Multidrug Resistance to HIV-1 Protease Inhibition Requires Cooperative Coupling between Distal Mutations†

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Received June 17, 2003; Revised Manuscript Received August 21, 2003

ABSTRACT: The appearance of viral strains that are resistant to protease inhibitors is one of the most serious problems in the chemotherapy of HIV-1/AIDS. The most pervasive drug-resistant mutants are those that affect all inhibitors in clinical use. In this paper, we have characterized a multiple-drug-resistant mutant of the HIV-1 protease that affects indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir. This mutant (MDR-HM) contains six amino acid mutations (L10I/M46I/I54V/V82A/I84V/L90M) located within and outside the active site of the enzyme. Microcalorimetric and enzyme kinetic measurements indicate that this mutant lowers the affinity of all inhibitors by 2−3 orders of magnitude. By comparison, the multiiple-drug-resistant mutant only increased the K_m of the substrate by a factor of 2, indicating that the substrate is able to adapt to the changes caused by the mutations and maintain its binding affinity. To understand the origin of resistance, three submutants containing mutations in specific regions were also studied, i.e., the active site (V82A/I84V), flap region (M46I/I54V), and dimerization region (L10I/L90M). None of these sets of mutations by themselves lowered the affinity of inhibitors by more than 1 order of magnitude, and additionally, the sum of the effects of each set of mutations did not add up to the overall effect, indicating the presence of cooperative effects. A mutant containing only the four active site mutations (V82A/I84V/M46I/I54V) only showed a small cooperative effect, suggesting that the mutations at the dimer interface (L10I/L90M) play a major role in eliciting a cooperative response. These studies demonstrate that cooperative interactions contribute an average of 1.2 ± 0.7 kcal/mol to the overall resistance, most of the cooperative effect (0.8 ± 0.7 kcal/mol) being mediated by the mutations at the dimerization interface. Not all inhibitors in clinical use are affected the same by long-range cooperative interactions between mutations. These interactions can amplify the effects of individual mutations by factors ranging between 2 and 40 depending on the inhibitor. Dissection of the energetics of drug resistance into enthalpic and entropic components provides a quantitative account of the inhibitor response and a set of thermodynamic guidelines for the design of inhibitors with a lower susceptibility to this type of mutations.

One of the most serious side effects associated with the therapy of HIV-1[1] infection is the appearance of viral strains that exhibit resistance to protease inhibitors (*1*−*8*). One approach to alleviate the effects of drug resistance has been to tailor antiretroviral therapy according to viral genotypes or to switch therapies to regimes that include inhibitors that are not affected or only mildly affected by the viral mutations found in a patient. For those reasons, a disturbing new development has been the emergence of mutations that confer resistance to all protease inhibitors currently in clinical use, indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir. Multidrug resistance is triggered by mutations located in different regions of the protease molecule: Leu 10, Met 46, Ile 54, Val 82, Ile 84, and Leu 90 (*9*) (Figure 1). At these positions, the following mutations have been observed with high probability in patients: L10I (18%); M46I (7%); I54V (8%); V82A (12%); I84V (3%); L90M (11%) (*10*). These mutations are located in different regions of the protease: the active site (V82A and I84V), the flap region (M46I and I54V), and the dimerization region (L10I and L90M).

In this paper, we report the thermodynamics of the binding of indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir to the wild-type HIV-1 protease and the multiple-drug-resistant mutant L10I/M46I/I54V/V82A/I84V/L90M. To identify the origin of multidrug resistance, the binding thermodynamics of the inhibitors to the submutants V82A/I84V (active site), M46I/I54V (flap region), and L10I/L90M (dimerization region) were also measured. The results of this investigation indicate that multidrug resistance occurs at two levels, a primary level characterized by the loss of inhibitor/protease interactions due to the presence of individual mutations and a global level triggered by cooperative interactions between distal sites in the protease structure.

† Supported by a grant from the National Institutes of Health (GM 57144).

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[1] Abbreviations: HIV-1, human immunodeficiency virus, type 1; ITC, isothermal titration calorimetry; IPTG, isopropyl β-D-thiogalactoside; EDTA, (ethylenedinitrile)tetraacetic acid; DMSO, dimethyl sulfoxide; ME, 2-mercaptoethanol; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; MDR-HM, multiple-drug-resistant hexamutant L10I/M46I/I54V/V82A/I84V/L90M; MDR-QM, multiple-drug-resistant tetramutant V82A/I84V/M46I/I54V.

10.1021/bi0350405 CCC: $25.00
Published on Web 10/30/2003

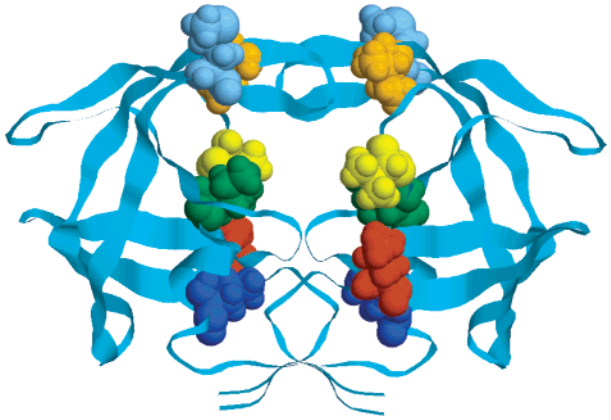

FIGURE 1: Structure of the HIV-1 protease showing the location of mutations associated with multidrug resistance: M46I (light blue), I54V (orange), V82A (yellow), I84V (green), L10I (red), and L90M (blue).

