ABSTRACT 49: EMERGENCE OF A NOVEL LOPINAVIR RESISTANCE MUTATION AT CODON 47 CORRELATES WITH ARV UTILIZATION

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XII International HIV Drug Resistance Workshop 10-14 June 2003, Los Cabos, Mexico

Abstract

OBJECTIVES: Multiple primary PR mutations are required to produce a high level of resistance to lopinavir. These mutations may be selected through ARV therapy with other PIs, leading to LPV cross resistance. We identified a novel I47A LPV-resistant variant and found that the emergence of mutations at codon 47 is highly correlated with increasing clinical LPV prescribing.

METHODS: Genotypes and mutational frequencies were obtained from records of DNA sequencing of clinical samples from the Quest Diagnostics HIV-1 sequence database. Quarterly ARV prescription data were obtained from Scott Levin Source™ Prescription Audit. Phenotypes were determined by the Antivirogram™ assay. Changes in binding energy were calculated from models of mutant PR-inhibitor complexes built by amino acid substitutions in the wild type complex with subsequent energy minimization of the ligand and PR binding site residues.

RESULTS: We identified the I47A mutation in 45/51,983 clinical specimens genotyped after LPV became available in late 2000. None of 24,426 samples genotyped prior to Q4'2000 had the I47A mutation (χ^2 = 19.7 p < 0.0001). Phenotypic data obtained for five I47A mutants showed high levels of LPV resistance (86x to >110x) that was not predicted by genotypic analysis. Molecular modeling and energy calculations for these variants showed binding energy changes (ΔE_{bind}) for LPV between 1.9 and 3.1 kcal/mol, consistent with high levels of LPV resistance (20x to >100x). I47V variants had smaller ΔE_{bind} changes (0.2 to 2.0 kcal/mol). In contrast to LPV, I47A variants were hypersusceptible to SQV, and showed improved binding to the ligand in PR-SQV complex models. I47V is associated clinically with resistance to amprenavir (APV) but not to LPV. I47V frequency has increased 4x from 2000 to 2002. Spearman correlations between APV or LPV prescription utilization and I47V frequencies were not significant for APV (r_s= 0.34, P = 0.21) but were highly significant for LPV (r_s= 0.95, P < 0.0001). I47V results from an A -> G nucleotide change, whereas I47A requires two nucleotide changes (ATA -> GCA). A single substitution is required for I47V -> I47A (GTA -> GCA). We identified five I47A mutants in specimens with prior sequences containing I47V, suggesting that the I47A variant emerges via a two-step pathway. In the PR-LPV complex model, the Ile side chain of residue 47 is positioned close to the LPV phenoxyacetyl moiety and its VDW interactions may contribute to the ligand binding. However, these interactions are lost for the smaller A47. The I47A PR-SQV complex models showed tighter packing of the PR flap around SQV, which results in additional hydrogen bonding interactions.

CONCLUSIONS:

We have identified a second pathway to a high level LPV resistance that does not result from cross resistance to other PIs. Increased usage of LPV correlates with increased frequencies of the I47V mutation leading to the stepwise emergence of the LPV-resistant I47A variant. The apparent increased susceptibility of these variants to SQV may have clinical implications for PI therapy. Surveillance of emerging resistance in large clinical databases may be facilitated by structural phenotypic methods that enable rapid computational determinations of the significance of newly identified mutational patterns.

Introduction

HIV-1 protease is essential for the cleavage of the viral polyprotein precursor of the *gag* and *pol* viral proteins (Kohl et al 1988). Antiviral drugs targeting the active site of HIV-1 protease have proven to be potent inhibitors of viral replication in infected individuals (Pallela et al 1998). Incomplete suppression of viral replication can lead to the mutation of one or more PR amino acid residues resulting in PI resistance. Such mutations have been identified in the HIV-1 patient population for all commercially available PIs (Hirsch et al. 2000, D'Aquila et al. 2003). The new PI lopinavir, introduced into clinical use at the end of 2000, has shown significant antiretroviral potency in therapy naïve and in single PI experienced patients when co-administered with ritonavir (LPV/r; Murphy et al 2001, Benson et al 2002). Mutations occurring at 11 PR codons in clinical isolates have been associated with LPV resistance including positions 10, 20, 24, 46, 53, 54, 63, 71, 82, 84 and 90 (Kempf et al 2001). Increasing numbers of mutations at these positions correlates to increased fold changes in phenotypic IC_{50}S for LPV and a mutational index between four and six mutations often predicts a greater than tenfold increase in IC_{50} (Kempf et al 2001). Recent work has identified 12 additional PR positions correlated with clinically significant resistance to LPV (Isaacson et al 2001, Paulsen et al 2002, Parkin et al 2003). The PR mutations I47V and I47A have been selected through *in vitro* passaging experiments with increasing concentrations of LPV, and confer a high level resistance to LPV (Carrillo et al 1998). The I47A variant was also observed in two variants with high level LPV resistance in a clinical database (Parkin et al 2003). In this work, we report that the increasingly frequent appearance of the I47A mutation in clinical isolates correlates with LPV drug utilization trends and results in high levels of phenotypic LPV resistance as well as saquinavir hypersusceptibility.

