Secondary Mutations M36I and A71V in the Human Immunodeficiency Virus Type 1 Protease Can Provide an Advantage for the Emergence of the Primary Mutation D30N†

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ABSTRACT: Development of resistance mutations in enzymatic targets of human immunodeficiency virus 1 (HIV-1) hampers the ability to provide adequate therapy. Of special interest is the effect mutations outside the active site of HIV-1 protease have on inhibitor binding and virus viability. We engineered protease mutants containing the active site mutation D30N alone and with the nonactive site polymorphisms M36I and/or A71V. We determined the K_i values for the inhibitors nelfinavir, ritonavir, indinavir, KNI272, and AG1776 as well as the catalytic efficiency of the mutants. Single and double mutation combinations exhibited a decrease in catalytic efficiency, while the triple mutant displayed catalytic efficiency greater than that of the wild type. Variants containing M36I or A71V alone did not display a significant change in binding affinities to the inhibitors tested. The variant containing mutation D30N displayed a 2−6-fold increase in K_i for all inhibitors tested, with nelfinavir showing the greatest increase. The double mutants containing a combination of mutations D30N, M36I, and A71V displayed −0.5-fold to +6-fold changes in the K_i of all inhibitors tested, with ritonavir and nelfinavir most affected. Only the triple mutant showed a significant increase (>10-fold) in K_i for inhibitor nelfinavir, ritonavir, or AG-1776 displaying 22-, 19-, or 15-fold increases, respectively. Our study shows that the M36I and A71V mutations provide a greater level of inhibitor cross-resistance combined with active site mutation D30N. M36I and A71V, when present as natural polymorphisms, could aid the virus in developing active site mutations to escape inhibitor binding while maintaining catalytic efficiency.

The use of highly active antiretroviral therapy (HAART),[1] which involves combinations of reverse transcriptase and protease inhibitors, can lead to a decrease in viral load to nearly undetectable levels in human immunodeficiency virus 1 (HIV-1) infected individuals (*1*). The efficacy of combination therapy is hindered by the rapid appearance of resistance mutations, which tend to diminish the binding strength of the inhibitor, within the target enzymes (*2*). Our study focuses on the HIV-1 protease, which is involved in the processing of the gag and gag-pol polyproteins into the viral structural and enzymatic proteins (*3*), a process critical for the production of viable viral particles (*4*). A number of mutations that develop under the selective pressure of protease inhibitor therapy occur in the active site of the protease, also described as primary mutations. The effect that primary mutations have on the binding strength of the inhibitor can easily be rationalized because those amino acids make direct contact with the inhibitor. Most of the mutations, however, are found outside of the active site cavity, also described as secondary mutations, and their effect cannot as easily be rationalized. Currently, it has become clear that secondary mutations require serious attention since such mutations may also be involved in decreasing inhibitor binding strength (*5*−*8*). Secondary mutations can occur as natural polymorphisms in PR at positions including 10, 36, 63, 71, and 77.

We created a series of variants containing the primary mutation D30N and the secondary mutations/polymorphisms M36I and A71V. The positions of these residues in the HIV-1 protease are shown in Figure 1. Our rationale was to understand the effects of secondary mutations along with published data and to evaluate mutations/polymorphisms as a predictive measure for therapy development and effectiveness. We chose D30N for two reasons. First, the substitution of asparagine for aspartic acid at position 30 occurs only in response to nelfinavir therapy (*9*) (see the Stanford database, http://hivdb.stanford.edu). We anticipated that the D30N change would have a limited effect on the binding of other inhibitors and would permit our focusing attention on the interpretation of the data on the effects of the secondary mutations. Second, the D30N mutation also has been shown

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[1] Abbreviations used: HAART, highly active antiretroviral therapy; IPTG, isopropyl thio-D-galactopyranoside; A_{600}, absorbance at 600 nm; dTT, dithiothreitol; Nph, *p*-nitrophenylalanine; nL, norleucine; LAI^{wt}, the protease species from laboratory strain LAI; LAI^{30}, the D30N mutant of LAI; LAI^{36}, the M36I mutant of LAI; LAI^{71}, the A71V mutant of LAI; $LAI^{30/36}$, the D30N and M36I double mutant of LAI; $LAI^{30/71}$, the D30N and A71V double mutant of LAI; $LAI^{36/71}$, the M36I and A71V double mutant of LAI; $LAI^{30/36/71}$, the D30N, M36I, and A71V triple mutant of LAI.

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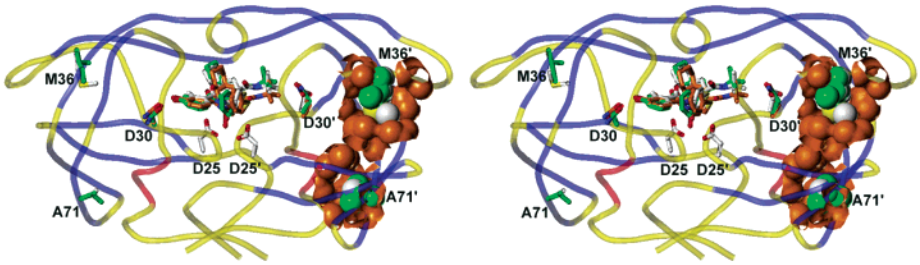