EXPERIMENTAL METHODS

Protease Mutants. The genes encoding HIV-1 protease pseudo wild type (with three autolytic sites protected, Q7K/L33I/L63I) were transferred to the pET24 vector (Novagen), where the expressions are under the control of the T7 promoter. Mutations at selected positions (L10I/M46I/I54V/V82A/I84V/L90M, M46I/I54V/V82A/I84V, V82A/I84V, M46I/I54V, and L10I/L90M) were introduced using an in vitro site-directed mutagenesis kit (QuikChange, Stratagene), and mutations were confirmed by DNA sequencing. Proteases were expressed in BL21/DE3 cells by adding IPTG to 1 mM once the culture density (as determined by absorbance at 600 nm) was 1.5 or greater as determined with an HP 8452 diode array spectrophotometer.

Protease Purification. Plasmid-encoded HIV-1 protease was expressed as inclusion bodies in *Escherichia coli* BL21/DE3 cells (*11*, *12*). Cells were suspended in extraction buffer (20 mM Tris, 1 mM EDTA, 10 mM 2-ME, pH 7.5) and broken with two passes through a French pressure cell (≥16000 psi). Cell debris and protease-containing inclusion bodies were collected by centrifugation (20000*g* at 4 °C for 20 min). Inclusion bodies were washed with three buffers. Each wash consisted of resuspension (glass homogenizer) and centrifugation (20000*g* at 4 °C for 20 min). In each step a different washing buffer was used: buffer 1 (25 mM Tris, 2.5 mM EDTA, 0.5 M NaCl, 1 mM Gly-Gly, 50 mM 2-ME, pH 7.0), buffer 2 (25 mM Tris, 2.5 mM EDTA, 0.5 M NaCl, 1 mM Gly-Gly, 50 mM 2-ME, 1 M urea, pH 7.0), buffer 3 (25 mM Tris, 1 mM EDTA, 1 mM Gly-Gly, 50 mM 2-Me, pH 7.0). Protease was solubilized in 25 mM Tris, 1 mM EDTA, 5 mM NaCl, 1 mM Gly-Gly, 50 mM 2-ME, and 9 M urea at pH 9.0, clarified by centrifugation, and applied directly to an anion exchange Q-Sepharose column (Q-Sepharose HP, Pharmacia) previously equilibrated with the same buffer. The protease was passed through the column and acidified by adding formic acid to 25 mM immediately upon elution from the column. Precipitation of a significant amount of contaminants occurred upon acidification. Protease-containing fractions were pooled, concentrated, and stored at 4 °C at 5−10 mg/mL.

The HIV-1 protease was folded by 20-fold stepwise dilution into 10 mM formic acid at 0 °C. The pH was gradually increased to 3.8, and then the temperature was raised to 30 °C. Sodium acetate at pH 5.0 was added to 100 mM, and the protein was concentrated. Folded protease was desalted into 1 mM sodium acetate and 2 mM NaCl at pH 5.0 using a gel filtration column (PD-10, Pharmacia) and stored at either 4 or −20 °C (≥2.5 mg/mL) without loss of activity in several weeks. After folding, protease was estimated to be ≥99% pure.

Table 1: Catalytic Parameters for HIV-1 Protease Mutants Used in These Studies

protease	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, $s^{-1}\,\mu M^{-1}$
wild type	14 ± 1	8.1 ± 0.2	0.58
MDR-HM	32 ± 3	1.5 ± 0.1	0.047
MDR-QM	41 ± 5	2.0 ± 0.2	0.049
V82A/I84V	13 ± 2	3.0 ± 0.1	0.23
M46I/I54V	20 ± 3	4.0 ± 0.2	0.20
L10I/L90M	40 ± 3	3.4 ± 0.1	0.085

Protease Inhibitors. Clinical inhibitors indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir were purified from commercial capsules as described before (*8*, *13*). Briefly, purification was performed by HPLC (Waters Inc.) using a semipreparative C-18 reversed-phase column developed with 20−80% acetonitrile containing 0.05% trifluoroacetic acid. Purified inhibitors were lyophilized and stored at −20 °C as solids or dissolved in DMSO. KNI-272 and KNI-764 were kindly provided by Prof. Y. Kiso (Kyoto Pharmaceutical University, Kyoto, Japan). The latter compounds were used without further purification. Acetyl pepstatin (Bachem AG) was prepared as a 7.8 mM stock solution in 9 mM NaOH. The inhibitor solution used in the thermodynamic experiments was prepared in 10 mM sodium acetate, pH 5.0, the DMSO concentration being kept constant at 2%.

Enzymatic Assays. The catalytic activities of the HIV-1 proteases were tested prior to thermodynamic analysis by following the hydrolysis of the chromogenic substrate Lys-Ala-Arg-Val-Nle-nPhe-Glu-Ala-Nle-NH_2, where Nle stands for norleucine and nPhe stands for *p*-nitrophenylalanine (California Peptide Research Inc., Napa, CA).

In the spectrophotometric assay, protease was added to a 120 μL microcuvette containing substrate at 25 °C. Final concentrations in the standard assay were 30−60 nM active protease, 0−170 μM substrate, 10 mM sodium acetate at pH 5.0, and 1 M NaCl. The absorbance was monitored at six wavelengths (296−304 nm) using an HP 8452 diode array spectrometer (Hewlett-Packard) and corrected for spectrophotometer drift by subtracting the average absorbance at 446−454 nm. An extinction coefficient for the difference in absorbance upon hydrolysis (1800 M^{-1} cm^{-1} at 300 nm) was used to convert the absorbance change to reaction rates. Hydrolysis rates were obtained from the initial portion of the data, where at least 80% of the substrate remains free. The concentration of active protease was determined by performing active site titration with KNI-272, a very potent inhibitor (at pH 5.0, $K_i \approx 16$ pM), using protease concentrations much higher (~2 μM) than the corresponding K_i. The enzymatic parameters k_{cat} and K_m determined under identical conditions for all proteases used in these studies are summarized in Table 1.

