Amplification of the Effects of Drug Resistance Mutations by Background Polymorphisms in HIV-1 Protease from African Subtypes[†]

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ABSTRACT: The vast majority of HIV-1 infections worldwide are caused by the C and A viral subtypes rather than the B subtype prevalent in the United States and Western Europe. Genomic differences between subtypes give rise to sequence variations in the encoded proteins, including those identified as targets for antiretroviral therapies. In the case of the HIV-1 protease, we reported earlier [Velazquez-Campoy et al. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98,* 6062−6067] that proteases from the C and A subtypes exhibit a higher biochemical fitness in the presence of widely prescribed protease inhibitors. In this paper we present a complete thermodynamic dissection of the differences between proteases from different subtypes and the effects of the V82F/I84V drug-resistant mutation within the framework of the B, C, and A subtypes. These studies involved four inhibitors in clinical use (indinavir, saquinavir, ritonavir, and nelfinavir) and a second-generation protease inhibitor (KNI-764). Naturally occurring amino acid polymorphisms found in proteases from the C and A subtypes lower the binding affinities of existing clinical inhibitors by factors ranging between 2 and 7.5 which by themselves are not enough to cause drug resistance. The preexisting lower affinity in the C and A subtypes, however, significantly amplifies the effects of the drug-resistant mutation. Relative to the wild-type B subtype protease, the V82F/I84V drug-resistant mutation within the C and A subtypes lowers the binding affinity of inhibitors by factors ranging between 40 and 3000. When the enzyme kinetic properties (k_{cat} and K_m) are included in the analysis, the biochemical fitness of the C and A subtype drug-resistant mutants can be up to 1000-fold higher than that of the wild-type B subtype protease in the presence of the studied inhibitors. From a thermodynamic standpoint, the combined effects of the drug-resistant mutations and the natural amino acid polymorphisms on the Gibbs energy are additive and involve significant alterations in the enthalpy and entropy changes associated with inhibitor binding. At the biochemical level, the combined effects of naturally existing polymorphisms and drug-resistant mutations might have important consequences on the long-term viability of current HIV-1 protease inhibitors.

The AIDS epidemic in Africa has achieved dramatic proportions (see ref *1* for a recent review). Of the 40 million people infected with HIV worldwide, 28 million are in Africa. While the adult prevalence rate in North America and Western Europe is around 0.5%, the overall figure reaches 8.4% in sub-Saharan Africa. However, the HIV-1 subtypes prevalent in Africa are not the same as those that are prevalent in North America and Western Europe. In North America and Western Europe the B subtype is responsible for the vast majority of HIV infections whereas in sub-Saharan Africa the A and C subtypes account for most of the infections. The A subtype predominates in the northern part of sub-Saharan Africa and the C subtype in southern Africa. Since protease inhibitors have been developed and tested against the HIV-1 B subtype, and proteases from other HIV-1 subtypes carry amino acid polymorphisms, some of which have been associated with drug resistance (*2*), two important questions need to be addressed at the biochemical level: (1) Are existing drugs equally effective against proteases from different HIV-1 subtypes? (2) How do drug-resistant mutations operate within the framework of proteases from non-B subtypes?

In a previous communication (*3*), we reported enzyme inhibition measurements and showed that naturally occurring polymorphisms in HIV-1 subtypes A and C lower the inhibitory ability (increase the inhibition constant, K_i) of protease inhibitors in clinical use by a factor of 2−7 (*3*). The effect of these polymorphisms is below the level required to trigger drug resistance, since the K_i's of these inhibitors still remain in the nanomolar level. These biochemical findings are in agreement with initial clinical reports describing the effectiveness of protease inhibitors in highly active antiretroviral therapies (HAART) in African patients (*4*). However, some naturally occurring amino acid polymorphisms in A and C HIV-1 subtype proteases are considered secondary resistant mutations in B subtype (i.e., M36I) (*2*) and may act at a later stage in the viral infection, following the onset of primary resistant mutations. A recent analysis of 248 drug-naïve HIV-1 patients in Italy suggests that the presence of M36I may be associated with an increased rate of failure in protease inhibitor therapies (*5*). Until today,

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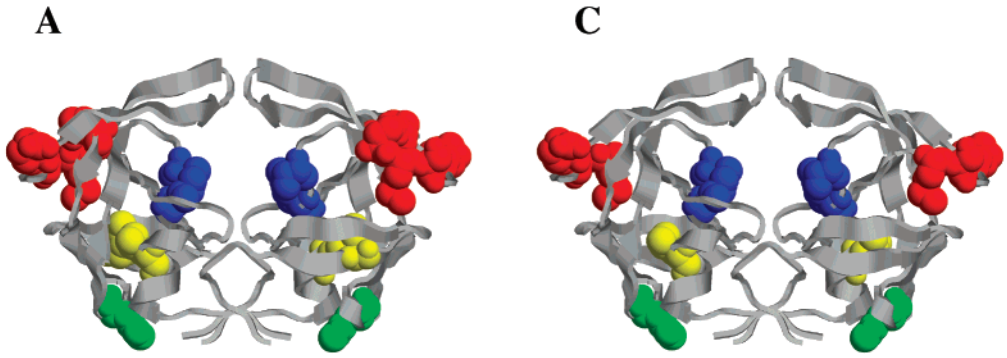