FIGURE 1: HIV-1 protease homodimer. Stereo transparent tube αC trace of the HIV-1 protease homodimer wild type (PDB file 1OHR) colored by secondary structure. In the active site is an overlap of nelfinavir, D25 and D25′, and D30 and D30′ in capped sticks in the wild type (white), 1OHR[30] (orange), and 1OHR[30/36/71] (green). Overlap of residues M36 and A71 in the wild type (white) and 1OHR[30/36/71] (green) are shown as capped sticks. Overlap of residues M36′ and A71′ in the wild type (white) and 1OHR[30/36/71] (green) is shown as a space filling structure. Atoms within 4 Å of residues M36′ and A71′ are shown as an orange Connolly surface. Structures were generated using PDB file 1OHR and Sybyl 6.9 modeling program.

to suppress the replicative fitness of the virus in vitro, but it does not affect the complete in vitro processing of the gag-pol polyprotein (*10*−*14*). The secondary mutations M36I and A71V were chosen for three reasons. First, position 36 is located in the interface between the enzyme core and the flap, while position 71 is located at the interface between the enzyme core and the terminal domain, as described by Rose et al. (*15*). Second, isoleucine at 36 or valine at 71 develop in response in vivo and in vitro to various protease inhibitors (*16*−*18*). Third, M36I and A71V occur as natural polymorphisms in protease prior to exposure to protease inhibitors (*8*, *19*−*22*). Our results indicated that the secondary mutations have a detrimental effect on the binding of clinically used inhibitors. As natural polymorphisms, the M36I and A71V substitutions could provide the virus with an advantage to develop primary mutations and maintain virus viability in the presence of inhibitors.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Protease. Cloning, expression, and purification procedures have been described (*23*, *24*). In brief, the HIV-1 PR from the HIV_{LAI} was subcloned into the pET23a expression vector from Novagen (*25*). The construct was transformed into the *Escherichia coli* strain BL21 Star DE3 PlysS from Invitrogen. Variants were created in the HIV_{LAI} PR background by introduction of point mutations using the QuikChange Mutagenesis Kit from Stratagene. Protease expression was initiated by addition of 1 mM IPTG to a culture grown in M9 media (6.8 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4SO_4, and 5 g of casamino acids were autoclaved together in 987 mL of H_2O, and then 1 mL of 0.1 M $CaCl_2$, 2 mL of 1.0 M $MgSO_4$, 10 mL of 20% glucose, and 50 μg/L ampicillin were added) at 37 °C when A_{600} reached 0.6. After 3 h, cells were harvested by centrifugation at 16000*g* for 5 min and resuspended in TN buffer (0.05 M Tris, 0.15 M NaCl, 0.001 M $MgCl_2$, pH 7.4). Inclusion bodies containing the protease were isolated through a 10% sucrose cushion and solubilized in 8 M urea. Protease was refolded by dialysis against 0.05 M sodium phosphate buffer (0.05 M Na_2HPO_4, 0.005 M EDTA, 0.3 M NaCl, 0.001 M dTT, pH 7.3), purified by ammonium sulfate precipitation and gel filtration chromatography using a Superdex 75 16/60 column (Amersham/Pharmacia) attached to an FPLC LCC 500 Plus (Pharmacia), and eluted using potassium phosphate buffer (50 mM K_2HPO_4, 2 mM EDTA, 150 mM NaCl, 2 mM dTT, 5% glycerol, 5% 2-propanol, pH 7.3).

Protease Activity and Inhibitor Constants. Nelfinavir and AG1776 (also called KNI764 or JE2147) were gifts from Agouron (now Pfizer San Diego). KNI272 was a gift from Professor Yoshiaki Kiso, Kyoto Pharmaceutical University. Ritonavir was a gift from Abbott Laboratories, and indinavir was obtained from Merck.

Michaelis−Menten constants k_{cat}, K_m, and k_{cat}/K_m were determined for each variant using the chromogenic substrate K-A-R-V-L#Nph-E-A-nL-G that mimics the p2-CA cleavage site. Cleavage of the substrate was monitored using a Hewlett-Packard 8452A spectrophotometer equipped with a seven-cell sample handling system (*26*). K_m and V_{max} values were determined from the initial rates of six different substrate concentrations assayed at 37 °C in sodium acetate buffer (0.05 M sodium acetate, 0.15 M NaCl, 0.002 M EDTA, 0.001 M dTT, pH 4.7). The resulting Michaelis−Menten curve was fit using the following equation:

$$v = \frac{V_{max}S}{K_m + S}$$

The values for k_{cat} were determined using the following equation:

$$k_{cat} = \frac{V_{max}}{E_t}$$

The total active enzyme concentration (E_t) was determined by active site titration using the tight binding inhibitors ritonavir and AG1776 for LAI^{wt} and the mutants, respectively. The inhibition constant K_i was determined by monitoring the inhibition of hydrolysis of the chromogenic substrate as describe by Bhatt et al. 2000 (*27*). The K_i values for inhibitors exhibiting K_i values greater than 10 nM were determined using the following equation:

$$v = \frac{V_{max}}{1 + (K_m/S)(1 + I/K_i)}$$

The K_i values for tight binding inhibitors (<10 nM) were determined using the following equation:

$$\frac{v}{v_o} = \{E_t - I_t - (K_i((S/K_m) + 1)) + [(E_t - I_t - (K_i((S/K_m) + 1)))^2 + 4E_t(K_i((S/K_m) + 1))]^{1/2}\}/2E_t$$

All kinetic values were determined using the SigmaPlot enzyme kinetics module.