Isothermal Titration Calorimetry. Isothermal titration calorimetry experiments were performed using a high-

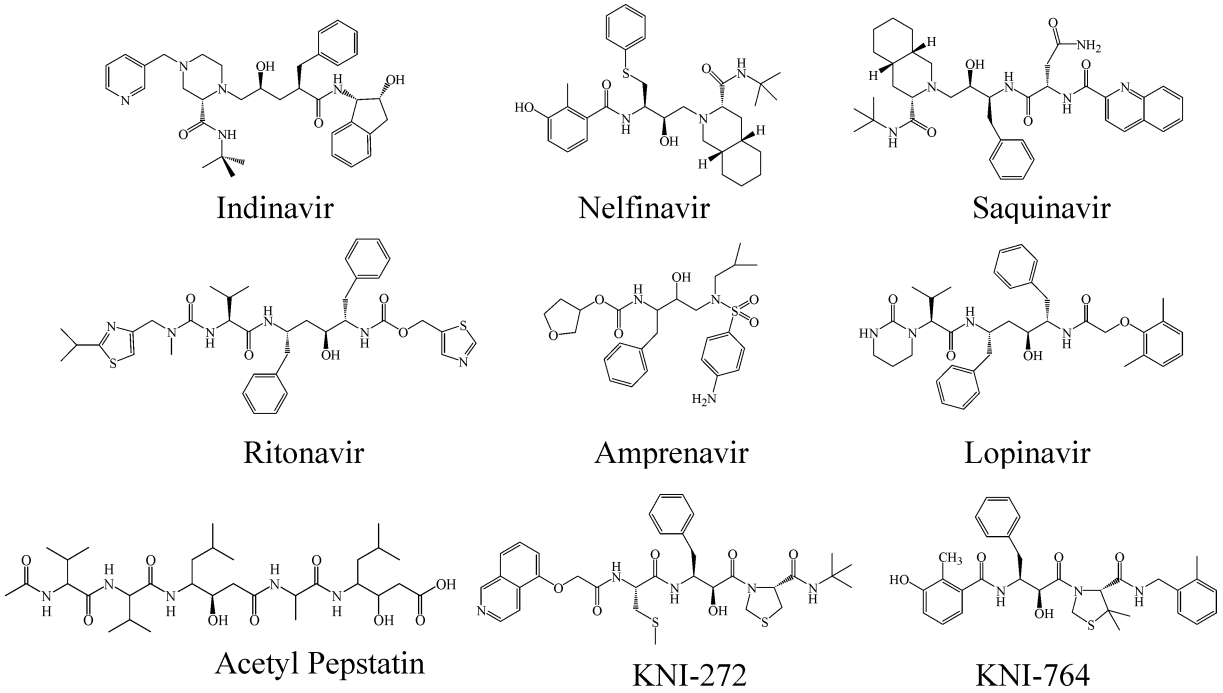

FIGURE 2: Chemical structures of the six protease inhibitors currently in clinical use (top two rows), acetyl pepstatin, and two experimental inhibitors (KNI-272 and KNI-764).

precision VP-ITC titration calorimetric system (Microcal Inc.). The enzyme solution in the calorimetric cell was titrated with indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, lopinavir, KNI-272, and KNI-764 or acetyl pepstatin dissolved in the same buffer. The inhibitor concentration was estimated by nitrogen content determination (acetyl pepstatin) or from stoichiometric determination with a standardized protease solution. The heat evolved after each inhibitor injection was obtained from the integral of the calorimetric signal. The heat from the binding reaction between the inhibitor and enzyme was obtained as the difference between the heat of reaction and the corresponding heat of dilution. The binding affinities were determined by using ITC displacement experiments. Acetyl pepstatin was selected as the weak inhibitor in the displacement titration because this inhibitor is endothermic and amplifies the signal of a high-affinity exothermic inhibitor when displaced. The measured binding enthalpies and binding affinities of acetyl pepstatin for the different HIV-1 proteases used in this paper are summarized in Table 3. Analysis of the data was performed using software developed in this laboratory.

RESULTS AND DISCUSSION

Binding Energetics of Protease Inhibitors to the Wild Type and Multiple-Drug-Resistant Mutant L10I/M46I/I54V/V82A/I84V/L90M. The binding energetics of the six inhibitors currently in clinical use, indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir (Figure 2), to wild-type HIV-1 protease are summarized in Table 2. Previously, we have reported the energetic parameters for indinavir, nelfinavir, saquinavir, ritonavir, and amprenavir (*8*, *14*), this being the first report of the binding energetics for lopinavir. The binding energetics was measured by isothermal titration calorimetry using the displacement technique as described before (*14*−*16*). This technique allows complete determination of binding thermodynamics for high-affinity ligands ($>\sim 10^9$ M^{-1}) that are beyond the range of determination by direct titrations. As shown in Table 2, of all the inhibitors currently in clinical use, lopinavir has the highest affinity for the wild-type protease (8.3 pM). In general, all inhibitors that exhibit affinities in the picomolar range (<100 pM) achieve that threshold by combining favorable enthalpic and entropic contributions to the Gibbs energy of binding. On the contrary, inhibitors in which the enthalpy change is unfavorable cannot achieve that threshold (*14*, *16*).

