The Contribution of Naturally Occurring Polymorphisms in Altering the Biochemical and Structural Characteristics of HIV-1 Subtype C Protease†

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ABSTRACT: Fourteen subtype B and C protease variants have been engineered in an effort to study whether the preexistent baseline polymorphisms, by themselves or in combination with drug resistance mutations, differentially alter the biochemical and structural features of the subtype C protease when compared with those of subtype B protease. The kinetic studies performed in this work showed that the preexistent polymorphisms in subtype C protease, by themselves, do not provide for a greater level of resistance. Inhibition analysis with eight clinically used protease inhibitors revealed that the natural polymorphisms found in subtype C protease, in combination with drug resistance mutations, can influence enzymatic catalytic efficiency and inhibitor resistance. Structural analyses of the subtype C protease bound to nelfinavir and indinavir showed that these inhibitors form similar interactions with the residues in the active site of subtype B and C proteases. It also revealed that the naturally occurring polymorphisms could alter the position of the outer loops of the subtype C protease, especially the 60's loop.

The genetic variability encountered in the human immunodeficiency virus (HIV) poses a major challenge for prevention and diagnostic strategies, therapy response, and vaccine development (*1*−*3*). There is extensive and growing literature on sequence data from untreated and treated persons infected with HIV-1 subtype B viruses (*1*, *4*). This plethora of information has led to increasingly accurate, though complex, interpretations of HIV-1 subtype B drug resistance (*4*−*6*). Such data are generally not available for non-B subtypes. More information needs to be gathered and analyzed in order to fully understand the important role that differences in protease (PR[1]) sequences of non-B subtypes, including subtype C, play in the interaction of enzyme with the substrates and the inhibitors.

This work investigates the role that the eight polymorphic residues have in altering the enzymatic and structural features of subtype C PR as compared to subtype B PR. These residues are located in the elbow of the flaps (M36I, and S37A), at the base of the enzyme (H69K, and I93L), and within the PR core (T12S, I15V, L19I, and L89M) (Figure 1). The aim of this study is to analyze whether these baseline polymorphisms engender a decrease in the affinity of the PR for the clinically used inhibitors, or confer an increased catalytic activity and thus possibly a greater biochemical fitness in the presence of the inhibitors.

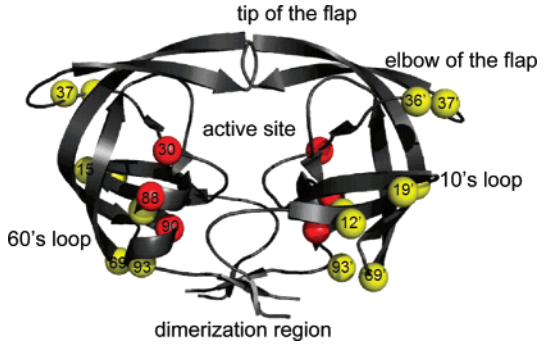

FIGURE 1: Cartoon representation of HIV-1 subtype C PR. The naturally occurring polymorphisms and the drug resistance mutations in subtype C PR, represented as yellow and red spheres, respectively, are superposed onto the crystal structure of HIV-1 subtype B PR (gray ribbon). Amino acid positions are as numbered.

Combinations of three major mutations (L90M, D30N, and N88D) that arise mainly in response to nelfinavir (NFV) treatment were engineered. NFV is a protease inhibitor (PI) preferably employed, especially in developing countries, because of relatively lower cost and fewer side effects. Also, its relatively safe biochemical and pharmacodynamic profiles make NFV the first choice to treat HIV-positive pregnant women. Recent studies have shown that the most common primary mutation observed in treated patients infected with HIV-1 subtype C was L90M (*7*), a major mutation that confers multi-PI resistance especially to saquinavir (SQV), ritonavir (RTV) and NFV (*8*−*10*). The leucines at positions 90 and 90′ are not located in the active site of the PR, but are positioned on the either side of each catalytic Asp25, and therefore catalytic activity may be more sensitive to substitutions at position 90 (*11*). In addition, this position,

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[1] Abbreviations: APV, amprenavir; ARV, antiretroviral; ATV, atazanavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; PI(s), protease inhibitor(s); PR(s), protease(s); RTV, ritonavir; SQV, saquinavir; TPV, tipranavir.

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in the core of the PR dimer, might also affect the stability of the enzyme as shown by Xie et al. The L90M mutant seems to be more sensitive to urea denaturation than other mutants. Sedimentation equilibrium studies have also shown that L90M has reduced dimer stability at pH 7.0 relative to the wild type PR (*12*).

D30N is a primary mutation that renders the HIV-1 PR less susceptible to NFV, and does not exhibit cross-resistance with other PIs (*13*). The crystal structure of the HIV-1 PR complexed with a peptide containing the wild type sequence of CA/p2 cleavage site from P5 to P5′, Lys-Ala-Arg-Val-Leu*Ala-Glu-Ala-Met-Ser, showed that the Asp30 residue side chain is involved in hydrogen bonding to the GluP2′ side chain (*14*). This is the only direct hydrogen bonding interaction seen between the enzyme and a side chain of the substrate, and therefore this mutation in the PR likely results in a weaker hydrogen bond that destabilizes NFV binding (*15*).

Mutations at position 88 (N88D and N88S) commonly occur in patients receiving NFV and occasionally in patients receiving indinavir (IDV). By itself, a mutation at this position causes low-level (less than 5-fold increase of K_i value) resistance to NFV. However, when associated with D30N or M46I, the N88D mutation causes high-level NFV resistance (*16*, *17*). These three mutations are considered major mutations against NFV, and three patterns of mutational associations were identified. First, D30N was positively associated with N88D but negatively associated with N88S. Second, D30N and L90M were negatively associated except in the presence of N88D, which facilitated the co-occurrence of D30N and L90M. Third, D30N + N88D + L90M formed a stable genetic backbone for the accumulation of additional PI resistance mutations. In 16 patients having isolates with more than one combination of mutations at positions 30, 88, and 90, all exhibited one of the steps in the following progression: D30N → D30N + N88D → D30N + N88D + L90M → D30N + N88D + L90M + (L33F ± I84V or M46I/L ± I54V) (*18*).

The goals of this work are to understand the effects of preexistent baseline polymorphisms, by themselves or in combination with drug resistance mutations, on the biochemical and structural characteristics of HIV-1 subtype C PR and to evaluate mutations/polymorphisms as a predictive measure for therapy development and effectiveness, in conjunction with published data. Kinetic studies were performed for each variant. Michaelis−Menten constants and K_i values provide biochemical information about the differences in the binding affinity of different mutants to the substrates and clinically used inhibitors, and could represent a guide for further *in vitro* and *in vivo* studies, pinpointing which inhibitor works best for a certain mutation or combination of mutations. Also, the structures of HIV-1 subtype C PR complexed with NFV and IDV were solved and compared with that of subtype B PR.