FIGURE 1: Structure of the HIV-1 protease used in these studies, illustrating the positions at which protease subtypes A (left) and C (right) differ from protease subtype B. All of the amino acid polymorphisms are located outside of the binding site: (1) in the hinge of the flaps (E35D/M36I/S37N/R41K/R57K, shown in red), (2) in the loop connecting β-strands (H69K, shown in green), and (3) in the α-helix and the opposite β-strand (I13V/L89M, shown in yellow). Eight polymorphisms are present in protease subtype A, whereas protease subtype C contains only a subset (M36I/S37N/R41K/H69K/L89M) found in some subtype C and recombinant HIV-1 isolates. The figure also shows the drug-resistant double mutation (V82F/I84V, shown in blue).

however, very little is known regarding the long-term effectiveness of existing antiretroviral drugs and the impact of inhibitor-resistant mutations in non-B subtype HIV-1.

The double mutation V82F/I84V is a widespread primary resistant mutation reported in clinical isolates and is known to confer cross-resistance to all the inhibitors currently in clinical use (*6*−*8*). To investigate the response of A and C subtype proteases to resistant mutations and evaluate any differences with the B subtype, we decided to incorporate the V82F/I84V mutation within the framework of the three different HIV-1 subtype proteases and perform a complete thermodynamic characterization of the binding energetics of inhibitors to the wild types and the V82F/I84V drug-resistant mutations of the three proteases. In these studies, we considered four inhibitors in clinical use (indinavir, saquinavir, ritonavir, and nelfinavir) and a second-generation protease inhibitor (KNI-764) with a reported low susceptibility to the effects of protease mutations (*9*) and currently under preclinical development.

Naturally occurring amino acid polymorphisms in the A and C HIV-1 subtype proteases occur outside the active site (Figure 1). They are clustered in the hinge region of the flap (red), in the loop connecting the β-strands (green), and in the α-helix and opposite β-strand (yellow). The double resistant mutation V82F/I84V (blue) is an active site resistant mutation and acts by distorting the binding site geometry without altering its chemical nature or polarity (*10*). The A and C HIV-1 subtype proteases used in these studies carry eight and five mutations that have been incorporated within the framework of the B subtype protease [I13V/E35D/M36I/S37N/R41K/R57K/H69K/L89M for the A subtype and M36I/S37N/R41K/H69K/L89M for the C subtype) (*10*−*12*) (due to a typographical error S37N was not listed in ref *3*)]. The sequence of the A subtype protease was derived from the consensus sequence of subtype A protease from 14 antiretroviral naïve Ugandan adults (*2*, *13*) and is identical to the sequences of two Ugandan isolates (no. 225.706 and no. 230.706). The C subtype protease is based on a consensus sequence [92RW026 (Rwanda, GenBank Accession Number AF009410), C2220 (Ethiopia, GenBank Accession Number U461016), Z1226 (Zimbabwe, GenBank Accession Number AF083603), 96BW01 (Botswana, GenBank Accession Number AF110959), and C11 (Zambia, GenBank Accession Number AF107378)]. The results of these studies indicate that the combined effects of naturally occurring amino acid polymorphisms and drug-resistant mutations might have significant consequences on the viability of current protease inhibition therapies.

MATERIALS AND METHODS

Protease Preparation. HIV-1 proteases containing the mutations I13V/E35D/M36I/S37N/R41K/R57K/H69K/L89M for the A subtype and M36I/S37N/R41K/H69K/L89M for the C subtype were generated from the B subtype protease (*10*−*12*) by PCR mutagenesis. The gene encoding the B subtype HIV-1 protease (with one autolytic site protected, Q7K) was transferred to the pET24 vector (Novagen), where the expression is under control of the T7 promoter. Mutations at the positions indicated above were introduced by using an in vitro site-directed mutagenesis kit (Stratagene), and mutations were confirmed by DNA sequencing. Proteases were expressed in BL21/DE3 cells by addition of isopropyl β-D-thiogalactoside (IPTG) to 1 mM once the culture density (as determined by absorbance at 600 nm) was 1.5 or greater. The drug-resistant mutations V82F/I84V were introduced into each subtype protease by using an in vitro site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. The expression and purification procedure was the same for wild-type and mutant proteases.

Protease Purification. Plasmid-encoded HIV-1 protease was expressed as inclusion bodies in *Escherichia coli* 1458 (*10*−*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 ($\geq$16000 psi). Cell debris and protease-containing inclusion bodies were collected by centrifugation (20000*g* for 20 min at 4 °C). Inclusion bodies were washed with three buffers. Each wash consisted of resuspension (glass homogenizer, sonication) and centrifugation (20000*g* for 20 min at 4 °C). In each step a different washing buffer was employed: 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), and 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, 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 then 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 10-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, pH 5.0, was added up to 100 mM, and the protein was concentrated. Folded protease was desalted into 1 mM sodium acetate 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, the protease was estimated to be ≥99% pure.