The binding thermodynamics of the inhibitors to the multiple-drug-resistant mutant (MDR-HM) were also measured by isothermal titration calorimetry (Table 2). MDR-HM lowers the binding affinity of indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir by factors of 200, 210, 2000, 1500, 85, and 660, respectively. The loss in binding affinities is due to a large loss in the binding enthalpy of all inhibitors (Figure 3). The figure also provides important clues regarding the origin of resistance in terms of the enthalpic and entropic binding components for each inhibitor. The loss of favorable interactions cannot be overcome by the compensatory change in the binding entropy. As a result the Gibbs energy of binding becomes 2.6−4.5 kcal/mol less favorable, which is equivalent to a loss in binding affinity of 85−2000-fold. The significant loss in binding affinity for all inhibitors should be contrasted with the small increase in the K_m of the substrate (Table 1), indicating that this molecule is able to adapt to the changes imposed by the mutations without losing significant affinity. The catalytic constant of MDR-HM, on the other hand, drops by a factor of 5.4, resulting in a 10-fold overall loss in catalytic efficiency. The biochemical fitness of a drug-resistant mutant compared to the wild type in the presence of inhibitor can be expressed as (*13*, *17*, *18*)

$$\text{relative vitality} = \frac{K_d k_{cat}/K_m}{k_{d,ref} k_{cat\ wt}/K_{m\ wt}}$$

Table 2: Thermodynamics of the Binding of Inhibitors in Clinical Use to Multiple-Drug-Resistant HIV-1 Protease Mutant MDR-HM and Constituent Submutants

protease	ΔG, kcal mol^{-1}	ΔH,[a] kcal mol^{-1}	$-T\Delta S$, kcal mol^{-1}	K_a, M^{-1}	K_d, nM	K_d ratio
			Indinavir			
wild type	−12.4	1.8	−14.2	$(1.3 \pm 0.1) \times 10^9$	0.76 ± 0.04	1
MDR-HM	−9.3	8.4	−17.7	$(6.5 \pm 0.3) \times 10^6$	150 ± 10	200
MDR-QM	−10.4	6.4	−16.8	$(4.5 \pm 0.4) \times 10^7$	22 ± 2	29
V82A/I84V	−10.8	3.7	−14.5	$(8.5 \pm 0.8) \times 10^7$	12 ± 1	15
M46I/I54V	−12.2	3.5	−15.7	$(8.9 \pm 0.7) \times 10^8$	1.1 ± 0.1	1.5
L10I/L90M	−11.8	3.0	−14.8	$(4.3 \pm 0.2) \times 10^8$	2.3 ± 0.1	3.0
			Nelfinavir			
wild type	−12.8	3.1	−15.9	$(2.2 \pm 0.2) \times 10^9$	0.44 ± 0.04	1
MDR-HM	−9.6	9.6	−19.2	$(1.1 \pm 0.1) \times 10^7$	93 ± 5	210
MDR-QM	−11.0	7.3	−18.3	$(1.2 \pm 0.1) \times 10^8$	8.1 ± 0.6	18
V82A/I84V	−12.4	3.2	−15.6	$(1.2 \pm 0.1) \times 10^9$	0.83 ± 0.08	1.9
M46I/I54V	−12.4	6.5	−18.9	$(1.2 \pm 0.1) \times 10^9$	0.83 ± 0.08	1.9
L10I/L90M	−12.1	4.8	−16.9	$(7.4 \pm 0.5) \times 10^8$	1.3 ± 0.1	3.0
			Saquinavir			
wild type	−13.0	1.2	−14.2	$(3.6 \pm 0.3) \times 10^9$	0.28 ± 0.02	1
MDR-HM	−8.5	10.4	−18.9	$(1.8 \pm 0.1) \times 10^6$	550 ± 20	2000
MDR-QM	−10.0	8.5	−18.5	$(2.3 \pm 0.2) \times 10^7$	43 ± 4	160
V82A/I84V	−11.8	3.7	−15.5	$(4.6 \pm 0.4) \times 10^8$	2.2 ± 0.2	7.9
M46I/I54V	−11.9	5.1	−17.0	$(5.1 \pm 0.3) \times 10^8$	2.0 ± 0.1	7.2
L10I/L90M	−12.0	3.6	−15.6	$(5.9 \pm 0.2) \times 10^8$	1.7 ± 0.1	6.1
			Ritonavir			
wild type	−13.7	−4.3	−9.4	$(1.0 \pm 0.1) \times 10^{10}$	0.098 ± 0.008	1
MDR-HM	−9.3	2.9	−12.2	$(6.7 \pm 1.3) \times 10^6$	150 ± 30	1500
MDR-QM	−10.7	3.3	−14.0	$(7.4 \pm 0.5) \times 10^7$	14 ± 1	140
V82A/I84V	−11.9	−3.6	−8.3	$(5.0 \pm 0.4) \times 10^8$	2.0 ± 0.2	21
M46I/I54V	−12.8	0.9	−13.7	$(2.3 \pm 0.2) \times 10^9$	0.44 ± 0.04	4.5
L10I/L90M	−12.9	−2.8	−10.1	$(2.7 \pm 0.2) \times 10^9$	0.37 ± 0.02	3.7
			Amprenavir			
wild type	−13.2	−6.9	−6.3	$(5.0 \pm 0.3) \times 10^9$	0.20 ± 0.01	1
MDR-HM	−10.6	1.6	−12.2	$(5.8 \pm 0.3) \times 10^7$	17 ± 1	85
MDR-QM	−11.6	−0.5	−11.1	$(3.2 \pm 0.1) \times 10^8$	3.1 ± 0.1	15
V82A/I84V	−12.2	−5.6	−6.6	$(9.2 \pm 0.5) \times 10^8$	1.1 ± 0.1	5.4
M46I/I54V	−12.8	−3.5	−9.3	$(2.4 \pm 0.2) \times 10^9$	0.41 ± 0.03	2.0
L10I/L90M	−12.4	−5.2	−7.2	$(1.3 \pm 0.1) \times 10^9$	0.79 ± 0.03	3.9
			Lopinavir			
wild type	−15.1	−3.8	−11.3	$(1.2 \pm 0.2) \times 10^{11}$	0.0083 ± 0.0014	1
MDR-HM	−11.3	4.3	−15.6	$(1.8 \pm 0.3) \times 10^8$	5.5 ± 0.8	660
MDR-QM	−12.8	2.8	−15.6	$(2.5 \pm 0.2) \times 10^9$	0.40 ± 0.03	48
V82A/I84V	−13.9	−2.1	−11.8	$(1.6 \pm 0.3) \times 10^{10}$	0.063 ± 0.012	7.6
M46I/I54V	−14.9	0.0	−14.9	$(8.1 \pm 1.2) \times 10^{10}$	0.012 ± 0.002	1.5
L10I/L90M	−14.9	−1.6	−13.3	$(8.7 \pm 0.7) \times 10^{10}$	0.011 ± 0.001	1.4