MATERIALS AND METHODS

Mutagenesis and Expression of Protease. A complete description of the cloning, expression, and purification procedures can be found in Goodenow et al. and Clemente et al. (*19*, *20*). In brief, the HIV-1 PR DNA for all the PR B and C variants was subcloned into the pET23a expression vector (Novagen). The HIV-1 subtype C PR clone p94IN476.104 was obtained through the AIDS Research and Reference Reagent program, Division of AIDS, NIAID, from Drs. Rodenburg, Gao, and Hahn (*21*). The construct was transformed into *Escherichia coli* strain BL21 Star DE3 PlysS (Invitrogen). The introduction of all mutations onto the B and C background was done using the Site-Directed Mutagenesis protocol (Stratagene). PR expression was performed at 37 °C in M9 minimal media (in 1 L: 6.8 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4SO_4, 5 g of casamino acids, 1 mL of 0.1 M $CaCl_2$, 2 mL of 1.0 M $MgSO_4$, 10 mL of 20% glucose, 50 μg/mL ampicillin, and 34 μg/mL chloramphenicol) and was initiated when the OD_{600} reached 0.8 by addition of 1 mM IPTG. Three hours after induction the cells were pelleted by centrifugation at 14300*g* for 10 min and resuspended in 30−50 mL of IB buffer (20 mM Tris pH 7.0, 2 mM EDTA, and 3 mM DTT). The cells were lysed using an SLM-Amico French Pressure cell at 1000 psi. Inclusion bodies containing the PR were isolated by centrifugation through a 27% sucrose cushion. The inclusion bodies were dissolved at 1 mg/mL in 8 M urea supplemented with 300 mM β-mercaptoethanol, 50 mM CAPS buffer pH 10, and 1 mM EDTA, and the PR was refolded by dialysis against sodium phosphate buffer (50 mM Na_2HPO_4 pH 7.0, 300 mM NaCl, 5 mM EDTA, and 1 mM DTT). The PR was purified through ammonium sulfate precipitation and gel filtration chromatography using a Superdex 75 Hi-Load 16/60 column (Amersham) attached to an FPLC LCC 500 Plus system (Pharmacia). The enzyme was eluted using potassium phosphate buffer (50 mM K_2HPO_4 pH 6.0, 2 mM EDTA, 100 mM NaCl, 2 mM DTT, and 5% glycerol,).

Protease Activity and Inhibitor Constants. The Michaelis−Menten constants k_{cat}, K_m, and k_{cat}/K_m and K_i values were determined for each variant as previously described (*22*). All HIV-1 subtype PR B and C variants were assayed kinetically in 50 mM sodium acetate buffer (50 mM NaOAc pH 4.7, 150 mM NaCl, and 2 mM EDTA), at 37 °C using the chromogenic substrates K-A-R-V-nL*Nph-E-A-nL-G, which mimics the CA/p2 cleavage site. (Norleucine at position P1 is used to mimic the volume occupied and hydrophobicity of methionine (Met) while preventing any problems due to oxidation of the sulfur). Cleavage of the substrate was monitored using a Cary 50 Bio Varian spectrophotometer equipped with an 18-cell multitransport system. The inhibition constants K_i were determined by monitoring the inhibition of hydrolysis of the chromogenic substrate as described by Bhatt et al. (*23*). The PIs tested were obtained through the AIDS Research and Reference Reagent program, Division of AIDS, NIAID.

Crystallization of a Protein−Inhibitor Complex. In order to enhance the stability of the subtype C PR, three additional mutations were introduced prior to crystallographic studies: Q7K, L33I, L63I. These mutations have been shown not to alter the catalytic activity or the PI susceptibility of the enzyme, but to suppress autodigestion (*24*). After HIV-1 PR C was purified, the enzyme was concentrated to 3 mg/mL (140 μM) using a 5 kDa VivaSpin 15R MWCO 3000 spin concentrator (VivaScience). Then the buffer was exchanged for sodium acetate buffer (30 mM NaOAc pH 4.7 and 2 mM DTT). NFV and IDV were dissolved at a concentration of 50 mM in 100% DMSO and mixed with C PR in a molar

Table 1: The Michaelis−Menten Constants for the HIV-1 Subtype B and C PRs

subtype-mutant	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
B	19.5 ± 2	10 ± 1	0.55 ± 0.06
B-D30N	38.6 ± 2	5 ± 0.5	0.13 ± 0.02
B-N88D	16 ± 1.5	12 ± 1.1	0.75 ± 0.10
B-L90M	10 ± 1	5.8 ± 0.5	0.58 ± 0.06
B-D30N/N88D	35 ± 3.5	12.4 ± 1	0.4 ± 0.04
B-D30N/L90M	61 ± 6	3.5 ± 0.4	0.06 ± 0.008
B-N88D/L90M	30 ± 2	10.2 ± 1	0.34 ± 0.04
B-30/88/90	30 ± 2.3	30.6 ± 7.5	1.0 ± 0.3
C	17 ± 1	5.6 ± 0.2	0.32 ± 0.02
C-D30N	64 ± 6	12 ± 1	0.18 ± 0.02
C-N88D	22 ± 3	4.3 ± 0.4	0.19 ± 0.03
C-L90M	23 ± 3	4.8 ± 0.3	0.2 ± 0.04
C-D30N/N88D	19 ± 1.6	3.2 ± 0.3	0.17 ± 0.02
C-D30N/L90M	23 ± 3	4 ± 0.2	0.17 ± 0.02
C-N88D/L90M	24 ± 3	13 ± 1.7	0.51 ± 0.09
C-30/88/90	35.5 ± 3	22 ± 2.4	0.6 ± 0.1

ratio of 3:1. The inhibitor and enzyme were allowed to equilibrate for 1 h at 4 °C, after which the enzyme−inhibitor mixture was spun at 14,000 rpm for 30 min to pellet any precipitation that might have occurred during incubation step. Two microliters of the enzyme−inhibitor complex was mixed with reservoir solution (30 mM citric acid pH 5.0 and 1 M NaCl) in a 1:1 (v/v) ratio on siliconized glass circle cover slides (Hampton). The droplet was suspended over 1 mL of the crystallization buffer. Platelike crystals formed overnight and grew at their full size in 7−10 days.

Data Collection, Structure Determination, and Refinement. Data were collected using a MAR CCD 225 detector at the SER-CAT beamline BM22 at the Advanced Photon Source, Argonne National Laboratory. The crystals were "quick-dipped" in 35% glycerol solution and flash cooled at 100 K prior to data collection. A total of 180° of data were collected (360 images) from single crystals of subtype C PRs complexed with IDV or NVF. The data were indexed, scaled, and reduced using DENZO and HKL2000 software (*25*). Complete data sets were collected to 2.3 Å resolution (*26*). Initial phases were calculated using the coordinates of B PR (PDB code 1W5Y) after removal of the inhibitor and solvent molecules. Standard methods of structure refinement were then employed using programs in the CNS suite (*27*). Electron density maps with $2F_o - F_c$ and $F_o - F_c$ coefficients were used to guide manual fitting of the PR and bound inhibitor using the molecular graphics program O, version 10.0.1 (*28*). The inhibitor and water molecules were added into $F_o - F_c$ electron density contoured at 3σ. Topology and parameter files for IDV and NFV were obtained from the HIC-UP website (*29*). Iterative cycles of refinement were performed in CNS including data between 15 and 2.3 Å resolution. The final R values converged to R_{work} of 25.3% and R_{free} of 28.4% and R_{work} of 21.1% and R_{free} of 23.8% for the NFV and IDV complexes, respectively. During model building and refinement, 5% of the data was reserved for cross validation of the refinement progress. The quality of the final refined structure was validated with PROCHECK (*30*). The atomic coordinates have been deposited in the Protein Data Bank (entries 2R5P, 2R5Q).

RESULTS

Analysis of HIV-1 Subtype C Protease Sequence. The HIV-1 subtype C PR sequence analyzed in this study comes from an HIV-positive patient from India, and the clone was provided by the NIH Research and Reference Reagent Resources Program (*21*). This clone contains eight amino acid differences (T12S, I15V, L19I, M36I, S37A, H69K, L89M, and I93L) from the subtype B PR (LAI sequence) (Figure 1). Two of these polymorphisms are located in the elbow of the flaps, three are located in the 10's loop, and three are situated within the loops at the base of the PR. All these regions harboring the subtype C PR polymorphic differences are located in the outside regions of the PR avoiding the active site, the flaps and the dimerization region. The initial clone contained one additional amino acid difference, N88D, considered a major mutation for NFV resistance. In this study, this enzyme is designated HIV-1 subtype C N88D variant and subtype C PR was obtained by back-mutating the aspartate (D) at the position 88 to the wild type residue, asparagine (N).