Clinical Inhibitor Purification. Clinical inhibitors, indinavir, saquinavir, ritonavir and nelfinavir, were purified from commercial capsules by HPLC (Waters, Inc.) using a semipreparative C-18 reversed-phase column developed with 0−100% acetonitrile in 0.05% TFA. Purified inhibitors were lyophilized and stored at −20 °C in the crystalline form (indinavir, nelfinavir) or as suspensions in DMSO (saquinavir, ritonavir). Acetylpepstatin (Bachem AG) and KNI-764 (gift from Dr. Kiso, Department of Medicinal Chemistry, Kyoto Pharmaceutical University, Kyoto, Japan) were used without further purification.

Determination of Kinetic Parameters. The catalytic activity of the HIV-1 proteases was monitored following the hydrolysis of the chromogenic substrate Lys-Ala-Arg-Val-Nle-nPhe-Glu-Ala-Nle-NH_2 (California Peptide Research Inc.). This substrate resembles the cleavage site ARVL/AEAM between the capsid protein and p2 in the *gag* protein precursor. This cleavage site is one of the most conserved between and within subtypes (>95%) according to the HIV Sequence Database. 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, and 1 M NaCl (pH 5.0). The absorbance was monitored at five wavelengths (296−304 nm) using a HP 8452 diode array spectrophotometer (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 absorbance change to reaction rates. Hydrolysis rates were obtained from the initial portion of the data, where at least 80% of the substrate remains unhydrolyzed.

Isothermal Titration Calorimetry. Isothermal titration calorimetry experiments were carried out using a high-precision VP-ITC titration calorimetric system (Microcal Inc.). The enzyme solution in the calorimetric cell was titrated with inhibitor solutions dissolved in the same buffer (10 mM sodium acetate, pH 5, 2% DMSO). In displacement titration experiments, inhibitors were injected into the calorimetric cell containing the protein prebound to a weak inhibitor (acetylpepstatin) as described before (*14*). The concentration of acetylpepstatin in the titration cell was 200 μM. Protein and inhibitor solutions were properly degassed and carefully loaded into the cells to avoid bubble formation during stirring. Exhaustive cleaning of the cells was undertaken before each experiment. The heat evolved after each ligand injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction between the inhibitor and the enzyme was obtained as the difference between the heat of reaction and the corresponding heat of dilution.

Table 1: Kinetic Parameters for HIV-1 Proteases Considered in This Paper

	subtype	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
wild type	B	8.9 ± 0.2	14 ± 1	0.64 ± 0.06
	C	7.7 ± 0.2	5.4 ± 0.4	1.4 ± 0.1
	A	7.8 ± 0.1	20 ± 2	0.39 ± 0.05
V82F/I84V	B	6.4 ± 0.1	28 ± 2	0.23 ± 0.02
	C	6.3 ± 0.1	15.9 ± 1	0.40 ± 0.03
	A	6.1 ± 0.1	31 ± 2	0.20 ± 0.02

RESULTS AND DISCUSSION

Enzymatic Characterization of HIV-1 Proteases. The catalytic rate constant, k_{cat}, and the Michaelis constant, K_m, were determined for the six protease molecules considered in this work in order to evaluate their intrinsic catalytic efficiency. Kinetic assays were performed using the chromogenic substrate Lys-Ala-Arg-Val-Nle-nPhe-Glu-Ala-Nle-NH_2 as described in Materials and Methods. Active site titrations were performed in order to determine the active protease concentration for an accurate estimation of k_{cat}. The kinetics parameters are summarized in Table 1. All of these proteases, wild types and resistant mutants, do not show significant variations in their catalytic activity, which ranges between 6.1 and 8.9 s^{-1} for all of them under the experimental conditions of these studies. The K_m's, on the other hand, vary between 5.4 and 31 μM for the chromogenic substrate used in these studies with lower K_m's observed for the wild-type proteases. Overall, the catalytic efficiencies range between 0.2 and 1.4 s^{-1} μM^{-1}. In general, the wild-type B, C, and A proteases have a catalytic efficiency 2.8, 3.5, and 2.0 times better than that of their corresponding V82F/I84V drug-resistant mutants.

Energetics of Inhibitor Binding to HIV-1 Wild-Type Subtype Proteases. Isothermal titration calorimetry experiments were performed in order to determine the binding affinity and the binding enthalpy of different inhibitors to the HIV-1 subtype A, B, and C proteases. The four inhibitors in clinical use (indinavir, ritonavir, nelfinavir, and saquinavir) and the second-generation inhibitor, KNI-764, are tight-binding inhibitors ($K_d \leq$ 1nM) of the HIV-1 protease, and therefore, standard titration experiments do not provide accurate estimates of the binding affinity, even though the binding enthalpy can be determined with high accuracy. To overcome this difficulty, calorimetric displacement titrations were carried out, allowing for the determination of the affinity and enthalpy of binding, as described before (*14*, *15*).