Relative Vitality. To better describe the effect resistant mutations have on virus viability in the presence of a specific inhibitor, Gulnik et al. introduced the vitality parameter that is described by the following equation (*28*):

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

Because some second-generation inhibitors have low picomolar binding constants, a 10-fold drop in affinity will still leave an inhibitor with a subnanomolar binding constant. This inhibitor would still be able to arrest viral maturation since it would be able to out-compete the substrate. To better describe the effects of these inhibitors, Velazquez-Campoy (*6*) introduced a modified vitality value, which is normalized to a reference inhibitor, termed relative vitality described by the following equation:

$$\text{relative vitality} = \frac{(K_i k_{cat}/K_m)_{mutant}}{(K_i)_{ref}(k_{cat}/K_m)_{wild\ type}}$$

Molecular Modeling and Docking. Unless otherwise stated default settings were used with all programs. The structure of HIV-1 PR bound to nelfinavir (PDB file 1OHR) was mutated at positions 30, 36 and 71 using the biopolymer module in the molecular modeling program Sybyl version 6.9 (Tripos Inc.). To relax all electrostatic and steric clashes due to the mutations, nelfinavir was removed from the active site and the enzyme was minimized using a two-step minimization protocol. First the protease was minimized using the steepest descent method and Tripos force field for 100 steps. The protease was then minimized using the Powell method and Tripos force field to an energy gradient of 0.05 kcal mol^{-1} Å^{-1}. Gasteiger−Huckel charges were used in both minimization steps (*29*). Nelfinavir was minimized using the Powell method and Tripos force field to an energy gradient of 0.05 kcal mol^{-1} Å^{-1} to remove all trace of binding history. Nelfinavir was then placed back into the active site of the mutant enzyme in the same orientation as in the reported structure. Docking simulations of nelfinavir into the active site of the mutated HIV-1 PR were done using the Sybyl6.9 FlexiDock module. FlexiDock allows for the assignment of flexible side chains in both the ligand and the enzyme. After docking, the ligand−enzyme complex with the lowest conformational energy was put through the minimization protocol previously described. The ligand was then redocked into the minimized structure, and the best five ranked structures were used for analysis of the docking conformation. Analysis of the changes in local intramolecular interactions surrounding the M36I and A71V residues and intermolecular interactions between nelfinavir and the D30N residue was done using the computational program HINT (*30*). The HINT program quantifies the favorable interactions, hydrogen bonding, hydrophobic, and acid−base, and unfavorable, hydrophobic−polar, base−base, and acid−acid, to give the total score of interaction.

RESULTS

Kinetic Analysis. Michaelis−Menten constants were obtained for a synthetic peptide representing the CA-p2 cleavage site in the HIV gag protein (Table 1). The P1′ position was replaced with *p*-nitrophenylalanine for monitoring cleavage. The P4′ position was replaced by norleucine for stability. The mutant protease containing the active site mutation D30N (LAI^{30}) showed a 51% decrease in k_{cat}/K_m from that of reference protease from HIV$_{LAI}$ (LAI^{wt}). This decrease in k_{cat}/K_m was attributed mainly to a 56% increase in K_m compared to that of the LAI^{wt} form of the enzyme. The mutants containing the secondary mutations M36I and A71V (LAI^{36} and LAI^{71}) showed a decrease in k_{cat}/K_m of 58% and 64%, respectively, from that of LAI^{wt}. For both mutants this was attributed to a large decrease in k_{cat}. Combination of mutation M36I or A71V with the D30N mutation (mutants LAI$^{30/36}$ and LAI$^{30/71}$) decreased k_{cat}/K_m 81% (LAI$^{30/36}$) or 74% (LAI$^{30/71}$) relative to that of LAI^{wt}. The decrease measured in k_{cat}/K_m for LAI$^{30/36}$ and LAI$^{30/71}$ was attributed to both an increase in K_m and a decrease in k_{cat}. The presence of mutations M36I and A71V further decreased k_{cat}/K_m compared to that of the mutant LAI^{30}, 30% for LAI$^{30/36}$ and 25% for LAI$^{30/71}$. This was mainly due to a decrease in k_{cat} compared to that of LAI^{30}. The double mutant containing mutations at positions 36 and 71 (LAI$^{36/71}$) showed a 78% decrease in k_{cat}/K_m. This decrease in k_{cat}/K_m was attributed to a large decrease in k_{cat}. The addition of mutations M36I and A71V to LAI^{30} (LAI$^{30/36/71}$) increased k_{cat}/K_m 40% above that of LAI^{wt}. This increase in k_{cat}/K_m was attributed to a 33% decrease in K_m.

Table 1: Michaelis−Menten Constants[a]

variant	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, s^{-1} μM^{-1}
LAI^{wt}	18 ± 2	21 ± 2	1.2 ± 0.2
LAI^{30}	28± 3	17 ± 2	0.59 ± 0.08
LAI^{36}	14 ± 1	7.2 ± 0.4	0.51 ± 0.04
LAI^{71}	11 ± 1	4.7 ± 0.2	0.43 ± 0.04
LAI$^{30/36}$	22 ± 2	4.9 ± 0.3	0.2 ± 0.03
LAI$^{30/71}$	26 ± 3	8.1 ± 0.5	0.31 ± 0.04
LAI$^{36/71}$	14 ± 1	3.7 ± 0.3	0.3 ± 0.03
LAI$^{30/36/71}$	12 ± 2	21 ± 2	1.7 ± 0.3

[a] Standard deviations are best fit values through multiple points done twice.