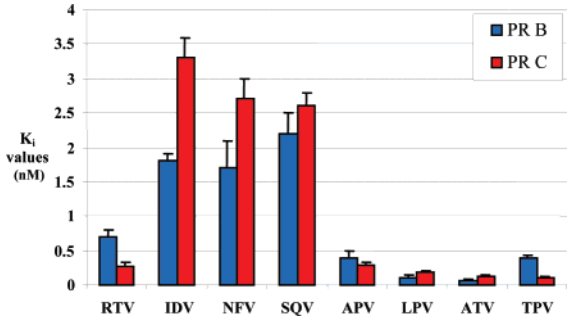

FIGURE 2: Kinetic analysis of HIV-1 subtype B and C PRs. The K_i values (nM) for subtype B and C wild type PRs. The blue and red bars designate subtype B PR and C PR, respectively.

Single, double and triple mutants, harboring various combinations of D30N, N88D, and L90M, were engineered in both subtype B and C PRs. There were a total of 14 mutants constructed and analyzed.

Kinetic Analysis of HIV-1 Subtype B and C Protease Variants: Michaelis−Menten Constants (K_m, k_{cat}). As seen in Table 1, K_m for the subtype C PR is similar to that of the subtype B PR, with values of 19.5 μM and 17 μM, respectively. However subtype C PR showed a 2-fold decrease in k_{cat} (5.6 s^{-1}) when compared to subtype B PR (10 s^{-1}). Accordingly, the catalytic efficiency, defined as k_{cat}/K_m, of the subtype C PR is about 60% of that of the subtype B PR.

The addition of the D30N mutation increased the K_m by to 2- and 3-fold for subtype B and subtype C PRs, respectively. However, the effect on the k_{cat} value was divergent with a 2-fold decrease for subtype B PR and 2-fold increase for subtype C PR. However, despite these varied effects on K_m and k_{cat} values, the enzymatic catalytic efficiency (k_{cat}/K_m) showed similar calculated values for both D30N subtype B and C mutants: 0.13 and 0.18 $s^{-1}\,\mu M^{-1}$, respectively (Table 1). The addition of the N88D mutation resulted in relatively unchanged K_m and k_{cat} values for subtype B PR with a 1.4-fold increase in the catalytic efficiency of the mutant. The HIV-1 subtype C N88D PR variant showed a slight increase in K_m and an approximate 2-fold decrease in the enzymatic catalytic efficiency. The variants harboring L90M mutation exhibited similar changes in their kinetic parameters when compared with the N88D variants.

Table 2: The K_i Values (nM) for HIV-1 Subtype B and C PRs[a]

subtype-mutant	RTV	IDV	NFV	SQV	APV	LPV	ATV	TPV
B	0.07 (0.01)	1.8 (0.1)	1.7 (0.4)	2.2 (0.3)	0.4 (0.1)	0.11 (0.03)	0.07 (0.01)	0.4 (0.04)
B-D30N	0.15 (0.01)	9.4 (1.1)	44.0 (5.5)	3.8 (0.3)	0.4 (0.02)	0.4 (0.05)	0.2 (0.02)	0.2 (0.03)
B-N88D	0.24 (0.05)	9.9 (0.8)	3.4 (0.3)	5.7 (0.7)	0.39 (0.05)	0.08 (0.02)	0.21 (0.02)	0.8 (0.1)
B-L90M	0.42 (0.07)	5.0 (0.4)	5.4 (0.5)	7.5 (0.9)	0.36 (0.05)	0.10 (0.03)	0.17 (0.04)	0.23 (0.04)
B-D30N/L90M	0.32 (0.05)	10 (1)	88 (10)	15 (2)	0.47 (0.07)	0.44 (0.07)	0.28 (0.04)	0.5 (0.1)
B-D30N/N88D	0.87 (0.17)	6.9 (0.6)	68 (6)	20 (2)	0.08 (0.02)	ND	0.09 (0.03)	ND
B-N88D/L90M	0.71 (0.13)	17.7 (1.3)	12 (1)	49 (4)	0.89 (0.11)	0.15 (0.02)	0.35 (0.05)	0.38 (0.04)
B-30/88/90	1.0 (0.2)	5 (1)	88 (7)	47 (6)	0.61 (0.07)	1.0 (0.2)	0.37 (0.05)	1.9 (0.2)
C	0.27 (0.06)	3.3 (0.3)	2.7 (0.3)	2.6 (0.2)	0.29 (0.03)	0.19 (0.02)	0.13 (0.01)	0.11 (0.02)
C-D30N	0.6 (0.1)	10.4 (0.3)	151 (4)	9.0 (0.1)	0.6 (0.1)	0.3 (0.03)	0.8 (0.1)	0.1 (0.01)
C-N88D	0.1 (0.02)	24 (2)	6.2 (0.7)	5.7 (0.1)	0.5 (0.2)	0.15 (0.01)	0.5 (0.1)	0.2 (0.03)
C-L90M	0.78 (0.05)	4.6 (0.4)	5.2 (0.5)	7.1 (0.8)	0.36 (0.05)	0.48 (0.04)	0.27 (0.04)	0.09 (0.02)
C-D30N/L90M	0.27 (0.06)	4.8 (0.1)	41.6 (4.3)	6.5 (0.2)	0.18 (0.02)	0.19 (0.05)	0.08 (0.01)	0.4 (0.08)
C-D30N/N88D	0.04 (0.01)	5.8 (0.4)	40.1 (3.4)	10.9 (0.8)	0.16 (0.03)	0.18 (0.03)	0.06 (0.01)	0.19 (0.03)
C-N88D/L90M	1.0 (0.15)	50 (4)	14 (2)	109 (13)	1.5 (0.2)	0.43 (0.08)	0.54 (0.08)	0.15 (0.05)
C-30/88/90	2.2 (0.3)	8.1 (0.1)	141 (17)	47 (4)	0.39 (0.08)	0.8 (0.2)	0.29 (0.03)	0.39 (0.06)

[a] The errors are in parentheses. RTV: ritonavir. IDV: indinavir. NFV: nelfinavir. SQV: saquinavir. APV: amprenavir. LPV: lopinavir. ATV: atazanavir. TPV: tipranavir.

The subtype C PR double mutants D30N/N88D and D30N/L90M exhibited an approximate 2-fold decrease in their catalytic activity. The D30N/L90M double mutants of subtype B PR showed more significant changes, retaining only about 10% of the enzymatic activity of the wild type enzyme. The double mutant N88D/L90M of subtype B PR showed slight changes in the K_m and k_{cat} values: 24 μM and 13 s^{-1}, with 60% catalytic activity of that of the wild type subtype B PR. The introduction of the same combination of mutations in HIV-1 subtype C PR engendered a 2-fold and 4-fold increase in K_m and k_{cat} values respectively, resulting in doubling the variant catalytic activity when compared to the wild type. Both variants of subtype B and C PRs harboring the triple combination D30N/N88D/L90M showed a 2-fold increase in their catalytic efficiencies.

Kinetic Analysis of HIV-1 Subtype B and C Protease Variants: K_i Values. The K_i values for subtype B and C PRs were calculated for eight PIs used in clinical settings: SQV, RTV, IDV, NFV, amprenavir (APV), lopinavir (LPV), atazanavir (ATV), and tipranavir (TPV). The results obtained for all of the inhibitors are summarized in Table 2. There are three PIs, SQV, IDV and NFV, that have slightly higher K_i values than the other five inhibitors (Figure 2). Even if the K_i values are comparable between subtype B and subtype C PRs, the naturally occurring polymorphisms within subtype C PR seem to have differential effect for different inhibitors. They increase the K_i value in subtype C PR for IDV by 2-fold and by 1.6-fold for NFV compared to that of subtype B. On the other hand, the influence on the change in the K_i values is in the opposite direction for RTV and TPV with a decrease in K_i values for subtype C PR by 2.6- and 3.6-fold, respectively, when compared to those of subtype B PR. However, even with the differences described above, the K_i values for these enzymes with all of the inhibitors tested were within the subnanomolar to low nanomolar range, in agreement with other studies analyzing both subtype B and C PRs (*31*, *32*).