[a] The experimental error in the enthalpy determination is ±0.2 kcal/mol.

where $K_{d,ref}$ is the reference affinity constant, which for convenience is chosen to be the one for ritonavir to the wild-type protease. This relation shows that the biochemical fitness or vitality for the multiple-drug-resistant mutant MDR-HM can be up to 450 times higher than that of the wild type in the presence of inhibitor: actual values for the clinical inhibitors are 125, 74, 450, 121, 14, and 4.5 times that of the wild type for indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir, respectively; for the wild-type protease, the relative vitalities are 7.7, 4.5, 2.8, 1.0, 2.0, and 0.08, respectively. On the other hand, the biochemical fitness of the mutant in the absence of drug will be about 12 times less than that of the wild type, as the relation reduces to the ratio between the catalytic efficiencies of the mutant and wild type. This is consistent with the observation that suppression of antiretroviral therapy leads to the reemergence of the wild type as the predominant viral form.

Energetics of the Binding of Protease Inhibitors to the Active Site Submutant V82A/I84V. The active site mutations by themselves lower the binding affinity of indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir by factors of 15, 1.9, 7.9, 21, 5.4, and 7.6, respectively. The K_m for the substrate remains essentially unchanged, but the catalytic constant drops by a factor of 2.7. In isolation, these two mutations only affect significantly the binding affinities of indinavir and ritonavir. For indinavir the effect of the mutation is almost exclusively enthalpic, whereas for ritonavir the effect involves a combination of enthalpic and entropic losses (Figure 4). The inhibitors that are not affected significantly by this mutation either lose very little binding enthalpy (nelfinavir) or are able to entropically compensate enthalpic losses with entropic gains (saquinavir, amprenavir, lopinavir) (Figure 4).

Energetics of the Binding of Protease Inhibitors to Flap Submutant M46I/I54V. The double mutation within the flap region (M46I/I54V) lowers the binding affinity of indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir by factors of 1.5, 1.9, 7.2, 4.5, 2.0, and 1.5, respectively. The K_m for the substrate increases by a factor of 1.4 and the catalytic constant drops by a factor of 2, resulting in an

Table 3: Thermodynamics of the Binding of Experimental Inhibitors to Multiple-Drug-Resistant HIV-1 Protease Mutant MDR-HM and Constituent Submutants

protease	ΔG, kcal mol^{-1}	ΔH, kcal mol^{-1}	$-T\Delta S$, kcal mol^{-1}	K_a, M^{-1}	K_d, nM	K_d ratio
			KNI-272			
wild type	−13.3	−5.4	−7.9	$(6.0 \pm 0.2) \times 10^9$	$0.17 \pm <0.01$	1
MDR-HM	−9.1	2.0	−11.1	$(4.6 \pm 0.6) \times 10^6$	220 ± 30	1300
MDR-QM	−10.9	0.0	−10.9	$(9.0 \pm 0.5) \times 10^7$	11 ± 1	67
V82A/I84V	−11.6	−3.4	−8.2	$(3.1 \pm 0.2) \times 10^8$	3.3 ± 0.2	20
M46I/I54V	−13.0	−2.9	−10.1	$(3.6 \pm 0.2) \times 10^9$	0.28 ± 0.01	1.7
L10I/L90M	−12.9	−5.2	−7.7	$(2.9 \pm 0.1) \times 10^9$	0.35 ± 0.02	2.1
			KNI-764			
wild type[a]	−14.3	−7.6	−6.7	$(3.1 \pm 0.2) \times 10^{10}$	0.032 ± 0.002	1
MDR-HM	−12.5	−2.0	−10.5	$(1.3 \pm 0.1) \times 10^9$	0.75 ± 0.05	23
MDR-QM	−13.7	−3.9	−9.8	$(1.1 \pm 0.1) \times 10^{10}$	0.094 ± 0.008	2.9
V82A/I84V	−13.8	−6.6	−7.2	$(1.3 \pm 0.2) \times 10^{10}$	0.075 ± 0.009	2.4
M46I/I54V	−14.5	−6.1	−8.4	$(4.1 \pm 0.2) \times 10^{10}$	0.024 ± 0.001	0.8
L10I/L90M	−14.5	−6.6	−7.9	$(4.5 \pm 0.4) \times 10^{10}$	0.022 ± 0.002	0.7
			Acetyl Pepstatin			
wild type[b]	−8.7	8.0	−16.7	$(2.3 \pm 0.1) \times 10^6$	440 ± 20	1
MDR-HM	−6.9	17.5	−24.4	$(1.2 \pm 0.1) \times 10^5$	8200 ± 800	19
MDR-QM	−7.6	12.2	−19.8	$(4.1 \pm 0.3) \times 10^5$	2500 ± 200	5.6
V82A/I84V	−8.0	10.4	−18.4	$(7.8 \pm 0.5) \times 10^5$	1300 ± 100	2.9
M46I/I54V	−8.8	9.6	−18.4	$(3.0 \pm 0.3) \times 10^6$	330 ± 40	0.7
L10I/L90M	−8.2	8.6	−16.8	$(1.1 \pm <0.1) \times 10^6$	950 ± 30	2.1

[a] Velázquez-Campoy et al. (*16*). [b] Ohtaka et al. (*14*).