In calorimetric displacement titrations, the high-affinity inhibitor is titrated into a protease sample prebound to a weaker inhibitor. Under these conditions, the apparent binding affinity of the tight inhibitor diminishes in a manner proportional to the affinity and concentration of the weak inhibitor and becomes calorimetrically measurable. Two

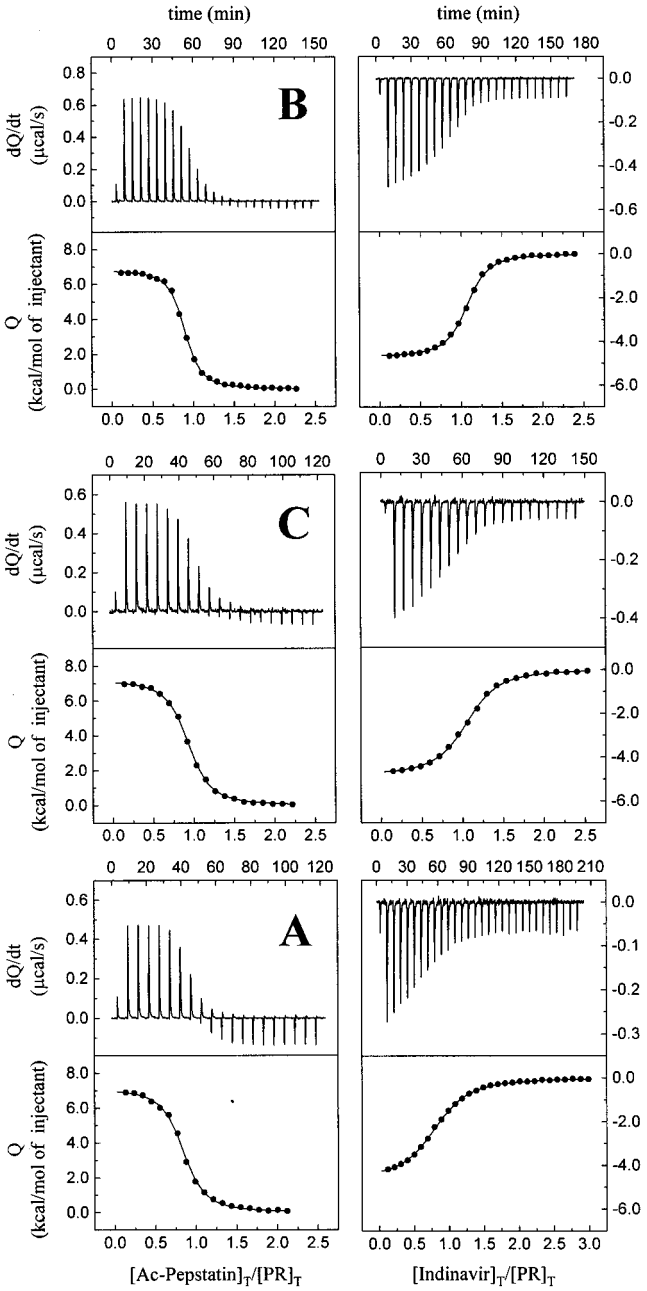

FIGURE 2: Typical set of ITC displacement experiments. Titrations of acetylpepstatin (300 μM) into a protease solution (20 μM) (left panels) and titrations of indinavir (290 μM) into a solution of protease (20 μM) prebound to acetylpepstatin (200 μM) (right panels) were performed. Experiments were done with protease subtype B (top), subtype C (center), and subtype A (bottom). The experiments were performed in 10 mM sodium acetate buffer, pH 5.0, and DMSO (2%) at 25 °C. Data were analyzed as explained elsewhere (*14*). The presence of acetylpepstatin in the calorimetric cell reduces the apparent affinity of indinavir by a factor of 740, 500, and 450 in the case of protease subtypes B, C, and A, respectively.

calorimetric titrations are required to solve the binding competition equations and determine the binding affinity and binding enthalpy of the tight binding inhibitor: the titration of the weak inhibitor into the protease and the titration of the high-affinity inhibitor into the protease prebound to the weak inhibitor (*14*, *15*). Figure 2 shows typical calorimetric displacement titrations for indinavir into wild-type B, C, and A HIV-1 proteases. The left panels show the titration with the low-affinity inhibitor acetylpepstatin and the right panels

Table 2: Thermodynamic Parameters for Binding of Inhibitors to HIV-1 Protease Wild-Type Subtypes B, C, and A[a]