Methods

Sequence analysis and phenotyping: HIV-1 RNA was extracted from plasma samples submitted for RT and PR genotype determination by Quest Diagnostics. The PR and the RT genes were amplified by RT PCR and sequenced on an ABI 3700 capillary sequencer. Sequence data was assembled and compared to the subtype B consensus sequence using Sequencher™ (Genecodes Corp.) software. Antivirogram™ HIV-1 phenotype assays were carried out by Tibotec-Virco NV (http://www.tibotec-virco.com).

Data analysis: ARV drug utilization data was obtained from Scott Levin Source™ Prescription Audit. Spearman correlations and chi-square tests were performed with Analyse-it™ for Microsoft Excel™ (http://www.analyse-it.com).

Structural analysis: Molecular models of the wild-type HIV-1 PR–inhibitor complexes were built starting from their crystal structures (Shenderovich et al 2001, 2003). Molecular modeling and simulations were performed using the ICM program, version 2.7 (Abagyan et al, 1994; ICM Manual, 1999). Modeling of mutant HIV protease – inhibitor complexes and binding energy calculations for PR-inhibitor complexes was performed as previously described (Shenderovich et al 2001, 2003).

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Figure 1: Increasing frequency of I47[VA] mutations and LPV prescribing

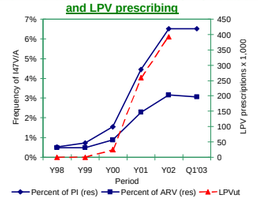

Fig. 1: proportion of variants submitted for testing between 1998 and the first quarter of 2003 with valine or alanine substitutions at PR codon 47. Variants with no predicted ARV resistances were excluded from this analysis. Filled squares: percent of I47V/A variants resistant to at least one ARV (PI or RTI). Filled diamonds: percent of I47V/A variants resistant to at least one PI. Triangles: the number of LPV prescriptions x 1,000 for the given period.

Table 1: Atypical LPV-resistant and SQV hypersusceptible genotypes

	Fold Phenotypic Resistance						
sample	*APV*	*IDV*	*LPV*	*NFV*	*RTV*	*SQV*	*PR mutations detected*
166701	26.4	15.1	**>91.5**	31.0	10.9	**0.3**	**K20R,V32I,E35D,M36I,N37D,I47A,I62V,L63P,A71TA ,L90M,I93L**
genotype	*S*	*R*	*S*	*R*	*R*	*R*	
164917	13.3	NA	**>102.5**	5.7	0.5	**0.3**	**L10F,T12P,K14R,I15V,G16E,K20T,V32I,E34Q,I47A,I 62V,L63H,A71T,V77I,L90M,I93L**
genotype	*S*	*R*	*S*	*R*	*R*	*R*	
165220	21.2	1.0	**86.2**	2.7	2.8	**0.2**	**T12P,I13V,N37D,M46L,I47A,R57K,L63P,A71T,V82I**
genotype	*S*	*S*	*S*	*S*	*S*	*S*	
175236	7.7	2.3	**>109.6**	7.2	5.7	**0.1**	**I13V,V32I,L33F,R41K,K43R,K45R,I47A,L63P,A71T, G73A,V77I,L90M,I93L**
genotype	*S*	*R*	*S*	*R*	*R*	*R*	
175848	>47.3	>95.0	**>95.8**	>45.3	14.0	**0.7**	**T4A,L10F,I13V,K20T,V32I,L33V,M46I,I47A,I62V, L63P,H69Y,A71V,L89V,L90M,I93L**
genotype	*S*	*R*	*P*	*R*	*R*	*R*	