As expected, the most significant effect of D30N mutation was on NFV binding for both subtype B and C PRs, with a fold-increase of K_i values of 26 and 89, respectively (Figure 3A). The other PI significantly affected by D30N is ATV with a 12-fold increase of K_i value for subtype C PR and only 3-fold increase for subtype B variant. HIV-1 subtype C D30N variant also exhibited a 9-fold increase in K_i value against RTV, while the same mutant of subtype B PR showed only 2-fold increase. However, the K_i values remain in the subnanomolar range, indicating tight binding inhibition. The subtype B and C N88D mutants exhibited a 2-fold and 4-fold increase of K_i values when tested with NFV. The N88D mutation is not selected by other clinically used inhibitors but decreases the binding affinity for SQV (3-fold) and IDV (6-fold and 13-fold) for subtype B and C PRs, respectively. The L90M mutation alone introduces, in both subtypes, slight increases (between 1.5- and 3.5-fold) in K_i values for almost all clinically used inhibitors tested in this study, except RTV where there is a 6-fold and 11-fold increase in K_i for subtype B and C PRs, respectively. The newer PIs APV, LPV, and TPV seem unaffected by any single mutations introduced within subtype B and C PRs backbone.

As expected, the double mutants exhibited larger increases in K_i values when compared to the single mutants. The addition of both D30N and N88D in subtype B and C PRs resulted, as expected, in a similar increase in K_i values for IDV, NFV, and SQV. RTV showed 11-fold increase of K_i value for subtype B PR and almost no change for subtype C variant. APV showed a 5- and 2.5-fold decrease in K_i values for subtype B and C variants, respectively (Figure 3B).

The subtype B PR D30N/L90M double mutant exhibited an increased in K_i values for all PIs tested in this study when compared with the same variant of subtype C PR. The most significant differences were observed for SQV, IDV, and NFV with an increase of K_i values by 7-, 6-, and 52-fold and 3-, 3-, and 24-fold for subtype B and C PRs, respectively. Upon the acquisition of N88D/L90M, there was noted an increase in K_i values for all the inhibitors analyzed except TPV, which showed a decrease in K_i values for both subtypes, thus indicating an increased affinity for the enzymes harboring the N8D/L90M combination. The highest fold-changes were observed for SQV, RTV, IDV with a 22-, 10-, and 10-fold increase for subtype B PR and a 50-, 14-, and 28-fold increase for subtype C PR.

The increase in K_i values is comparable between the triple mutants with the most significant differences for RTV and NFV with 14- and 52-fold increase and 31- and 83-fold increase for HIV-1 subtype B and C variants respectively (Figure 3C).

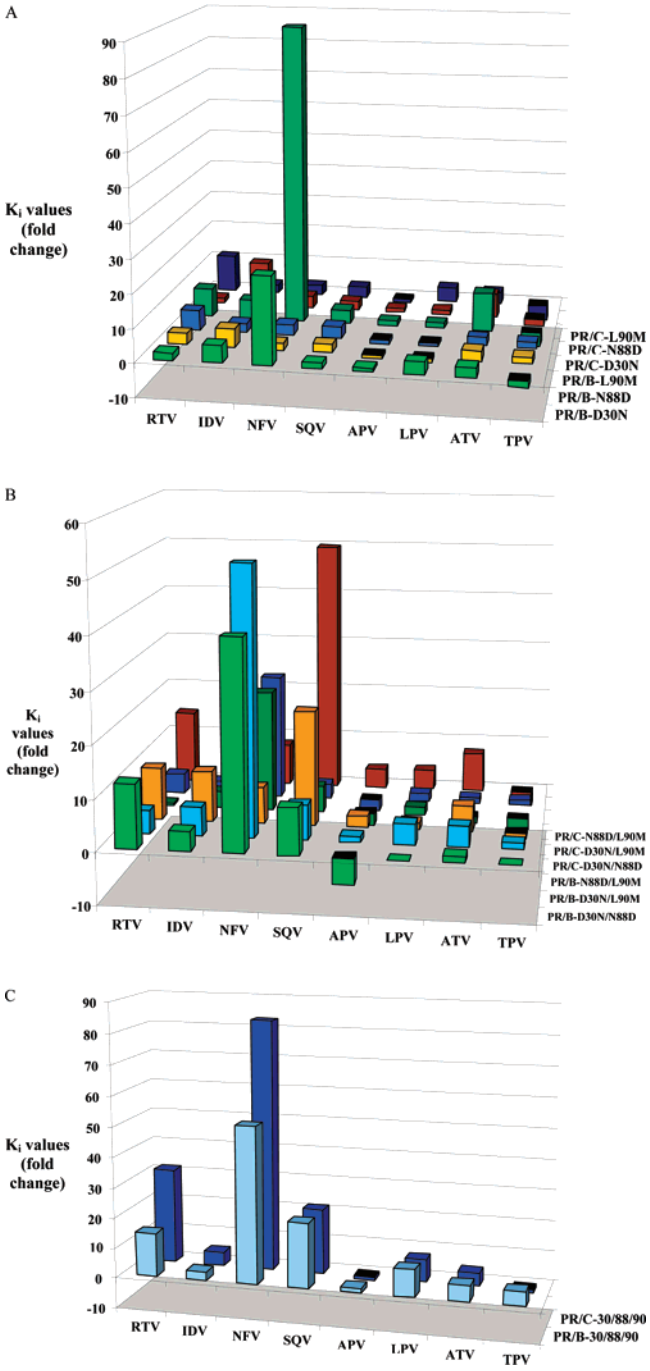

FIGURE 3: Kinetic analysis of HIV-1 subtype B and C mutants. (A) The K_i values for the single mutants of the subtype B and C wild type PRs. (B) The K_i values for the double mutants of the subtype B and C wild type PRs. (C) The K_i values for the triple mutants of the subtype B and C wild type PRs. The K_i values are shown as fold change when compared with K_i values of subtype B PR.

Structure Analysis of the Drug-Bound HIV-1 Subtype C PR. As expected, given the high amino acid sequence identity, the two crystal structures of the enzyme complexed with IDV and NFV had the overall shape and fold of HIV-1 subtype B PR.

Both inhibitor-bound structures of subtype C PR were determined to 2.3 Å resolution in the $P2_1$ space group with two homodimers in the unit cell. In these structures, the PR exhibited a closed conformation with the inhibitor buried in the active site, covered by the two flaps. The solvent for the final models included 169 waters and 2 sodium ions for IDV-bound and 136 water molecules for NFV-bound PRs. The average *B* factors for the main chain of the IDV- and NFV-bound structures were 28.5 and 31.5 Å^2, respectively (Table 3). In each structure the inhibitor, either IDV or NFV, was observed to have a single orientation.

Table 3: Refinement Statistics

	subtype C PR	
	complexed with IDV	complexed with NFV
PDB code	2R5Q	2R5P
space group	$P2_1$	$P2_1$
resolution (Å)	2.3	2.3
R values (%):[a] R_{work}, R_{free}	21.2, 23.8	25.3, 28.4
no. of amino acids, water molecules, inhibitors per PR dimer	198, 169, 1	198, 136, 1
rmsd for bond length (Å), angle (deg)	0.2, 0.12	0.27, 0.04
Ramachandran statistics (%): most favored, allowed regions	96.2, 3.8	95.9, 4.1
B factors (Å^2): av main chain, av side chain, solvent, inhibitor atoms	29.8, 32.1, 35.9, 27.5	32.5, 34.3, 32.8, 27.5

[a] $R_{work} = \sum(|F_o| - |F_c|)/\sum|F_o| \times 100$; R_{free} is identical to R_{work} for 5% of data omitted from refinement.

The least-squares superposition of C^α atoms of the IDV- and NFV-complexed subtype C PR and subtype B PR (PDB code 1SDT) was performed in Pymol (DeLano Scientific) and revealed an rms deviation of 0.30 Å and 0.39 Å for IDV- and NFV-bound structures respectively, with the highest divergence of 1.3 Å observed in the outer loops (residues 65−69) (Figure 4A, B).