	HIV-1 PR B	HIV-1 PR C	HIV-1 PR A
K_d (nM)			
indinavir	0.48 ± 0.01	1.41 ± 0.05	3.2 ± 0.1
saquinavir	0.40 ± 0.01	0.77 ± 0.02	1.11 ± 0.04
nelfinavir	0.26 ± 0.005	0.59 ± 0.02	0.71 ± 0.02
ritonavir	0.0293 ± 0.001	0.160 ± 0.005	0.222 ± 0.005
KNI-764	0.0110 ± 0.0005	0.0122 ± 0.0005	0.0161 ± 0.0006
ΔG (cal/mol)			
indinavir	−12700	−12100	−11600
saquinavir	−12800	−12400	−12200
nelfinavir	−13100	−12600	−12500
ritonavir	−14400	−13400	−13200
KNI-764	−14900	−14900	−14700
ΔH (cal/mol)			
indinavir	2100	2400	2600
saquinavir	1900	2100	2200
nelfinavir	2600	2900	3000
ritonavir	−3700	−3100	−2900
KNI-764	−8000	−7500	−7400
$-T\Delta S$ (cal/mol)			
indinavir	−14800	−14500	−14200
saquinavir	−14700	−14500	−14400
nelfinavir	−15700	−15500	−15500
ritonavir	−10700	−10300	−10300
KNI-764	−6900	−7400	−7300

[a] Experiments were done in 10 mM sodium acetate and 2% DMSO, pH 5.0, at 25 °C. Errors in ΔH are estimated to range between 100 and 200 cal/mol.

the titrations of indinavir into the HIV-1 proteases prebound to acetylpepstatin. The experiments shown in the left panels were used to determine the binding affinities and binding enthalpies of acetylpepstatin to each of the proteases. The K_d values were 244, 360, and 400 nM, and the binding enthalpies 6.8, 7.2, and 7.2 kcal/mol for the B, C, and A proteases, respectively. Similar experiments were performed with the remaining four inhibitors and the results analyzed as described before (*14*). The thermodynamic data obtained from the experiments with the three wild-type proteases and the five inhibitors are summarized in Table 2. In agreement with inhibition studies reported earlier (*3*), the affinity of the clinical inhibitors is reduced by a factor of 2−7.5 when confronted with the A and C proteases. By itself, this decrease in binding affinity is not enough to elicit drug resistance and suggests that, from a biochemical standpoint, existing protease inhibitors should perform reasonably well against the wild-type A and C proteases. Notably, the binding affinity of the second-generation inhibitor is only reduced by a factor of 1.5 and 1.1 for the A and C proteases, consistent with a better capacity to adapt to the presence of amino acid polymorphisms.

Figure 3 presents the thermodynamic dissection of the effects of naturally occurring subtype polymorphisms on the binding affinity of inhibitors. Indinavir and ritonavir experience the highest loss in Gibbs energy, especially against the A subtype protease. For all inhibitors, the loss of binding energy is due to a combination of enthalpic and entropic losses. The loss is roughly similar for the enthalpy and entropy changes against the C subtype protease. Against the A subtype protease and, to a lesser extent, against the C subtype protease, nelfinavir and ritonavir lose twice as much enthalpy than entropy contributions to the binding affinity. The second-generation inhibitor KNI-764, on the other hand, loses only 0.07 and 0.24 kcal/mol against the C and A subtype proteases. Interestingly, its enthalpy loss is of the

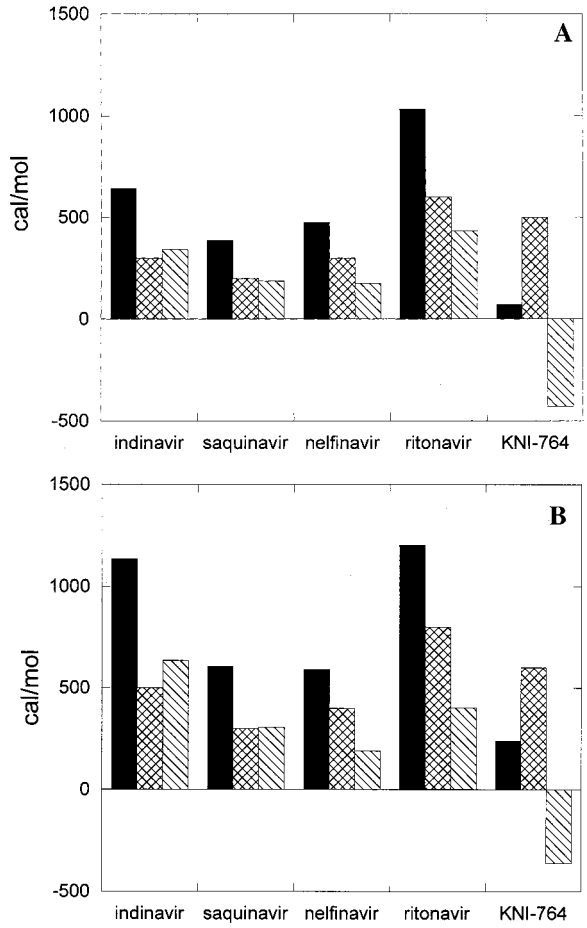

FIGURE 3: Panel A shows the relative changes (considering the wild-type B subtype protease as reference) in the Gibbs energy, $\Delta\Delta G$ (solid bars), the enthalpy, $\Delta\Delta H$ (crosshatched bars), and the entropy, $-T\Delta\Delta S$ (hatched bars) ($\Delta\Delta X = \Delta X_{C,wt} - \Delta X_{B,wt}$), associated with the binding of different inhibitors to the C subtype wild-type protease. Panel B presents the same analysis for the A subtype wild-type protease ($\Delta\Delta X = \Delta X_{A,wt} - \Delta X_{B,wt}$).