Table 1: PI phenotypic fold resistances (Virco Antivirogram™), genotypic predictions (Quest Diagnostics Jan-03 algorithm) and PR mutations identified in five variants with the I47A PR mutations. S: susceptible; R: resistant; P: probable resistance

Fig. 2: Spearman correlation of LPV or APV utilization and I47V/A mutation frequency

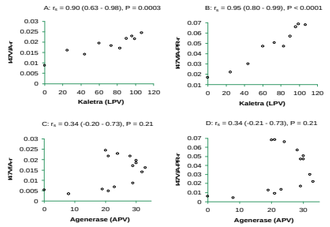

Fig. 2: Frequency of the I47V/A PR variants in the Quest database plotted against ARV utilization (thousands of prescriptions; Scott Levin Source™ Prescription Audit). I47V/A-r: I47V or A variants as a proportion of samples with any predicted ARV resistance. I47V/A-PR-r: I47V or A variants as a proportion of samples with at least one genotypically predicted PI resistance.

Fig. 3: LPV bound to superimposed I47 and A47 PR Variants

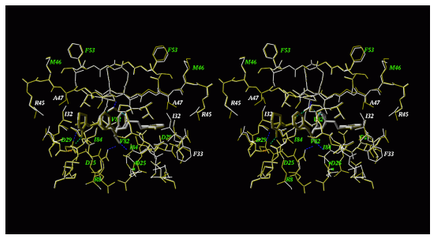

Fig. 3: Superimposed stereo views of the Lopinavir complexes with WT PR (a model derived from the crystal structure; yellow) and the 175236_47A mutant PR variant (white). The ligand and the flap water molecule are shown as stick models. Mutated residues are labeled in white, other important residues are labeled in green. Note that 3D structure of the PR-LPV complex shows little change upon the I47A mutation, which mainly reduces the energy of van der Waals ligand-protein interactions.

Results

We noted a high degree of phenotypically-predicted lopinavir (LPV) and amprenavir (APV) resistance in several clinical HIV-1 PR variants, which was not predicted from the PR genotype of these same variants (**Table 1**). Four of the five variants showed phenotypic hypersensitivity to SQV (fold resistance from 0.1 to 0.3) yet also demonstrated multiple PR mutations predictive of SQV resistance, including the L90M mutation. The I47A mutation was present in all five variants. We investigated its possible involvement in this unusual resistance profile. Forty five of 51,983 samples sequenced from the fourth Quarter of 2000 through early 2003 had an I47A mutation but none of 24,426 samples genotyped prior to Q4'2000 had the I47A mutation (χ^2 = 19.7 p < 0.0001). The first I47A variant appeared in the Quest Diagnostics database dates in July 2001, nine months after the approval of lopinavir for clinical use. A valine substitution at residue 47 has previously been associated with APV resistance and often occurs together with V32I (Maguire et al 2002, Wu et al 2003). APV was approved for clinical use in April 1999, and I47V substitutions became increasingly frequent beginning in 2000 (**Fig. 1**).

The increasing frequency of I47V/A mutations was tested for correlation with ARV prescribing of APV and LPV. No significant correlation between APV utilization and I47V/A mutation frequency (**Fig. 2c,d**: r_s= 0.34, P = 0.21) was noted, however I47V/A mutation frequency was highly correlated with LPV utilization (**Fig. 2a,b**: r_s= 0.95, P < 0.0001). The V32I mutation was found with I47A and I47V variants at comparable frequencies (77% and 80%). However, mutations at position 54, found in 628/942 I47V variants were only detected in 2/45 I47A variants (χ^2 = 26.1, P < 0.0001).