The profiles of mean main-chain *B* factors of both subtype C PR-bound structures are similar to that of bound subtype B PR, indicating a similar behavior of these proteins upon inhibitor binding. However, there were several differences in the *B* value profiles (Figure 5) such as in the 80's loop that shows lower than average *B* values for subtype C PRs and higher for subtype B PR. It was also noticed that the flap regions in the bound subtype B PR showed a ~2-fold increase in the *B* values when compared with the bound subtype C PRs. This is a perplexing finding, as the closed flaps, due to decreased flexibility and atomic motion, would be expected to exhibit low *B* values. The observed difference between the *B* factors of these two structures might be due to crystallization conditions, crystal quality and/or crystal packing environment. The bound subtype B PR structure was solved at 1.3 Å resolution in the $P2_12_12_1$ space group while both the bound subtype C PR structures were solved in the $P2_1$ space group at 2.3 Å resolution.

The IDV atoms were in well-defined electron density in the active site for the subtype C PR structure (Figure 6A). The superposition of the bound subtype B and C PRs showed that the IDV structures superposed very well and the number of subtype C PR−IDV van der Waals contacts showed only a small variation between the subtype C and B PR crystal structures. Also these crystal structures showed a very similar arrangement of PR−IDV hydrogen bond interactions, including the same water-mediated interactions. Six water molecules that mediate interactions between IDV and the subtype C PR were observed in subtype C PR-bound structure forming 14 hydrogen bonds, while there are 7 water molecules that form 16 hydrogen bonds in the subtype B PR. The number of hydrogen bonds between the inhibitor

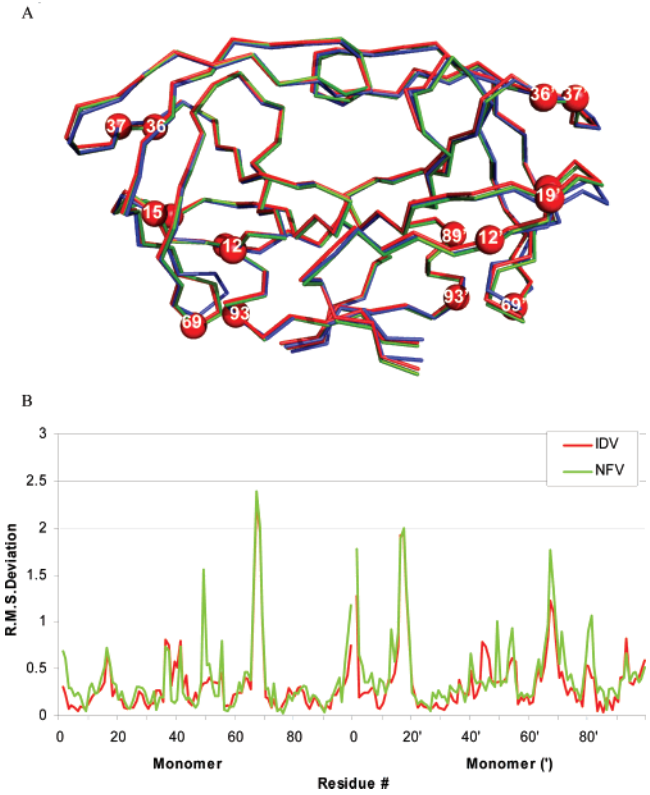

FIGURE 4: Superposition of the IDV- and NFV-bound subtype C PR with the IDV-bound subtype B PR. (A) Ribbon representations of IDV- (red) and NFV- (olive) bound subtype C PR and superposed over IDV-bound B PR (blue). The naturally occurring polymorphisms in subtype C PR are represented as red spheres. (B) The rms differences (Å) per residue are plotted for the C^{α} atoms of bound B PR compared with the bound subtype C PR. The residues in the two subunits are labeled 1−99 and 1′-99′.

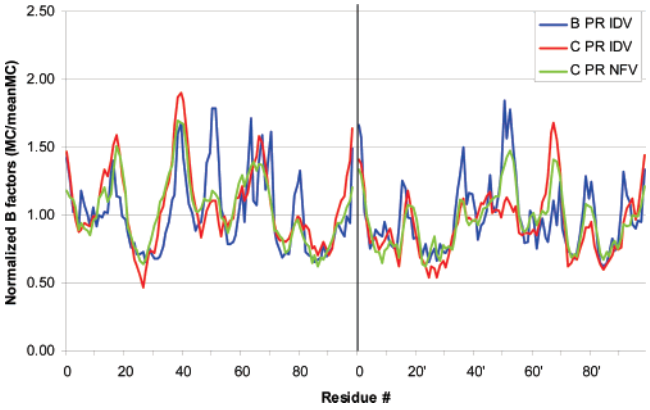

FIGURE 5: The normalized mean *B* values for the main chain (MC) atoms of the IDV- and NFV-bound subtype C and B PRs. The normalized B values are plotted for the residues of IDV- (red) and NFV- (olive) bound subtype C PR and B PR (blue). Normalization was done by dividing the *B* values for the main chain of each residue by the average *B* values for the entire PR molecule. The residues in the two subunits are labeled 1−99 and 1′-99′.

and the active site aspartate residues is 3 for both structures compared in this study.

In the NFV-bound subtype C PR structure, the PI atoms are in well-defined electron density (Figure 6B). Attempts were made to perform the same comparison of the NFV-bound subtype C PR with a similar subtype B PR structure. But, despite the fact that NFV is one of the PIs frequently used to treat HIV-positive patients and NFV-induced drug

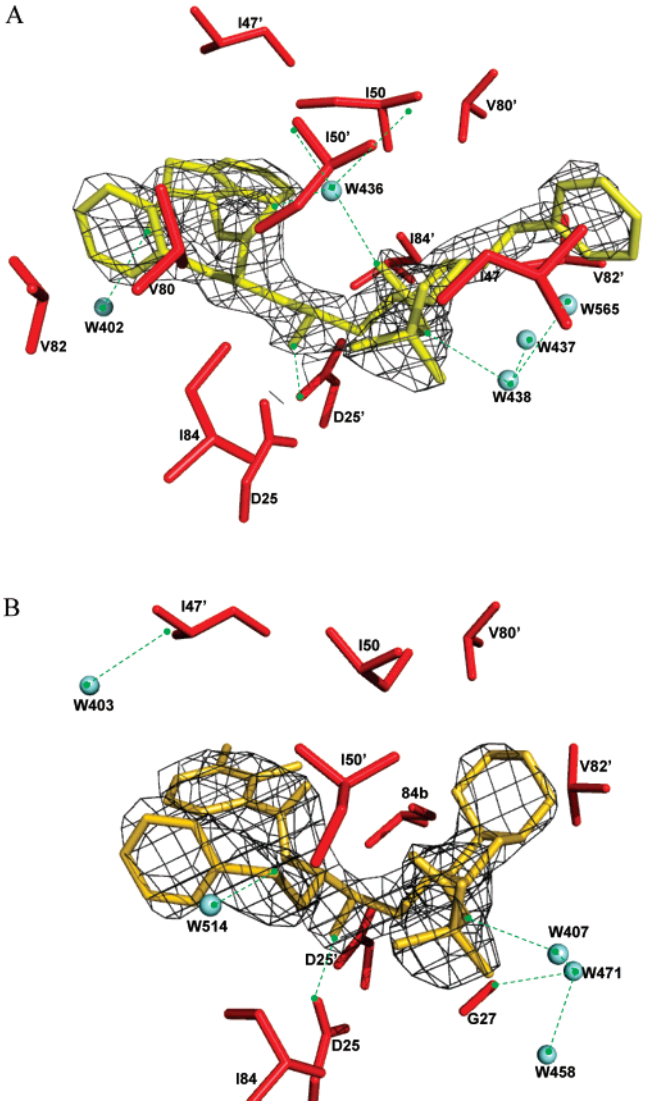

FIGURE 6: The omit electron density maps for IDV (A) and NFV (B) in the active site of HIV PR. The contour level is 2σ. Stick representation of the IDV (yellow) and NFV (sand) in the active site of the subtype C (red) PR. The dashed green lines represent the hydrogen bonds. Water molecules are shown as cyan spheres. Figures rendered with Pymol (Delano Scientific).

resistance mutations, such as D30N, L90M, N88D, have been extensively studied, only one NFV-bound subtype B PR is deposited in the Protein Data Bank (PDB code 1OHR). This structure was deposited in 1997, crystallized in the $P2_12_12_1$ space group and was solved at 2.1 Å resolution. However, when superposed, the monomer B of the subtype B PR structure showed a spatial shift of ~0.5 Å relative to the subtype C PR-bound structure, apparently due to a rotational movement at the dimerization region at the base of the subtype B PR. Consequently a satisfactory comparison could not be performed.