Inhibitor Binding. The dissociation constant (K_i) was determined for the clinically used inhibitors ritonavir, indinavir, and nelfinavir, and for two second-generation inhibitors, AG1776 and KNI-272 (Table 2) (*31*). The D30N mutation (LAI^{30}) had the greatest effect on binding of nelfinavir as the K_i value increased by >450%. D30N affected the other inhibitors tested to a lesser extent with percent changes ranging from −200% to +295% compared to that of LAI^{wt}. M36I (LAI^{36}) had its greatest affect on the binding of AG1776, increasing the K_i value by 424% above that of the wild type, but it should be noted that the K_i for AG1776 was still subnanomolar (Table 2). LAI^{36} showed no significant change in K_i values for the other inhibitors, with percent changes ranging from −75% to −25%. Mutation A71V (LAI^{71}) produced no significant increase in K_i for any of the inhibitors tested, but did cause a 29%, 82%, or 36% decrease in the K_i value of ritonavir, nelfinavir, or KNI-272, respectively. The combination of D30N and M36I (LAI$^{30/36}$) produced an increase in K_i values for all inhibitors from 65% to 543% above that of LAI^{wt}, with nelfinavir being the most affected. The combination of D30N and A71V (LAI$^{30/71}$) created a binding profile similar to that of LAI$^{30/36}$,

Table 2: K_i Values (nM)

variant	ritonavir	indinavir	nelfinavir	AG 1776	KNI 272
LAI^{wt}	0.7 ± 0.1	3.1 ± 0.1	1.2 ± 0.2	0.021 ± 0.004	0.58 ± 0.08
LAI^{30}	0.23 ± 0.04	4.9 ± 0.1	6.8 ± 0.9	0.083 ± 0.015	0.9 ± 0.2
LAI^{36}	0.15 ± 0.02	2.7 ± 0.1	0.9 ± 0.1	0.11 ± 0.02	0.6 ± 0.1
LAI^{71}	0.5 ± 0.1	2.8 ± 0.2	0.22 ± 0.04	<0.021[a]	0.37 ± 0.05
$LAI^{30/36}$	4.5 ± 0.3	5.1 ± 0.6	6 ± 1	0.11 ± 0.02	1.7 ± 0.3
$LAI^{30/71}$	2.4 ± 0.1	5.1 ± 0.2	6.1 ± 0.2	<0.021[a]	1.1 ± 0.1
$LAI^{36/71}$	1.1 ± 0.3	2.0 ± 0.3	0.63 ± 0.04	0.030 ± 0.008	0.38 ± 0.07
$LAI^{30/36/71}$	13 ± 2	7.6 ± 0.2	26 ± 3	0.32 ± 0.09	1.7 ± 0.1

[a] These constants were below the sensitivity level of our assay.

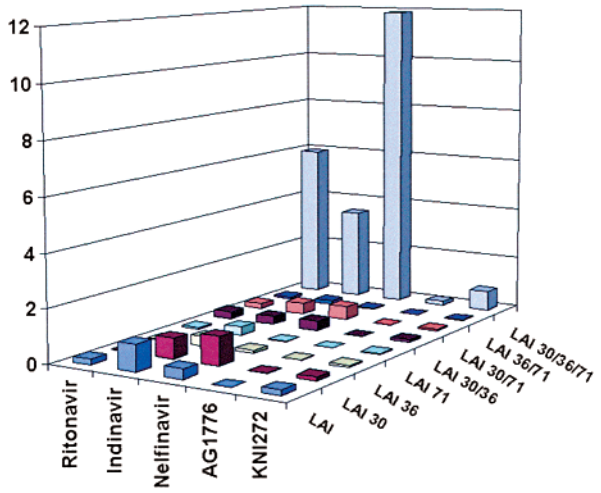

FIGURE 2: Relative vitality. Relative vitality values using indinavir and wild-type enzyme as reference for all variants with each inhibitor tested. Relative vitality values were calculated using the chromogenic substrate K-A-R-V-L*Nph-E-A-nL-G.

again with nelfinavir being the most affected. The triple combination of D30N, M36I, and A71V ($LAI^{30/36/71}$) brought about a significant increase (>1000%) in the K_i values of ritonavir (1757%), nelfinavir (2067%), and AG1776 (1424%) compared to that of LAI^{wt}. AG1776 was the only inhibitor that retained a subnanomolar K_i for the triple mutant. $LAI^{30/36/71}$ displayed only a slight increase in the K_i value of indinavir and KNI-272 of 145% and 193%, respectively, compared to LAI^{wt}.

Relative Vitality Values. Relative vitality values (Figure 2) were calculated for each mutant with each inhibitor using the chromogenic substrate. Relative vitality values give a measure of the viability of a mutant in the presence of a particular inhibitor compared to a reference inhibitor and the wild-type enzyme. In our study we chose indinavir as our reference inhibitor because it showed the least potency to the LAI^{wt} protease. Using nelfinavir as the standard would require the other inhibitors to overcome their binding superiority to the wild-type enzyme when comparing the ability of the mutant enzyme to survive in the presence of the tighter binding inhibitors. A value greater than one would be predictive of a virus able to survive in the presence of that particular inhibitor. The triple mutant $LAI^{30/36/71}$ showed a large increase in relative vitality with nelfinavir, ritonavir, and indinavir. The other mutants showed no increase in relative vitality.

DISCUSSION

In the process of escaping inhibitor binding, many of the mutations that accumulate in HIV PR will compensate for a replication defect and/or increase drug resistance. Many of these mutations occur outside of the protease active site and are termed secondary mutations. Among these, others have studied positions 10, 20, 35, 46, 54, 63, 77, 88, and 90 (*8*, *10*, *17*, *32*−*35*). We analyzed the secondary mutations M36I and A71V in the absence and presence of the active site mutation D30N because (a) these two residues are located at the interfaces between structural subdomains, (b) they arise in both in vitro and in vivo studies in response to treatment with several protease inhibitors, and (c) they occur as natural polymorphisms. The D30N mutation is also termed a primary mutation because it is associated with causing a substantial decrease in the binding affinity of inhibitors and is associated with decreasing the catalytic efficiency of the enzyme. To analyze the catalytic efficiency of the mutants, kinetic analyses were performed using a synthetic chromogenic substrate that mimics the CA-p2 site. This site has been shown to be important for the ordered cleavage and maturation of virus particles (*36*, *37*).