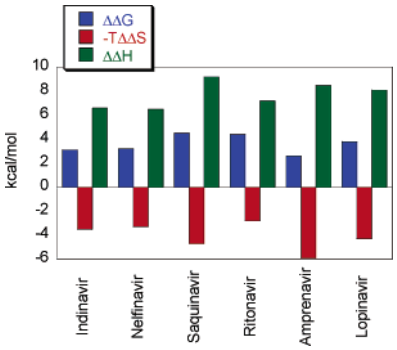

FIGURE 3: Thermodynamic dissection of the loss of affinity of the clinical inhibitors against the multiple-drug-resistant mutant L10I/M46I/I54V/V82A/I84V/L90M.

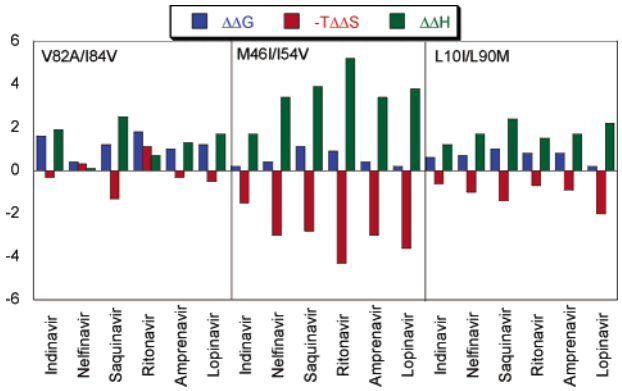

FIGURE 4: Thermodynamic dissection of the effects of individual mutations within the active site (V82A/I84V), flap region (M46I/I54V), and dimerization region (L10I/L90M).

almost 3-fold loss in catalytic efficiency. In isolation, these two flap mutations only affect significantly the binding affinity of saquinavir (Table 2 and Figure 4). In general, these two mutations induce large enthalpy/entropy compensation effects, resulting in small changes in the binding affinity of most inhibitors.

Energetics of the Binding of Protease Inhibitors to Dimer Interface Submutant L10I/L90M. The double mutation at the dimerization region (L10I/L90M) lowers the binding affinity of indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir by factors of 3.0, 3.0, 6.1, 3.7, 3.9, and 1.4, respectively. The K_m for the substrate increases by a factor of 2.9 and the catalytic constant drops by a factor of 2.4, resulting in a 6.8-fold loss in catalytic efficiency. Interestingly, these mutations at the dimer interface are the ones that have the largest effect on the K_m of the substrate. In fact, the effect on the K_m of the substrate is very similar to the effect on the K_d of the inhibitors, suggesting that these two mutations that are distal from the binding site might influence binding by affecting the conformational change associated with binding. This conformational change involves closing of the flaps and rotation along the dimer interface (*19*). In isolation, the binding affinity changes induced by these two mutations are small and involve largely compensatory enthalpy/entropy changes. Despite their intrinsic small effects, these two mutations appear to play a major role in amplifying the effects of the mutations at the active site. Mutations at the dimer interface (specifically L90M) have previously been shown to reduce the catalytic activity of the protease and to lower its structural stability (*20*).

Cooperative Amplification by Mutations at Dimer Interface. The results presented above indicate that the effects of MDR-HM on inhibitor binding are much larger than the sum of the effects of the individual mutations, indicating that cooperative interactions amplify multidrug resistance. The effects are not the same for all inhibitors, suggesting the possibility of identifying compounds that maintain significant affinity against a set of mutations such as the ones present in MDR-HM. Cooperative interactions in MDR-HM affect inhibitors currently in clinical use from a low factor of 2 (amprenavir) to a high factor of 40 (lopinavir). Indinavir, nelfinavir, saquinavir, and ritonavir are affected by factors of 3, 17, 8, and 5, respectively; i.e., the effects of cooperative

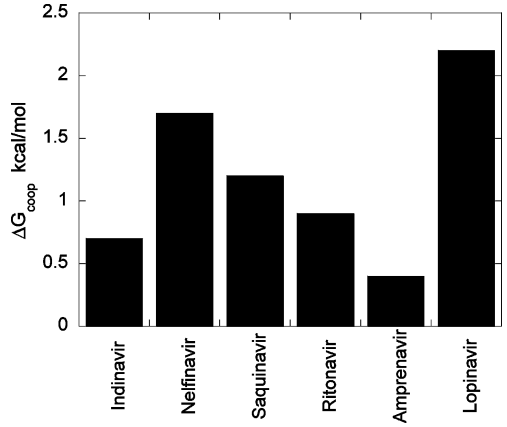

FIGURE 5: Cooperative energy elicited by the dimerization region mutations (L10I/L90M) on the loss of affinity of each inhibitor.

interactions are comparable and often larger than the effects of the individual pairs of drug-resistant mutations (Table 2). To evaluate the magnitude of the cooperative role of the mutations at the dimer interface (L10I/L90M), a protease mutant (MDR-QM) containing only the active site and flap mutations (M46I/I54V/V82A/I84V) was constructed. The results are shown in Tables 1 and 2. The loss of inhibitor potency against this quadruple mutant is very close to the sum of the effects of the isolated active site (V82A/I84V) and flap (M46I/I54V) mutations, indicating that little if any cooperativity exists among those sites. Since these mutations are chemically conservative, they probably act by introducing distortions in the geometry of the binding site cavity. Addition of the mutations at the dimerization interface (L10I/L90M) potentiates the effects of the active and flap mutations even though by themselves they do not have a major effect on inhibitor potency. The magnitudes of the cooperative energies responsible for the amplification of the effects of active site and flap mutations are shown in Figure 5. They range in value between 0.4 and 2.2 kcal/mol and amplify the loss in inhibitor affinity by 2−42-fold. Two of the inhibitors, indinavir and amprenavir, appear to be less affected by the synergistic effect among mutations, suggesting that some functional characteristics of these compounds render them less susceptible to these effects.