DISCUSSION

The discovery of such a diverse panel of non-B subtypes of HIV-1 in patients infected around the world has posed the question if the currently used methods of prevention, diagnostic, and treatment are as effective as they are for HIV-1 subtype B, the subtype these methods have been originally developed to combat. A large number of groups

and numerous resources have been allocated to investigate the matter. One of the most researched topics is the efficacy of the treatment and the development of drug resistance to currently available PIs, which have been designed based on biochemical and structural information on subtype B PR. The main concern is that the preexisting naturally occurring polymorphisms within non-B subtypes PRs might increase the speed of which highly resistant viruses are selected, promoting a poorer response to the clinically used PIs.

This research is of interest because many *in vivo* and *in vitro* studies (*20*, *32*−*37*) advanced the hypothesis that the naturally occurring polymorphisms play a role in modulating antiretroviral (ARV) drug susceptibility with the possibility of faster development of drug resistance during therapy. In this study, the structural parameters of the subtype C PR complexed with IDV and NFV were analyzed and also compared to available structures of subtype B PR in an attempt to understand the structural differences due to the baseline polymorphisms and their implications in ARV drug resistance/susceptibility.

The aim of this study is to analyze the contributions to the catalytic efficiency and inhibitor resistance by naturally occurring polymorphisms and therapy-selected, active and nonactive site, residue changes in subtype C compared to subtype B PRs. As mentioned before, even though the baseline polymorphisms found in subtype C PR have arisen in the absence of PI therapy, residue changes in C PR at positions 36, 69, and 89 have been associated with PI resistance *in vivo* and *in vitro* in B PRs (*22*, *38*−*40*). Thus, these polymorphisms can potentially influence substrate processing and the binding of currently available PIs and/or facilitate the development of resistance.

In the PR sequence analyzed in this study, all naturally occurring polymorphism changes represent conservative amino acid changes, except at position 37 where a hydrophilic amino acid, serine, is replaced by a small hydrophobic residue, alanine. Two polymorphic residues (M36I and S37A) are located in the flap making contact with the solvent molecules. More precisely, M36I lies in the interface between the enzyme core and the flap and develops as a secondary drug resistance mutation in subtype B PR, decreasing the enzyme affinity for several clinically used PIs (*22*, *41*). The S37A is rarely encountered in clinical isolates (*42*) and has an unknown role if any in the development of drug resistance but in *in vitro* experiments seems to decrease the enzyme solubility in polar solvents and to promote precipitation (data not published). Three other polymorphisms are located in the 10's loop: T12S, I15V, and L19I. This combination is highly specific for subtype C PR (*42*). The role of these polymorphisms was not fully explored, but the I15V mutation has been described to arise in association with TPV treatment. However, there is no clear link between the occurrence of this polymorphism and treatment failure to TPV (*38*). H69K is located in the loop at the base of the PR, and together with M36I was documented to predict decreased phenotypic susceptibility or diminished antiviral responses to TPV (*41*, *43*). L89M is located at the base of the PR, and Sanches et al. hypothesized that this polymorphism may lead to early development of drug resistance in patients infected with non-B HIV subtypes (*35*). Also, recent studies inferred that the L89M mutation in subtype F viruses is a high genetic barrier to the accumulation of the L90M resistance mutation and can function as a resistance mutation, depending on the presence of other polymorphisms in the subtype F PR backbone (*44*).

The I93L polymorphism is located within a hydrogen-bonded turn immediately upstream from the PR/RT cleavage site, in close proximity to L89M and H69K and the dimerization domain. It seems to play a dual role, depending on the ARV regimen administered to the HIV-positive patients. A higher relative risk for developing treatment failure was observed with the presence of I93L in a subgroup of patients treated with IDV, RTV or SQV in several studies (*45*, *46*). Also, although the I93L substitution has been described as a naturally occurring polymorphism in subtype B PR as well, the frequency of this substitution appears to be substantially higher in the treated population than in PI-naive patients (40% vs 20%) (*47*). On the other hand, I93L seems to increase the susceptibility of subtype C PR to LPV as observed by Gonzalez et al. (*48*).

The results of all these studies and others not mentioned here demonstrate the need for further investigating the role of naturally occurring polymorphisms in the development of drug resistance and their contribution in evading drug inhibition and in preservation of the catalytic activity of the PR upon accumulation of major drug resistance mutations.

The kinetic data presented here on the wild type subtype B and C PRs showed that there are no significant differences in the substrate affinity constant between these two enzymes, both having K_m values within the 17−19 μM range. This lack of a significant effect in K_m probably reflects the fact that the sequence variations found in subtype C PR are located outside the active site and that the chemical environment surrounding the aspartyl dyad remains unchanged. Also, even if the naturally occurring polymorphism, such as L89M and I93L, located in the hydrophobic core of the enzyme would change the shape of the substrate-binding cleft, the flexibility of the substrate seems to overcome this effect.

From these data it is also apparent that the naturally occurring polymorphisms in subtype C PR by themselves do not provide an advantage for substrate catalysis when compared to subtype B PR, with the catalytic enzymatic efficiency of subtype C PR being 60% of that of subtype B PR. These results come in contrast to the data reported earlier for subtype C PR. Velazquez-Campoy et al. showed that the subtype C and A PRs exhibit catalytic advantage over B PR, with the C subtype PR displaying lower K_m values against two different substrates and resulting in a higher (2.4-fold) catalytic efficiency than the subtype B PR (*49*). These different results can be explained by the differences in the amino acid sequences between the subtype C PRs analyzed in theses studies. The enzyme analyzed in this studies harbors five additional polymorphisms: T12S, I15V, L19I, S37A, and I93L, whose overall effects on the biochemical characteristic of the HIV PR have not been studied in detail.

The inhibition kinetic studies conducted with eight clinically used PIs showed that the K_i values for all the inhibitors tested on subtype B and C wild type PRs are comparable between theses two enzymes and have low nanomolar and subnanomolar ranges, indicating high affinity of currently used PIs for these enzymes. These data are in agreement with previous clinical studies that report that, at least at the

initiation at ARV treatment, drug-naive patients infected with subtype C viruses respond to ARV treatment as well as HIV-1 subtype B infected patients (*50*−*55*). However, several studies showed that differences in response to ARV treatment, measured as viral load or CD4 cells count, seem to arise after 1−2 years of treatment, with non-B subtypes infected patients performing worse than subtype B infected patients (*56*−*59*). But other factors, such as adherence to treatment, ethnicity, psychosocial conditions, should be taken into consideration as well when analyzing these differences. However, to date there are no long-term clinical studies (more than 5 years) to exploring the efficacy of the existing PIs in patients infected with HIV-1 non-B subtypes, including subtype C (*52*). Due to the severity of this disease and its life-long implications, together with the use of ARV therapy becoming more widespread across Africa, it is imperative to characterize baseline molecular variability and subtype-specific peculiarities of drug targets in non-subtype B HIV-1 infection.