same order and even higher than that of indinavir, saquinavir, and nelfinavir; however, this inhibitor is able to compensate the enthalpy loss by an actual gain in entropic contributions to the binding energetics. This type of entropic compensation of binding enthalpy losses appears to be a common characteristic of second-generation inhibitors when confronted with inhibitor-resistant mutations and other amino acid polymorphisms (*14*, *16*). The entropy gain is due to an increase in the number of degrees of freedom in the system, resulting either from an augmented conformational dynamics within the complex or to a larger number of water molecules released from the complex upon binding. In both cases, the observation can be traced back to a higher flexibility or to the presence of asymmetric functional groups in the inhibitor molecules that exhibit this behavior (*16*, *17*). Mutations or amino acid polymorphisms usually cause direct or indirect alterations in the binding site that result in a loss of van der Waals contacts, hydrogen bonds, or other inhibitor/protein interactions. The loss of these interactions is immediately reflected in a loss of binding enthalpy and, in the case of conformationally constrained inhibitors, in a potential increase in the solvent exposure of previously buried atoms and a loss of entropic contributions to the binding affinity. Inhibitor molecules that exhibit conformational flexibility in the regions facing the enthalpy losses are able to search for a new energy minimum. Asymmetric functional groups in these regions allow the inhibitor to present different interacting surfaces against the variable regions of the protease. Even if the inhibitor is not able to establish new energetically favorable contacts with the protein, it may bury additional atoms from the solvent or gain degrees of freedom and compensate the loss of enthalpic interactions with an entropy gain.

Energetics of Inhibitor Binding to HIV-1-Resistant Mutant Protease V82F/I84V. By themselves, the amino acid polymorphisms found in the proteases from the C and A HIV-1 subtypes do not appear to be sufficient to induce drug resistance. For the inhibitors in current clinical use, the onset of drug resistance requires a drop in binding affinity of at least 1 order of magnitude. The highest drops that we have measured are about 7-fold for ritonavir and indinavir against the A subtype. However, it is within this already weaker binding background that drug-resistant mutations take place. Until now, it is not known if the effects of naturally occurring amino acid polymorphisms and drug-resistant mutations are additive or show some degree of positive or negative synergism. To answer this question, we incorporated a well-characterized drug-resistant mutation within the framework of the three proteases. The double mutation V82F/I84V was chosen because it has been reported in clinical isolates and is known to confer cross-resistance to all the inhibitors currently in clinical use (*6*−*8*). It is classified as an active site resistant mutation (Figure 1), since it is located within the binding site. It acts by distorting the binding site geometry without altering its chemical nature or polarity. The V82F/I84V drug-resistant mutations were incorporated into the B, C, and A HIV-1 subtype proteases, and the binding thermodynamics of protease inhibitors was measured by high-sensitivity isothermal titration calorimetry.

The inhibitor-resistant mutation V82F/I84V causes a drop in the binding affinity of all the inhibitors tested in these studies. For the inhibitors currently in clinical use, the drop is sufficient to allow measurement of their binding thermodynamics by direct calorimetric titrations. As an example, Figure 4 shows the calorimetric titrations corresponding to the binding of indinavir to the V82F/I84V drug-resistant mutant of the C subtype protease and saquinavir to the V82F/I84V drug-resistant mutant of the B subtype protease. The thermodynamic results for all inhibitors and drug-resistant proteases are summarized in Table 3.

When the binding Gibbs energies corresponding to each mutant subtype are compared with their own wild type, it is evident that the effect of the V82F/I84V mutation is the same irrespective of the preexisting naturally occurring polymorphism (Table 4). For any given subtype, each inhibitor loses the same binding energy as a result of the V82F/I84V mutation, indicating that the effects of this resistant mutation are additive, i.e., independent of the existing background. While the overall effect in ΔG is the same, the effects of the mutation on individual enthalpic and entropic contributions are not the same, indicating the presence of enthalpy/entropy compensation. For example, indinavir loses 2.5 kcal/mol in binding Gibbs energy. Against the B subtype it is the result of a loss of 1.4 kcal/mol in binding enthalpy and 1.1 kcal/mol in the entropic contribution. Against the A subtype, on the other hand, the loss in binding enthalpy is only 0.3 kcal/mol, but the loss in entropic contributions

