K-60 enzyme where 100% of the binding energy is due to the non-active site amino acids (i.e. the addition of the active site V82F change has no effect on binding (see Table II)). One possible explanation is that the negative effects of introducing the bulky phenyl group to residue 82 in the S1 binding pocket of the enzyme active site are counteracted by the introduction of added binding interaction with the quinoline moiety of saquinavir in that pocket. Molecular modeling of the protease active site has revealed that it is possible for the aromatic quinoline of saquinavir to form additional van der Waals interactions similar to that reported for DMP323 (17).

Active Site versus Non-active Site Substitutions—In previous reports, different effects on catalysis have been assigned to active site amino acid mutations and several selected non-active site substitutions of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site amino acid mutations and several selected non-active site substitutions of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site of the HIV-1 protease (2, 3). Active site changes such as V82I and I84V have been found (3) at the active site of the HIV-1 protease (2, 3).

ser-37, ile-64, val-77, ile-93) in which the incidence of change does not correlate statistically or significantly with phenotypic resistance to indinavir. Residues 37 and 63 exhibit a high frequency of polymorphism in untreated patients (6). The predominant side chains for residue 37 are Ser, Asp, or Asn; Cys is sometimes seen. The predominant side chains for residue 63 are Leu and Pro; Ala, Ser, His, and Thr are often seen.

### Table I

| Protein   | Leu-10 | Lys-20 | Leu-24 | Met-36 | Ser-37 | Met-46 | Ser-57 | Val-58 | Ala-71 | Gln-73 | Val-77 | Val-82 | Ile-84 | Leu-90 | Ile-93 |
|-----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Wild-type | Met    | Val    | Ile    | Met    | Ser    | Met    | Arg    | Gly    | Leu    | Leu    | Ser    | Ala    | Ile    | Pro    | Val    |
| A-44      | Glu    | Pro    | Val    | Asn    | Ile    | Val    | Pro    | Val    | Ser    | Pro    | Val    | Ser    | Pro    | Val    |
| K-60      | Glu    | Pro    | Val    | Asn    | Ile    | Val    | Pro    | Val    | Ser    | Pro    | Val    | Ser    | Pro    | Val    |
| V-18      | Glu    | Pro    | Val    | Asn    | Ile    | Val    | Pro    | Val    | Ser    | Pro    | Val    | Ser    | Pro    | Val    |

For the inhibitors, we emphasize that the data do not imply that a similar magnitude of change in the Gibbs free energy of binding ($\Delta G_b$) would necessarily be observed for the binding of these inhibitors to individual mutants having a single active site amino acid change within the context of the wild-type protease. The reason is that the ground state energetics of the active site revertants and the wild-type HIV-1 protease are unlikely to be the same.
wild-type activity (data not shown).\(^5\) Thus, the point mutation in the active site of the protease remains a major factor in the loss of catalytic prowess of the enzyme as well as the loss of binding affinity to the inhibitors. What the current data reveal, in addition, is that apparently multiple mutations outside the active site are involved not only in stabilizing the transition-state binding of substrate \((i.e. \text{ES}^1)\), as is already known (3, 19), but also in contributing significantly to the destabilization of inhibitor binding. Thus, non-active site mutations of the protease that emerge under the selective pressure of a drug are capable of raising resistance via two mechanisms. Because all four protease drugs select for many of the same non-active site amino acid mutations \(\text{in vivo}\) (1, 12), our data also suggest that the non-active site changes cannot be ignored when considering resistance and cross-resistance issues relating to HIV-1 protease.

The relationship at the molecular level of the two mechanisms of resistance caused by the non-active site mutations is unclear at present. We note that the overall combination of inhibitor-bound structures of a mutant of the HIV-1 protease that contains nine point mutations, eight of which are away from the active site of the enzyme, is not noticeably different from those of the wild type at greater than 2.5 Å resolution.\(^6\)

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\(^5\) We have noted previously (3) that a full assessment of changes in the catalytic properties of the HIV-1 protease should be made with the panel of eight substrates representing all the cleavage sites of the HIV-1 polypeptide. Activity data have been estimated in this work using only one peptide analog that mimics the cleavage site (SQNY.PIV) between the MA and CA domains of the HIV-1 polypeptide (see "Materials and Methods"). Thus, the change in \(k_{\text{cat}}/K_M\) for the three mutant proteases and their corresponding active site revertants are only rough indications of the effect of amino acid substitutions in and away from the active site of the enzyme.

\(^6\) S. Munshi, B. Galvin, Y. Li, D. Olsen, Z. Chen, and L. C. Kuo, submitted for publication.