The crystal structure of the HIV-1 protease complexed with a peptide containing the wild-type sequence of the CA-p2 cleavage site from P5 to P5′, Lys-Ala-Arg-Val-Leu*Ala-Glu-Ala-Met-Ser, showed that the D30 residue side chain was involved in hydrogen bonding to the GluP2′ side chain (*38*). This is the only direct hydrogen-bonding interaction detected between the enzyme and a side chain of the substrate. Mahalingam et al. showed the D30N variant has a slightly increased k_{cat}/K_m for a peptide containing the wild-type sequence of the CA-p2 site shown above due to a decrease in K_m (*39*). In our study, modification of position 30 of the protease from an Asp to Asn produced a 51% decrease in the k_{cat}/K_m of the enzyme due to a 50% increase in K_m and a 20% decrease in k_{cat}. It had been shown that residue changes in both the peptide and the enzyme subsites, including position 30, will influence the specificity of the enzyme for adjacent or juxtaposed substrate residues (*40*, *41*). The crystal structure of the peptide Arg-Val-Leu*Phe-Glu-Ala-Nleu in the active site of the wild-type enzyme (PDB file 1DW6) and D30N mutant enzyme (PDB: file 1FFF) is shown in Figure 3 (*42*,*43*). This peptide contains a Phe group in the P1′ site and norleucine in the P4′ instead of the wild-type residues alanine and methionine, respectively. Analysis of residues 30 and 30′ in the structures of the wild-type and mutant enzymes showed an RMSD of 0.68 and 0.17 Å, respectively. Analyses of the side chains of residues V82 and V82′, which are key residues in the S1 and S1′ pockets, showed an RMSD of 1.2 and 1.3 Å, respectively. The side chains of the peptide in the active site of the two structures between the P2 and P2′ residues showed a range in RMSD of 0.1−0.2 Å. This suggested that the

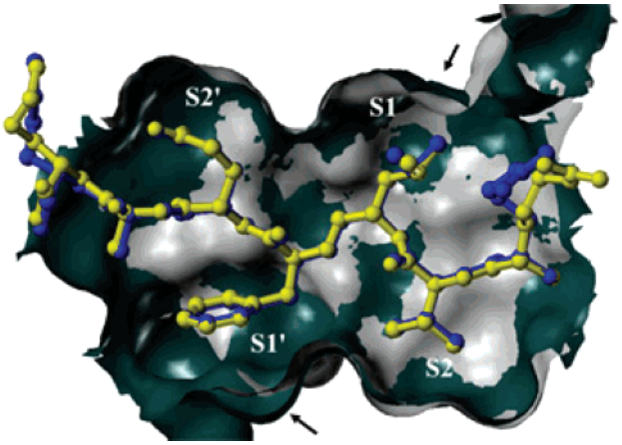

FIGURE 3: Substrate binding. Bird's-eye view of the crystal structures of HIV-1 protease (PDB file 1DW6, resolution 1.88 Å (*39*)) and D30N mutant protease (PDB file 1FFF, resolution 1.9 Å (*43*)) active site overlap. The active site floor and sides are shown as a Connolly surface in dark gray for the 1DW6 structure and light gray for the 1FFF structure. The reduced peptide inhibitor, R-V-L-r-F-E-A-Nleu, in the active site is shown as a ball-and-stick model in yellow for the 1DW6 and blue for the 1FFF structure. Arrows point to surface differences in the structure of the S1 and S1′ pockets. This figure is further described in the text.

D30N mutation in the S2 and S2′ pockets has a structural effect on the packing of the S1 and S1′ pockets, but did not affect the position of the core fragment of the substrate, allowing for efficient cleavage. These results suggested that the changes in kinetics due to the D30N mutation determined in our study are due not to the loss of side chain hydrogen bonding between Asn 30 and the P2′ Glu, but to structural changes in the active site which affect the efficiency of the catalytic process. The presence of the pNO_2-Phe at the P1′ position in our chromogenic substrate increases the catalytic efficiency of the wild-type enzyme compared to the wild-type substrate sequence due to a better fit in the S1′ pocket (*44*). It is possible that the changes in packing of the S1 and S1′ pockets of the D30N mutant, as mentioned above, have greater effects for binding of the bulky pNO_2-Phe group compared to the Ala found in the wild-type substrate peptide.