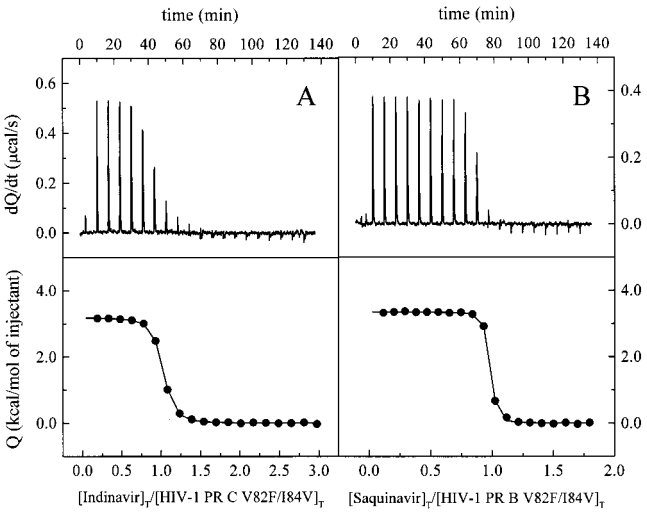

FIGURE 4: (Left panel) Titration corresponding to indinavir binding to HIV-1 C V82F/I84V and (right panel) titration corresponding to saquinavir binding to HIV-1 B V82F/I84V. The concentrations employed were 23 μM for the protease and 480 μM for indinavir or 290 μM for saquinavir. The experiments were performed in 10 mM sodium acetate buffer, pH 5.0, and DMSO (2%) at 25 °C.

Table 3: Thermodynamic Parameters for Binding of Inhibitors to HIV-1 Protease Double Mutant V82F/I84V Subtypes B, C, and A[a]

	HIV-1 PR B	HIV-1 PR C	HIV-1 PR A
K_d (nM)			
indinavir	32 ± 1	100 ± 3	192 ± 4
saquinavir	8.3 ± 0.2	16.1 ± 0.4	24 ± 1
nelfinavir	5.3 ± 0.1	12.0 ± 0.2	16.1 ± 0.3
ritonavir	11.2 ± 0.05	62 ± 2	83 ± 2
KNI-764	0.28 ± 0.01	0.33 ± 0.01	0.50 ± 0.02
ΔG (cal/mol)			
indinavir	−10200	−9500	−9200
saquinavir	−11000	−10600	−10400
nelfinavir	−11300	−10800	−10600
ritonavir	−10900	−9800	−9700
KNI-764	−13000	−12900	−12700
ΔH (cal/mol)			
indinavir	3500	3100	2900
saquinavir	3300	3000	2900
nelfinavir	4400	4200	4100
ritonavir	−700	−1100	−1200
KNI-764	−5800	−6000	−6000
$-T\Delta S$ (cal/mol)			
indinavir	−13700	−12600	−12100
saquinavir	−14300	−13600	−13300
nelfinavir	−15700	−15000	−14700
ritonavir	−10200	−8700	−8500
KNI-764	−7200	−6900	−6700

[a] Experiments were done in 10 mM sodium acetate and 2% DMSO, pH 5.0, at 25 °C. Errors in ΔH are estimated to range between 100 and 200 cal/mol.

amounts to 2.1 kcal/mol. In general for all inhibitors, the losses in enthalpic contributions are smaller for the C and A subtypes than for the B subtype. Conversely, the losses in entropic contributions to binding are larger in the C and A subtypes. The origin of this effect could be related to existing differences in structural stability between the B, C, and A proteases (*3*). The protease undergoes a conformational change upon binding, and the energetics of this change, which is part of the observed binding energy, might have different enthalpy/entropy components. The conformational change primarily involves the closing of the flaps on top of the inhibitor molecule (*12*, *18*). Notably, the C and A

Table 4: Relative Changes in Thermodynamic Parameters for Binding of Inhibitors to HIV-1 Protease Double Mutant V82F/I84V Subtypes B, C, and A, Considering HIV-1 Protease Wild-Type Subtypes B, C, and A as Reference, Respectively[a]

	HIV-1 PR B	HIV-1 PR C	HIV-1 PR A
K_d ratio			
indinavir	68	70	60
saquinavir	21	21	22
nelfinavir	20	20	22
ritonavir	368	381	383
KNI-764	25	27	30
$\Delta\Delta G$ (cal/mol)			
indinavir	2500	2500	2400
saquinavir	1800	1800	1800
nelfinavir	1800	1800	1800
ritonavir	3500	3500	3500
KNI-764	1900	1900	2000
$\Delta\Delta H$ (cal/mol)			
indinavir	1400	700	300
saquinavir	1400	900	700
nelfinavir	1800	1300	1100
ritonavir	3000	2000	1700
KNI-764	2200	1500	1400
$-T\Delta\Delta S$ (cal/mol)			
indinavir	1100	1800	2100
saquinavir	400	900	1100
nelfinavir	0	500	700
ritonavir	500	1500	1800
KNI-764	−300	400	600

[a] Experiments were done in 10 mM sodium acetate and 2% DMSO, pH 5.0, at 25 °C.

subtypes contain polymorphisms in the hinge region of the flaps (Figure 1) which may alter the structural and energetic details of this change.

Implications for Drug Resistance. The calorimetric experiments indicate that the changes in binding affinity induced by the V82F/I84V mutation are similar, independently of the subtype background. From the point of view of the effectiveness of protease inhibitors, a most appropriate comparison is against the wild-type B subtype protease as summarized in Table 5. Inhibitors were developed against the wild-type B subtype, and it is against this standard that the effects of mutations are compared. Within the B subtype, the V82F/I84V mutation lowers the binding affinity by a factor of ∼20 for saquinavir and nelfinavir, 68 for indinavir, and 368 for ritonavir. In the C and A subtypes, these numbers are amplified by the preexisting lower affinity observed with these proteases. For the C subtype protease, the V82F/I84V mutation lowers the binding affinity by a factor of ∼40 for saquinavir and nelfinavir, 210 for indinavir, and 2188 for ritonavir. For the A subtype protease, the V82F/I84V mutation lowers the binding affinity by a factor of ∼60 for saquinavir and nelfinavir, 400 for indinavir, and 2900 for ritonavir.