It has been hypothesized that the effects of naturally occurring polymorphisms on the enzymatic catalytic activity and inhibitor binding affinity of subtype C PR are augmented by the acquisition of major drug resistance mutations (*20*, *32*, *33*). As a first step to explore this possibility, a series of single mutants containing one PI-induced mutation, that is, D30N, N88D or L90M, were engineered.

For most PIs analyzed, when comparing the effects of various combinations of mutations between subtype B and C PRs, the results of this study showed that the trends in the changes in K_i values are similar, exhibiting either concurrent increases or decreases against the clinically used PIs. The difference consists in the magnitude of the change that varies between subtype B and C PRs. The most significant fold increase in K_i values was noted for NFV when tested against PRs harboring the D30N mutation. Subtype B variants showed a 26-fold increase when compared to the wild type, while D30N subtype C PR exhibited an 89-fold increase, and thus a significant decrease in the binding affinity toward NFV. The catalytic efficiencies for both variants were similar when compared as absolute values. However, when compared to the wild type enzyme, D30N subtype B PR preserved only 25% of the catalytic efficiency of the wild type, while D30N subtype C PR maintained 50% catalytic efficiency. These results do not bring a biochemical explanation to the previous observation that HIV-1 subtype C viruses preferentially select L90M over D30N when exposed to NFV treatment (*60*). However, other clinical studies also could not corroborate with the Grossman et al. findings, as no differences were observed in the frequency of development of resistance mutations L90M and D30N in B and non-B viruses, including subtype C, upon NFV treatment (*42*, *55*, *61*).

Subtype C PR harboring D30N also exhibited a larger decrease in the susceptibility to PIs, such as RTV and ATV inhibition when compared to a similar mutant of subtype B PR. Although this might predict a predisposition to developing resistance to these two inhibitors by the subtype C variants, it does not seem to be a clear marker of resistance, because both PIs maintained a binding strength sufficient for effective inhibition of the PR.

Both single mutants, N88D and L90M, of subtype B PR showed a slight decrease in the K_m values and a preservation of the enzymatic catalytic efficiency when compared to the wild type subtype B PR. The similar mutants of the subtype C PR exhibited a slight decrease in substrate affinity and in enzymatic catalytic efficiency. Both mutations are located on each side of the 89 position occupied by a Leu in subtype B PR and a Met in subtype C PR. The differences in enzyme activity could be explained by slight distortions that a larger 89M could induce in the subtype C PR core. The same hypothesis was recently advanced by Sanches et al. after structural characterization of subtype F PR which also contains the L89M polymorphisms (*35*). Upon acquisition of N88D, the only significant change in binding affinity was observed for IDV, with a higher increase in K_i values for subtype C PR (13-fold increase in K_i value, compared to 6-fold increase for subtype B PR mutant). This study demonstrates that N88D by itself does not provide higher level of resistance for NFV in both subtype B and C PRs. L90M mutants exhibited slight decreases in binding affinity for SQV, IDV and RTV, for both subtype B and C PRs.

So far, the data on single mutants for subtype B and C PRs confirmed some of the previous studies on subtype B PR and highlighted several differences in the kinetic profile between these two enzymes. These differences are minor for newer PIs such as APV, LPV, ATV, and TPV and of greater amplitude for SQV, RTV, IDV, and NFV.

The variants harboring the double D30N/N88D combination of mutations exhibited similar kinetic behavior for both subtype B and C PRs. The subtype B mutant maintained 70% of the catalytic efficiency when compared with the wild type, much better than the mutant harboring D30N alone. Previous studies showed that there is a strong association between D30N and N88D in subtype B PR, with the latter probable having the role to stabilize the enzyme by compensating for the negative charge lost with D30N substitution (*18*, *62*). The D30N/N88D subtype C PR preserved 55% of the wild type enzymatic activity. The K_i value profile is comparable between these two mutants with, as expected, a significant decrease in binding affinity for NFV.

For the D30N/L90M double mutants, a more notable increase in K_i value for NFV for subtype B PR (52-fold) was observed when compared to subtype C mutant (24-fold). However, a significant decrease of almost 10-fold in the catalytic efficiency of the D30N/L90M subtype B mutant was also noticed. On the other hand, upon acquisition of this combination of mutation, subtype C PR exhibited only a 50% decrease in its enzymatic activity. The kinetic results presented in this work appear to be in accordance with previous studies that showed that subtype B mutants carrying D30N/L90M combination are only rarely found *in vivo*, and generally have additional, potentially compensatory, mutations. Interestingly, a major loss of replicative capacity for a subtype B mutant clone harboring both D30N and L90M was also described (*63*−*65*). It can be inferred that in the context of this combination of major resistance mutations, the naturally occurring polymorphisms within subtype C PR could act as compensatory mutations having a role in preserving the catalytic efficiency of the enzyme.

N88D/L90M combines two nonactive site mutants: N88D, which has high structural stability, with L90M, which shows poor structural stability (*12*, *66*). Both N88D and L90M are not part of the substrate-binding site, but they can influence the catalytic process through indirect interactions. This

combination of mutations appeared to be very favorable in the context of the naturally occurring polymorphisms of subtype C PR when compared to subtype B PR. The subtype C mutant exhibited increased catalytic efficiency when compared with both the wild type subtype C PR and the similar variant of subtype B PR. The inhibition profile showed higher K_i values for subtype C PR when compared to subtype B PR mutant for all PIs tested in this study. Not surprisingly the fold increase in K_i value for NFV was modest, but the K_i values for SQV and IDV showed 50- and 28-fold increase, respectively, for N88D/L90M subtype C PR and 22- and 10-fold increase, respectively, for subtype B variant.

Surprisingly, the mutants harboring the triple combination D30N/N88D/L90M showed improved catalytic efficiencies for both subtypes and increased K_i values for NFV, SQV, and RTV. The fold change was higher for NFV in subtype C PR (83-fold) versus subtype B PR (52-fold).

This data also demonstrated that the D30N mutation in combination with D88N and L90M provides a higher level of cross-resistance for subtype C PR, specifically to RTV. This is of interest as no such combination was described in response to RTV in subtype B PR. This result could argue for a new mutational pathway in developing resistance to RTV in subtype C PR. However, despite the high fold increase, the absolute K_i value remains low, indicating that RTV maintains sufficient binding strength to effectively inhibit the D30N/N88D/L90M mutant of subtype C PR. For these reasons the use of pretherapy and therapy resistance testing, especially in cases of early failure, is crucial for providing optimum treatment potency.

An interesting observation is that the kinetic parameters of subtype C PR harboring the D30N mutation do not improve in the same way as happens for subtype B PR upon acquisition of other drug resistance mutations. Specifically, D30N subtype C PR does not improve its catalytic efficiency upon acquisition of N88D, as happens for subtype B PR. Also, the K_i values for NFV for D30N subtype C PR do not change after combination with N88D and L90M. A possible interpretation of these results is that the naturally occurring polymorphisms in subtype C PR have already set a favorable context in which the acquisition of D30N by itself is sufficient enough to attain a higher level of resistance (more than 5−10 fold increase in K_i values), while in subtype B PR other major mutations are required to develop a similar degree of resistance. It is important to mention that this effect can be limited to the combination of baseline polymorphisms in subtype C PR and the type of major mutations induced by the ARV treatment. No attempt is made to make the same assumption for other subtype PRs, due to the fact that they harbor different combinations of polymorphisms that might overall not have the same effect on the biochemical and structural characteristics of the PR. This is another reason why the exploration of each subtype PR is important in the context of designing the best ARV treatment strategy, especially for patients who have failed multiple drug regimens.