Response of Experimental Protease Inhibitors. HIV-1 protease inhibitors based upon the allophenylnorstatine scaffold have been under development for several years (*21*−*23*). Some of these compounds such as KNI-272 exhibit picomolar affinities against the wild-type protease but have been shown to be severely affected by common mutations associated with drug resistance (*24*). Other compounds such as KNI-764 have demonstrated low susceptibility against the same mutations because they have flexible functionalities that allow them to adapt to the binding site distortions associated with the mutation (*16*). The results obtained with these inhibitors as well as the generic aspartic protease inhibitor acetyl pepstatin are shown in Table 3. As expected, KNI-272 loses significant binding affinity (1300-fold) against the multiple-drug-resistant mutation, while the affinity of KNI-764 drops by only a factor of 23. In all, KNI-764 and amprenavir, which loses affinity by a factor of 85 against MDR-HM, are the synthetic inhibitors less affected by MDR-HM. Both amprenavir and KNI-764 bind to wild-type protease with large favorable enthalpy (−6.9 and −7.6 kcal/mol, respectively) as a result of strong interactions with the binding site. In addition, the entropic contribution to the affinity is similar for the two drugs.

Acetyl pepstatin, the naturally occurring generic aspartyl protease inhibitor, provides a different scenario; this peptidic inhibitor is known to inhibit all aspartic proteases with varying affinities (*25*−*28*). The wild-type HIV-1 protease is only weakly inhibited by acetyl pepstatin (K_d of 440 nM) in a process characterized by a largely unfavorable enthalpy change (Table 3). The thermodynamic signature of acetyl pepstatin is certainly different from that of KNI-764 (binding enthalpies of +8.0 and −7.6 kcal/mol, respectively); however, both inhibitors show the same low susceptibility to the multiple-drug-resistant mutation (K_d ratios of 19 and 23, respectively).

Characteristics of Inhibitors with Low Susceptibility to Multiple-Drug-Resistant Mutation. Overall the set of six mutations L10I, M46I, I54V, V82A, I84V, and L90M in MDR-HM lowers the binding affinity of inhibitors by up to 3 orders of magnitude; however, some inhibitors are affected more than others. Are there any features common to those inhibitors that are affected the least? Important clues can be obtained by analyzing the way they bind to the wild-type enzyme. At the thermodynamic level this analysis requires the dissection of the binding energetics into its enthalpic and entropic components.

Not all inhibitors bind to the protease with the same thermodynamic signature. While in principle many combinations of enthalpy and entropy values can give rise to the same Gibbs energy of binding ($\Delta G = \Delta H - T\Delta S$), experimentally there is a clear correlation between binding affinity and binding enthalpy. Figure 6a summarizes the thermodynamic data at 25 °C for all the HIV-1 protease inhibitors that have been measured calorimetrically and the data reported in the literature. As illustrated in the figure, all inhibitors with affinities better than 0.3 nM are characterized by favorable binding enthalpies. There is not a single reported case of an entropically driven HIV-1 protease inhibitor with an affinity better than 0.3 nM.

Is there any correlation between binding affinity and susceptibility to MDR-HM? The data do not support any correlation. Some high-affinity inhibitors (e.g., KNI-272 or lopinavir) are highly susceptible, while others (amprenavir, KNI-764) are not. Acetyl pepstatin, a low-affinity inhibitor, is not very susceptible to MDR-HM. Inhibitors derived from the same chemical scaffold (KNI-764 and KNI-272) do not necessarily share similar susceptibilities even though they share a common binding mode (*21*, *29*). The same lack of correlation is observed between susceptibility to MDR-HM and binding enthalpy. Acetyl pepstatin and KNI-764 exhibit similar susceptibilities to MDR-HM but are characterized by binding enthalpies of opposite sign.

The only correlation that we have been able to find is between susceptibility and the relative enthalpic or entropic contribution to the Gibbs energy of binding. If the loss in binding affinity for each inhibitor (log(K_d ratio)) is plotted as a function of the proportion in which the enthalpy change contributes to the overall Gibbs energy of binding ($\Delta H/\Delta G$), an intriguing pattern with two distinguishable regimes is observed. As shown in Figure 6b, enthalpically favorable and enthalpically unfavorable inhibitors appear to display opposite trends. The solid lines in the figure are the linear