To compare the selective advantage of different protease mutations in the presence of specific inhibitors, Gulnik et al. (*19*, *20*) introduced an empirical parameter, called vitality, which provides a measurement of the biochemical fitness of a specific mutation in the presence of a given inhibitor. Since different inhibitors have different binding affinities, a similar drop in binding affinity does not have the same effect in the vitality of the virus. For example, a drop of 10 in the affinity of a picomolar inhibitor is not the same as the same drop in a nanomolar affinity. From the point of view of arresting viral maturation, the first inhibitor is still effective

Table 5: Relative Changes in Thermodynamic Parameters for Binding of Inhibitors to HIV-1 Protease Double Mutant V82F/I84V Subtypes B, C, and A, Considering HIV-1 Protease Wild-Type Subtype B as Reference[a]

	HIV-1 PR B	HIV-1 PR C	HIV-1 PR A
K_d ratio			
indinavir	68	210	404
saquinavir	21	41	61
nelfinavir	20	44	60
ritonavir	368	2188	2917
KNI-764	25	30	45
$\Delta\Delta G$ (cal/mol)			
indinavir	2500	3200	3600
saquinavir	1800	2200	2400
nelfinavir	1800	2200	2400
ritonavir	3500	4600	4700
KNI-764	1900	2000	2300
$\Delta\Delta H$ (cal/mol)			
indinavir	1400	1000	800
saquinavir	1400	1100	1000
nelfinavir	1800	1600	1500
ritonavir	3000	2600	2500
KNI-764	2200	2000	2000
$-T\Delta\Delta S$ (cal/mol)			
indinavir	1100	2200	2800
saquinavir	400	1100	1400
nelfinavir	0	600	900
ritonavir	500	2000	2200
KNI-764	−300	0	300

[a] Experiments were done in 10 mM sodium acetate and 2% DMSO, pH 5.0, at 25 °C.

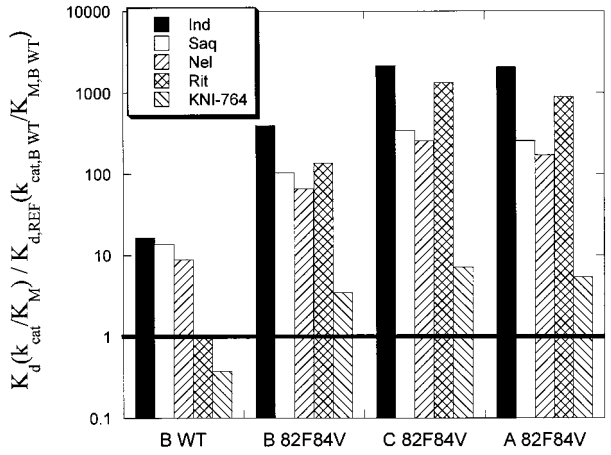

FIGURE 5: Relative vitality (as defined in the text) for B, C, and A subtype V82F/I84V drug-resistant mutant proteases in the presence of indinavir, saquinavir, nelfinavir, ritonavir, and KNI-764. Also, B subtype wild-type protease is shown for comparison. The horizontal line marks the reference value (i.e., relative vitality for B subtype wild-type protease in the presence of ritonavir).

whereas the second one is not. A better descriptor of the biochemical fitness of the proteases is given by a modified vitality function, normalized to a reference inhibitor:

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

We chose the widely prescribed protease inhibitor ritonavir as the reference inhibitor and the wild-type B subtype protease as the reference protease. Figure 5 shows the relative vitalities for the B, C, and A subtype V82F/I84V drug-resistant mutants. In this graph the wild-type B subtype protease has a normalized vitality of 1 in the presence of ritonavir. Since ritonavir has a higher binding affinity than the remaining clinical inhibitors, the vitality of the same protease increases to ~10. On the other hand, in the presence of KNI-764 that has a higher affinity than ritonavir, the vitality drops to ~0.4. The double mutation V82F/I84V improves the relative vitality of the protease to ~100 in the presence of all clinical inhibitors. The effect is more pronounced for the C and A subtypes in which the relative vitality might approach and even exceed 1000.

CONCLUSIONS

The results presented in this paper demonstrate that, at the biochemical level, the combined effects of naturally existing polymorphisms and drug-resistant mutations might have important consequences on the viability of current HIV-1 protease inhibitors. It is apparent that the optimization of drug candidates against highly variable targets, like retroviruses, requires consideration of the most frequently observed polymorphisms in the target proteins. Amino acid polymorphism need not be in the active site to influence the binding thermodynamics of inhibitors, and they may amplify the effects of drug-resistant mutations.

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