As would be predicted, the D30N mutation had its greatest effect on the binding strength of nelfinavir, which could be due to loss of the H-bond interaction of D30 with the *m*-phenol group of the 2-methyl-3-hydroxybezamide side chain of nelfinavir in the S2 pocket. To assess if this is indeed the cause of the decrease in binding strength, a docking simulation was performed using the FlexiDock module in the Sybyl 6.9 molecular modeling program. The crystal structure of HIV-1 protease complex with nelfinavir (PDB file 1OHR) was mutated at positions 30, 36, and 71 to Asn, Ile, and Val, respectively, and energy minimization was performed as described in the Experimental Procedures. Docking simulations were performed on enzyme containing D30N ($1OHR^{30}$) and enzyme containing D30N, M36I, and A71V ($1OHR^{30/36/71}$). All single nonaromatic bonds were allowed rotational freedom in the ligand. In the first docking simulation the N30 side chain was allowed rotational freedom. In the second docking simulation the N30 side chain was held fixed. From the second docking simulation the five best ranked structures were used for further analysis. These structures predicted that the D30N mutant would maintain the H-bonding interaction between 30N and 2-methyl-3-hydroxybenzamide since neither the N30 side chain nor the side chain of nelfinavir in the S2 pocket showed much deviation from the original structure. Figure 1 shows the best docking solution for $1OHR^{30}$ and $1OHR^{30/36/71}$. All structures maintained a hydrogen-bonding distance from 2.9 to 3.3 Å. Although hydrogen-bonding ability was maintained, analysis of the N30 interaction with nelfinavir using the computational program HINT predicts a decreased interaction strength of the N30 residue in both the single ($1OHR^{30}$) and triple ($1OHR^{30/36/71}$) modeled variants with the ligand at the P2 pocket due mainly to loss in hydrogen-bonding strength and acid−base interactions, Table 3.

Table 3: Hint Scores for Position 36, 71, and 30 Interactions[a]

residue	hydrogen bond	acid−base	hydrophobic	unfavorable interactions	total
10HR M36	0	51	232	−557	−274
10HR M36′	0	205	167	−748	−376
$10HR^{30/36/71}$ I36	0	0	369	−387	−18
$10HR^{30/36/71}$ I36′	0	191	321	−752	−240
10HR A71	0	0	287	−774	−487
10HR A71′	0	0	235	−772	−537
$10HR^{30/36/71}$ V71	0	0	253	−847	−594
$10HR^{30/36/71}$ V71′	0	0	245	−885	−640
10HR D30−Nel	837	260	0	−590	507
10HR D30′−Nel	0	0	21	−201	−180
$10HR^{30/36/71}$ N30−Nel	323	66	0	−296	93
$10HR^{30/36/71}$ N30′−Nel	0	0	0	−212	−212
$10HR^{30}$ N30−Nel	284	61	0	−236	109
$10HR^{30}$ N30′−Nel	0	0	0	−223	−223

[a] A = chain A, B = chain B. wt = wild type−nelfinavir structure 1OHR (PDB file). 30/36/71 = structure with mutations D30N, M36I, and A71V. D30N = structure with D30N mutation. Nel = nelfinavir. − = interaction. Unfavorable interactions, polar−polar, hydrophobic−polar, and acid−acid, are grouped into one value.

Positions 36 and 71 occupy regions in the protease that are highly mobile during flap opening and closing in the course of ligand binding (*45*). M36I and A71V may promote long-range structural changes to the active site or changes in the flexibility of the protease which may lead to the closed or open conformation of the protease being dominant. These mutations do not affect the structural integrity of the protease when analyzed by urea denaturation (results not shown). Analysis of the M36I and A71V mutation using the HINT program predicted an increase and decrease, respectively, in the interaction strength of I36 and V71 in chains a and b of the protease with the surrounding atoms, Table 3. Adopting the model that the I36 region is a fulcrum and the V71 region is a cantilever for flap opening and closing, these changes in interactions would alter the dynamics of opening and closing of the active site. Since inhibitors are rigid and are designed to bind the closed conformation, they would preferentially bind to enzymes that carry mutations that favor the closed conformation. The increase in the binding strength of the substrate and some inhibitors would suggest that the mutants LAI^{36}, LAI^{71}, and $LAI^{36/71}$ favor the closed conformation compared to LAI^{wt}. We also saw a decrease in k_{cat} for these mutants. It is expected that an enzyme that favors the closed conformation would show a decrease in k_{cat} due to a slow release of the cleavage products.

Surprisingly, the addition of mutation M36I or A71V to the D30N mutation did not further decrease the binding strength of nelfinavir compared to that of LAI^{30}. The mutants $LAI^{30/36}$ and $LAI^{30/71}$ showed no significant change in

inhibitor binding to the other inhibitors compared to the LAI^{wt} protease. These mutants did experience a drop in enzyme efficiency compared to LAI^{wt} and the rest of the mutants. The $LAI^{30/36/71}$ mutant showed an additive effect, significantly decreasing the binding strength of ritonavir, nelfinavir, and AG1776 compared to LAI^{wt} and the rest of the single and double mutants. The enzymatic efficiency of $LAI^{30/36/71}$ was higher than that of LAI^{wt} and significantly higher than those of the other mutants. The mutations M36I and A71V are nonpolar conserved changes, but lie buried in the protease and would require local rearrangements to accommodate the new residues, Figure 1, Table 3. These local rearrangements coupled with the structural positions of these residues would most likely induce a cooperative conformational adjustment that propagates to mobile regions of the protease, such as the flaps. Due to the effects on the dissociation constants of both the substrate and inhibitors upon the addition of the M36I and/or the A71V mutation to the mutant LAI^{30}, exemplified by nelfinavir (probably because the D30N mutation is specific for nelfinavir), we propose that these secondary mutations have an effect on the enzyme that causes a change in the dynamic interactions during ligand binding. Maschera et al. described the interaction of saquinavir and variants containing the mutations G48V and/or L90M. These variants experienced an increase in the off rate of saquinavir but maintained the same on rate as the wild-type enzyme (*46*). Our results suggest that, when added onto the D30N mutation, the M36I and A71V mutations promote the open conformation of the protease.