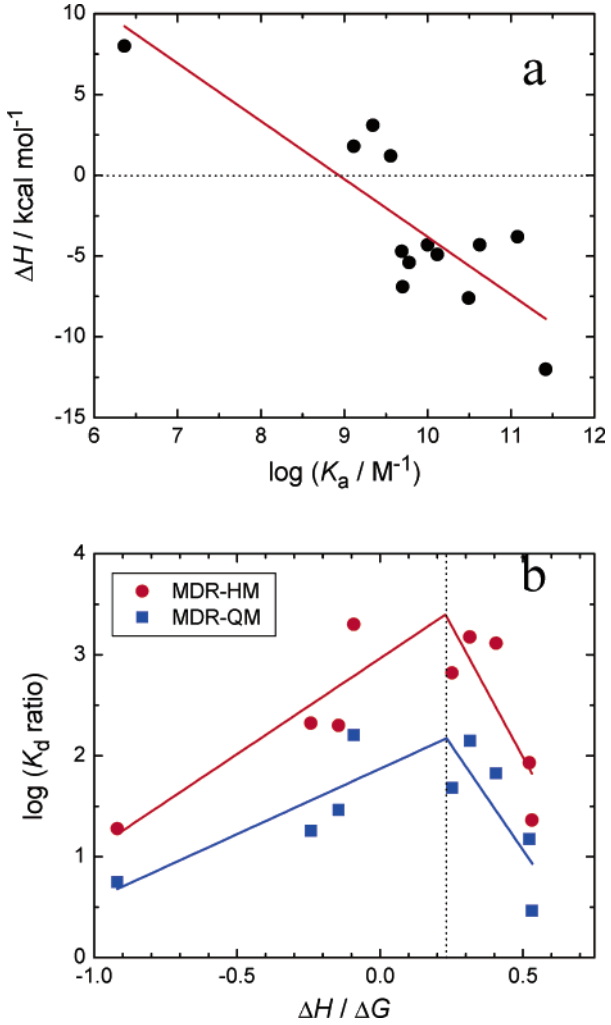

FIGURE 6: (a) Correlation between binding affinity and binding enthalpy for all inhibitors for which binding thermodynamics have been reported. In addition to the data reported in this paper, the figure also contains data for TMC-126 (*14*) and KNI-10050 and KNI-10055 (unpublished data from this laboratory). (b) Loss in binding affinity expressed as $\log(K_d \text{ ratio}) \equiv \log(K_{d,mutant}/K_{d,wild\ type})$ as a function of the proportion in which the enthalpy change contributes to the Gibbs energy ($\Delta H/\Delta G$) for the wild-type protease. Since ΔG is always negative, a positive value reflects a favorable enthalpy contribution and a negative value an unfavorable enthalpy contribution. The solid lines represent linear least-squares fits to the data for entropically driven ($\Delta H/\Delta G < 0$) and enthalpically favorable ($\Delta H/\Delta G > 0$) inhibitors.

least-squares fits to the data for entropic inhibitors ($\Delta H/\Delta G < 0$) and for enthalpically favorable inhibitors ($\Delta H/\Delta G > 0$). It is clear that the lowest susceptibilities to MDR-HM and MDR-QM are expected when the contributions to the Gibbs energy are either predominantly enthalpic or predominantly entropic. Inhibitors in which the enthalpy change contributes about 25% of the total Gibbs energy behave the worst.

CONCLUSIONS

The multiple-drug-resistant protease studied in this work includes a set of mutations that might directly affect binding interactions, those in the binding cavity and flaps, and a set that only indirectly affects binding, those at the dimerization interface. Mutations within the binding site cavity are expectedly conservative since the enzyme needs to maintain a reasonable affinity toward the substrate and a viable catalytic activity. These mutations introduce geometric distortions rather than chemical changes within the binding cavity. Mutations at the dimerization interface most likely have a similar effect either by sterically altering the bound conformation or by altering the location of the minimum energy within the subensemble of conformations accessible to the bound protease.

Since enthalpic and entropic contributions to the Gibbs energy reflect different types of interactions, inhibitors that are either predominantly enthalpic or predominantly entropic exhibit different binding modes. Enthalpic contributions reflect the strength of the inhibitor interactions with the protein (H-bonds, van der Waals interactions) relative to those with the solvent (water). This property does not require a higher polarity of the inhibitor molecule but a proper placement of H-bond donor and acceptor groups. For example, indinavir, KNI-764, nelfinavir, ritonavir, saquinavir, and lopinavir all have four H-bond donor groups; however, they bind with enthalpies ranging from −7.6 to +3.1 kcal/mol. Entropic contributions to the binding affinity, on the other hand, are mainly due to a large increase in solvent entropy arising from the burial of hydrophobic groups upon binding, combined with a minimal loss of conformational degrees of freedom in the inhibitor molecule due to the introduction of conformational constraints during the design process. Previously, we concluded that the introduction of nonconstrained asymmetric functional groups in regions facing mutation-prone areas of the protein provides adaptability to the inhibitor and a lower susceptibility to mutations (*8*, *14*, *16*, *30*). This strategy, however, requires a highly enthalpic inhibitor for both affinity and specificity, since the presence of nonconstrained groups will lower the entropic contribution to the binding energy (*8*, *14*, *16*, *30*). This idea is consistent with the data presented here. The novel idea revealed by the present data is that a purely entropic inhibitor, e.g., an inhibitor that does not establish any hydrogen bond and that binds to the target only through nonspecific hydrophobic contributions, could also be expected to have a low susceptibility to mutations that cause drug resistance. Even though this is an intriguing conjecture, its practical implementation appears uncertain since all known high-affinity inhibitors bind with favorable binding enthalpies. For enthalpically favorable inhibitors, it appears that the lowest susceptibility to mutations is achieved when the enthalpy contribution to the Gibbs energy is maximal. This conclusion is supported by the published data for TMC-126, the most enthalpically favorable inhibitor for which data are available (*14*, *31*). TMC-126 is characterized by a $\Delta H/\Delta G$ ratio of 0.53 and exhibits the lowest reported susceptibility to the active site mutation V82F/I84V (*14*). Yoshimura et al. (*31*) studied TMC-126 against a panel of eight multiple-drug-resistant mutants isolated from inhibitor-experienced patients. These mutants included up to 14 different mutations, some occurring at locations similar to those of the mutations studied here. TMC-126 was the best performing inhibitor when compared to ritonavir, indinavir, saquinavir, nelfinavir, and amprenavir, and was shown to never lose more than 8-fold potency against any of these multiple-drug-resistant mutations.

ACKNOWLEDGMENT

We thank Prof. Yoshiaki Kiso (Kyoto Pharmaceutical University, Kyoto, Japan) for providing samples of KNI-764 and KNI-272.

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BI0350405