In this study the binding affinities of the newer PIs, APV, LPV, ATV, TPV, seem less affected by the introduction of three mutations studied here. Most of the subtype C PR mutants analyzed in this study appear to exhibit a modest increase in the susceptibility to TPV. It might be due to the fact the newly designed PIs are able to evade drug resistance mutations better that the older PIs. This study does not include mutations that specifically arise upon treatment with these inhibitors, such as I50L for ATV or I84 V for LPV, but such studies are under way (data not shown). Also, the viruses isolated from patients that failed ARV treatment usually have PRs harboring a much larger number of mutations. So, in the long term, the minor effects introduced by the baseline polymorphisms, exemplified in these studies by modest changes in K_i values, could be even further accentuated by the multiplicative effect of addition of multiple drug resistance mutations (*20*, *67*). Even larger differences in K_i values between subtype B and C PRs are expected to be seen upon acquisition of a larger number of drug resistance mutations, as usually happens during ARV therapy.

Gonzalez et al. showed that the addition of three or four IDV drug resistance mutations has led to a highly resistant subtype C virus compared with its subtype B counterpart (*68*). The same phenomenon was observed with NFV. This means that the IDV drug resistance mutations impact was much more noticeable in subtype C than in subtype B viruses. Differences in behaviors between the subtype C and subtype B viruses observed *in vitro* in the Gonzalez study and in this study may have a clinical relevance, suggesting a lower genetic barrier in subtype C viruses than in subtype B isolates.

The PR clone used in this study does not harbor a frequent polymorphism found in subtype C PRs: R41K. It is one of the residues that appears to be involved in preserving or augmenting the catalytic efficiency of the subtype C PR and enhancing the viral fitness when compared to subtype B viruses (*17*, *49*). It also appears to decrease viral susceptibility to RTV and APV treatment. The purpose of this work was not to study the effects of each individual polymorphic residue but their influence as a group within the subtype C PR context.

In order to correlate the biochemical findings with structural data, the structures of subtype C PR bound to NFV and IDV were determined and analyzed. In the inhibitor-bound structures of subtype C PR the main differences, when compared with drug-complexed structure of subtype B PR, are seen in the outer loops, where the preexisting polymorphisms are located (Figure 4A). In a recent study, the effects of the naturally occurring polymorphisms on the overall structure of the unbound subtype C PR were described in detail (*69*). The overall structure of the PR is more compact and some of the differences seen between the unbound forms of the subtype B and C PRs are less obvious in the inhibitor-complexed enzymes. The overall superposition of inhibitor-bound structures of subtype B, F and C PRs (Figure 7) showed that the most significant differences are located in the 60's loop, at the base of the PR, where there is a shift similar to that seen in the unbound form of subtype C PR (*69*). The working hypothesis is that mutation of a shorter and bulkier histidine residue at position 69 to a longer lysine causes a displacement of the loop, resulting in ∼1.5 Å shift of the main chain of the 60's loop. The flaps in both subtype B and C enzymes are firmly closed over the active site enclosing the inhibitor within. The differences noted at the 20's loop and the elbows of the flap are of a lesser extent when compared with the unbound subtype C PR. These

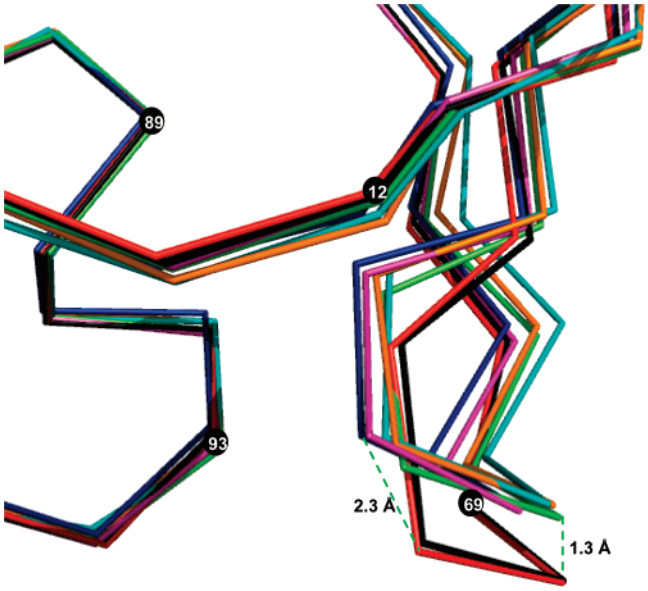

FIGURE 7: Superposition of 6 structures of inhibitor-bound PRs. The comparison includes the two structures reported here, 2R5P (2.3 Å IDV-bound subtype C HIV PR, black) and 2R5Q (2.3 Å NFV-bound subtype C HIV PR, red), and four previously reported structures, 2B7Z (2.2 Å IDV-bound subtype B PR, teal), 1SDT (1.3 Å IDV-bound subtype B PR, magenta), 1W5Y (1.9 Å BE6-bound subtype B PR, green), and 2P3C (2.1 Å TL-3-bound subtype F HIV PR, blue). The positions of baseline polymorphisms in subtype C PR are indicated by numbered black spheres.

findings suggest that PIs designed to specifically inhibit subtype C PR might work better if targeted against the open conformation of the enzyme that harbors the most significant structural changes when compared to subtype B PR

The IDV binding site analysis in subtype B and C PRs with LIGPLOT (*70*) revealed that there are no significant differences in the number of hydrogen bonds and hydrophobic interactions between the inhibitor and the active site of the PR. It is apparent that the naturally occurring polymorphisms do not affect the shape of the active site to the extent of hindering inhibitor binding. These results correlate well with previous kinetic data that showed that the K_i values for IDV and NFV for subtype B and C PRs are comparable, indicating that both enzymes bind the inhibitors with similar affinity.

In conclusion, the differences observed in this study between various mutants of subtype B and C PRs are due to the presence of the preexisting polymorphisms. This study showed that the presence at the start of therapy of the naturally occurring polymorphisms could give the virus an advantage in the rapid development of drug resistance while preserving the virus viability in the presence of a specific inhibitor. These results led to the hypothesis that the effects of the naturally occurring polymorphisms in subtype C PR are influenced by the PI choice for treatment and by the type of major mutations acquired upon drug treatment. The same is probably true for all other HIV-1 subtypes. These mutation patterns are complex and frequently overlapping. It is necessary to test the major groups of naturally occurring polymorphisms for each subtype in combination with several major drug resistant mutations to further be able to predict the response to treatment based on the combination of baseline polymorphisms. Also, determining the biochemical and biophysical properties of enzymes with these patterns of mutations will be important for designing new PIs that are less likely to trigger resistance or are effective against already drug-resistant isolates.

It is important to mention that studying the addition of PI-resistance mutations within the PR is limited only to their effect on the biochemical characteristics of the enzyme. The reported results from this experimental design prevent the study of the effects of the naturally occurring polymorphisms within the gag or the viral context. For example, several studies showed that there is an interrelation between acquisition of D30N and N88D and changes occurring within the rest of the gag and gag/pol polyproteins. It has been shown that mutations in the HIV-1 PR substrate cleavage-site p1−p6 covary with the D30N/N88D PR mutations. Aspartic acid at position 30 is important both to the binding of NFV and also likely to the recognition of the p1−p6 cleavage site. Structural analysis shows that both NFV and p1−p6 have atoms that protrude beyond the substrate envelope and contact Asp30. Thus, both the inhibitor and the p1−p6 substrate are likely to be affected by D30N mutation. This is a possible explanation for the particular coevolution of the p1−p6 cleavage site with the D30N-resistant mutation and also why no other coevolution with any of the other substrates occurs (*15*).

All these studies highlight the importance of further characterization of the development of resistance in non-subtype B viruses. For some PIs, the differences seen in baseline polymorphisms between subtypes may influence which mutational patterns develop. Subtle effects of such polymorphisms on drug susceptibility and replicative capacity may underlie such changes.

Despite the limitations of these studies, they verify and complement data obtained through phenotypic studies. While experiments involving virus cultures might require more time and are more expensive, these biochemical studies can be done quickly and at lower cost. Also biochemical studies are performed in a controlled environment, in which differences in response can be tracked down to a single variable. The results of this study should prove useful in the design of new and continuing therapy.

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