It is now clear that the use of protease inhibitors is one of the most promising therapies for controlling HIV-1 infection. The rapid development of protease-resistant codon variations under the selective pressure of inhibitors has become a tremendous challenge to drug discovery and development. With the presence of these mutations in therapy-naive patients and in the transmission of HIV-1-resistant variants at the time of infection, it is clear that a better understanding of these variations is required for treatment decisions. Secondary mutations (i.e., M36I and A71V) are commonly seen as wild-type polymorphisms in subtype B as well as in many non-B subtypes (*47*−*49*). The effect residue variations found in pretherapy non-B subtypes have on inhibitor binding is still controversial (*50*). Our study showed that the presence at the start of therapy or the addition of the secondary mutations M36I and A71V during therapy will give the virus an advantage in the rapid development of drug resistance and in the maintenance of said resistance, leading to virus viability in the presence of a specific inhibitor as shown by relative vitality values, Figure 2. In the context of D30N and nelfinavir our data are in agreement with a recent publication where nelfinavir-specific mutations are characterized (*14*). Our data also demonstrated that the D30N mutation in combination with M36I and A71V provides a high level of cross-resistance, specifically to ritonavir. This is of interest since these combinations of mutations are not seen in response to inhibitors other than nelfinavir. For these reasons the use of pretherapy and therapy resistance testing, especially in cases of early failure, is crucial for providing optimum treatment potency. Though our in vitro analysis is not without its limitations, it should prove useful in the design of new and continuing therapy.

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REFERENCES

1. Hammer, S. M., Squires, K. E., Hughes, M. D., Grimes, J. M., Demeter, L. M., Currier, J. S., Eron, J. J., Jr., Feinberg, J. E., Balfour, H. H., Jr., Deyton, L. R., Chodakewitz, J. A., and Fischl, M. A. (1997) *N. Engl. J. Med. 337*, 725−733.
2. Wilson, S. I., Phylip, L. H., Mills, J. S., Gulnik, S. V., Erickson, J. W., Dunn, B. M., and Kay, J. (1997) *Biochim. Biophys. Acta 1339*, 113−125.
3. Debouck, C. (1992) *AIDS Res. Hum. Retroviruses 8*, 153−164.
4. Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A., Scolnick, E. M., and Sigal, I. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4686−4690.
5. Olsen, D. B., Stahlhut, M. W., Rutkowski, C. A., Schock, H. B., vanOlden, A. L., and Kuo, L. C. (1999) *J. Biol. Chem. 274*, 23699−23701.
6. Velazquez-Campoy, A., Vega, S., and Freire, E. (2002) *Biochemistry 41*, 8613−8619.
7. Towler, E. M., Thompson, S. K., Tomaszek, T., and Debouck, C. (1997) *Biochemistry 36*, 5128−5133.
8. Smidt, M. L., Potts, K. E., Tucker, S. P., Blystone, L., Stiebel, T. R., Jr., Stallings, W. C., McDonald, J. J., Pillay, D., Richman, D. D., and Bryant, M. L. (1997) *Antimicrob. Agents Chemother. 41*, 515−522.
9. Patick, A. K., Duran, M., Cao, Y., Shugarts, D., Keller, M. R., Mazabel, E., Knowles, M., Chapman, S., Kuritzkes, D. R., and Markowitz, M. (1998) *Antimicrob. Agents Chemother. 42*, 2637−2644.
10. Martinez-Picado, J., Savara, A. V., Sutton, L., and D_Aquila, R. T. (1999) *J. Virol. 73*, 3744−3752.
11. Devereux, H. L., Emery, V. C., Johnson, M. A., and Loveday, C. (2001) *J. Med. Virol. 65*, 218−224.
12. Sugiura, W., Matsuda, Z., Yokomaku, Y., Hertogs, K., Larder, B., Oishi, T., Okano, A., Shiino, T., Tatsumi, M., Matsuda, M., Abumi, H., Takata, N., Shirahata, S., Yamada, K., Yoshikura, H., and Nagai, Y. (2002) *Antimicrob. Agents Chemother. 46*, 708−715.
13. Cabana, M., Fernandez, G., Parera, M., Clotet, B., and Martinez, M. A. (2002) *Virology 300*, 71−78.
14. Perrin, V., and Mammano, F. (2003) *J. Virol. 77*, 10172−10175.
15. Rose, R. B., Craik, C. S., and Stroud, R. M. (1998) *Biochemistry 37*, 2607−2621.
16. Gatanaga, H., Suzuki, Y., Tsang, H., Yoshimura, K., Kavlick, M. F., Nagashima, K., Gorelick, R. J., Mardy, S., Tang, C., Summers, M. F., and Mitsuya, H. (2002) *J. Biol. Chem. 277*, 5952−5961.
17. Condra, J. H., Schleif, W. A., Blahy, O. M., Gabryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M., and et al. (1995) *Nature 374*, 569−571.
18. Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H. M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G. R., Ho, D. D., Boucher, C. A., Leonard, J. M., Norbeck, D. W., and Kempf, D. J. (1996) *Nat. Med. 2*, 760−766.
19. Perno, C. F., Cozzi-Lepri, A., Balotta, C., Bertoli, A., Violin, M., Monno, L., Zauli, T., Montroni, M., Ippolito, G., and d'Arminio-Monforte, A. (2002) *Aids 16*, 619−624.
20. Coffin, J. M. (1995) *Science 267*, 483−489.
21. Perez, E. E., Rose, S. L., Peyser, B., Lamers, S. L., Burkhardt, B., Dunn, B. M., Hutson, A. D., Sleasman, J. W., and Goodenow, M. M. (2001) *J. Infect. Dis. 183*, 579−588.
22. Barrie, K. A., Perez, E. E., Lamers, S. L., Farmerie, W. G., Dunn, B. M., Sleasman, J. W., and Goodenow, M. M. (1996) *Virology 219*, 407−416.
23. Ido, E., Han, H. P., Kezdy, F. J., and Tang, J. (1991) *J. Biol. Chem. 266*, 24359−24366.
24. Goodenow, M. M., Bloom, G., Rose, S. L., Pomeroy, S. M., O'Brien, P. O., Perez, E. E., Sleasman, J. W., and Dunn, B. M. (2002) *Virology 292*, 137−149.

25. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., and Alizon, M. (1985) *Cell 40*, 9−17.
26. Dunn, B. M., Gustchina, A., Wlodawer, A., and Kay, J. (1994) *Methods Enzymol. 241*, 254−278.
27. Bhatt, D., and Dunn, B. M. (2000) *Bioorg. Chem. 28*, 374−393.
28. Gulnik, S. V., Suvorov, L. I., Liu, B., Yu, B., Anderson, B., Mitsuya, H., and Erickson, J. W. (1995) *Biochemistry 34*, 9282−9287.
29. Purcell, W. P., and Singer, J. A. (1967) *J. Phys. Chem. 71*, 4316.
30. Burnett, J. C., Botti, P., Abraham, D. J., and Kellogg, G. E. (2001) *Proteins 42*, 355−377.
31. Yoshimura, K., Kato, R., Yusa, K., Kavlick, M. F., Maroun, V., Nguyen, A., Mimoto, T., Ueno, T., Shintani, M., Falloon, J., Masur, H., Hayashi, H., Erickson, J., and Mitsuya, H. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8675−8680.
32. Mahalingam, B., Boross, P., Wang, Y. F., Louis, J. M., Fischer, C. C., Tozser, J., Harrison, R. W., and Weber, I. T. (2002) *Proteins 48*, 107−116.
33. Mammano, F., Trouplin, V., Zennou, V., and Clavel, F. (2000) *J. Virol. 74*, 8524−8531.
34. Nijhuis, M., Schuurman, R., de Jong, D., Erickson, J., Gustchina, E., Albert, J., Schipper, P., Gulnik, S., and Boucher, C. A. (1999) *Aids 13*, 2349−2359.
35. Velazquez-Campoy, A., Todd, M. J., Vega, S., and Freire, E. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 6062−6067.
36. Pettit, S. C., Moody, M. D., Wehbie, R. S., Kaplan, A. H., Nantermet, P. V., Klein, C. A., and Swanstrom, R. (1994) *J. Virol. 68*, 8017−8027.
37. Wiegers, K., Rutter, G., Kottler, H., Tessmer, U., Hohenberg, H., and Krausslich, H. G. (1998) *J. Virol. 72*, 2846−2854.
38. Prabu-Jeyabalan, M., Nalivaika, E., and Schiffer, C. A. (2000) *J. Mol. Biol. 301*, 1207−1220.
39. Mahalingam, B., Louis, J. M., Reed, C. C., Adomat, J. M., Krouse, J., Wang, Y. F., Harrison, R. W., and Weber, I. T. (1999) *Eur. J. Biochem. 263*, 238−245.
40. Ridky, T. W., Cameron, C. E., Cameron, J., Leis, J., Copeland, T., Wlodawer, A., Weber, I. T., and Harrison, R. W. (1996) *J. Biol. Chem. 271*, 4709−4717.
41. Lin, Y., Lin, X., Hong, L., Foundling, S., Heinrikson, R. L., Thaisrivongs, S., Leelamanit, W., Raterman, D., Shah, M., Dunn, B. M., and et al. (1995) *Biochemistry 34*, 1143−1152.
42. Weber, I. T., Wu, J., Adomat, J., Harrison, R. W., Kimmel, A. R., Wondrak, E. M., and Louis, J. M. (1997) *Eur. J. Biochem. 249*, 523−530.
43. Mahalingam, B., Louis, J. M., Hung, J., Harrison, R. W., and Weber, I. T. (2001) *Proteins 43*, 455−464.
44. Pazhanisamy, S., Stuver, C. M., Cullinan, A. B., Margolin, N., Rao, B. G., and Livingston, D. J. (1996) *J. Biol. Chem. 271*, 17979−17985.
45. Piana, S., Carloni, P., and Parrinello, M. (2002) *J. Mol. Biol. 319*, 567−583.
46. Maschera, B., Darby, G., Palu, G., Wright, L. L., Tisdale, M., Myers, R., Blair, E. D., and Furfine, E. S. (1996) *J. Biol. Chem. 271*, 33231−33235.
47. Frater, A. J., Beardall, A., Ariyoshi, K., Churchill, D., Galpin, S., Clarke, J. R., Weber, J. N., and McClure, M. O. (2001) *Aids 15*, 1493−1502.
48. Perno, C. F., Cozzi-Lepri, A., Balotta, C., Forbici, F., Violin, M., Bertoli, A., Facchi, G., Pezzotti, P., Cadeo, G., Tositti, G., Pasquinucci, S., Pauluzzi, S., Scalzini, A., Salassa, B., Vincenti, A., Phillips, A. N., Dianzani, F., Appice, A., Angarano, G., Monno, L., Ippolito, G., Moroni, M., and d'Arminio Monforte, A. (2001) *J. Infect. Dis. 184*, 983−991.
49. Palmer, S., Alaeus, A., Albert, J., and Cox, S. (1998) *AIDS Res. Hum. Retroviruses 14*, 157−162.
50. Frater, J., Dunn, D., Weber, J. N., and McClure, M. O. (2002) *J. Infect. Dis. 185*, 1376; author reply 1376−1377.

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