To investigate further the role of I47A in PI resistance, we constructed computer generated molecular models of these variants with the docked PI and calculated the changes in binding energy upon mutation (Shenderovich et al 2001, 2003). A refined crystal structure of the PR-LPV wild type complex (PDB: 1MUI), superimposed on a model of the I47A variant complex 175236_I47A, showed little variation of the 3D structure (**Fig. 3**). However, large changes in binding energy (ΔE_{bind}) for LPV were predicted for the I47A variants (2.7 +/- 0.5 kcal/mol) consistent with high levels of LPV resistance (**Table 2**). When 47V was substituted for 47A in the models, the binding energy change was significantly smaller, on average (1.3 +/- 0.75 kcal/mol), with a mean difference of -1.4 kcal/mol (95% CI: -1.9 - -0.90 kcal/mol) relative to I47A (paired t-test; P = 0.0014). These binding energy changes were principally due to the loss of van der Waals interactions (ΔE_{vw}) between the phenoxyacetyl moiety of LPV and the side chain of residue 47 (**Table 2** and **Fig. 3**).

Binding energy changes calculated for the PR-SQV complexes were negative, consistent with the apparent phenotypic hypersusceptibility to SQV shown by these variants (**Table 2**). The greatest change was observed for variant 17536, from ΔE_{bind} = + 0.9 kcal/mol for the I47 variant to ΔE_{bind} = - 1.1 kcal/mol for the A47 variant. We superimposed the model of 17536_47A PR-SQV complex on the wild type PR SQV complex and observed a tighter packing between the PR flap residues and SQV in the mutant complex (**Fig 4a**). The A47 variant formed two additional hydrogen bonds with the SQV ligand, between the central hydroxyl donor of SQV and the backbone carbonyl acceptor of the G27 residue, and between the backbone amide donor of the residue D29 and the carbonyl acceptor adjacent to the SQV quinoline moiety (**Fig. 4a**). When I47 and A47 PR-LPV complexes of the mutant variant 17536 were superimposed, the structural differences were less pronounced, however only the A47 variant formed the hydrogen bond between the residue D29 and the carbonyl acceptor adjacent to the SQV quinoline moiety (**Fig. 4b**).

The isoleucine at PR residue 47 is encoded by the codon AUA and two nucleotide substitutions would be required to yield the alanine codon, GCA. We identified five variants with two or more sequences sampled at various time points that harbored the I47V mutation and then exhibited the I47A mutation at a later time point. In 5/5 cases, the V47 variant was encoded by GUA and the subsequent I47A mutant variant was encoded by GCA, thus requiring only a single nucleotide change. We also identified a change from I47A → I47V/A in one case, as well as an I47V/A → I47V change in a second case. It is likely, therefore, that the I47A mutation emerges through a two-step process.

Table 2: Predicted PI binding energy change for I47 variants

	id	Geno	phenotype (fold)	ΔE_{el} (kcal/mol)[2]	ΔE_{vw} (kcal/mol)[2]	ΔE_{hb} (kcal/mol)[2]	ΔE_{ts} (kcal/mol)[2]	ΔE_{bind} (kcal/mol)[2]	SP fold
LPV	*164917_1 (47I)*	*S*	*NA*	*0.2*	*-0.7*	*0.4*	*0.1*	*-0.1*	1.2
	164917_2 (I47V)	*S*	*NA*	*0*	*0.8*	*0.2*	*0*	*0.9*	6.4
	164917_3 (I47A)	**S**	**>102.5**	**0.3**	**2.5**	**0.1**	**0**	**2.8**	**160**
	165220_1 (I47)	*S*	*NA*	*-0.3*	*2.2*	*0.3*	*0*	*2.1*	44.0
	65220s_2 (I47V)	*S*	*NA*	*-0.1*	*2*	*0.1*	*0.1*	*2.0*	36.5
	165220_3 (I47A)	**S**	**86.2**	**0**	**3**	**0.1**	**0**	**3.0**	**224**
	166701_1 (I47)	*S*	*NA*	*0.1*	*-0.2*	*0.4*	*0*	*0.2*	2.0
	166701_2 (I47V)	*S*	*NA*	*0.2*	*1.5*	*0.2*	*0*	*1.9*	31.3
	166701_3 (I47A)	**S**	**>91.5**	**0.2**	**2.7**	**0.3**	**0.1**	**3.1**	**244**
	175236_1 (I47)	*S*	*NA*	*0.2*	*-1.3*	*0.2*	*0.1*	*-0.9*	0.3
	175236_2 (I47V)	*S*	*NA*	*0.2*	*-0.1*	*0.1*	*0*	0.2	1.8
	175236_3 (I47A)	**S**	**>109.6**	**0.3**	**1.6**	**0**	**0**	**1.9**	**34.4**
	175848_1 (I47)	*P*	*NA*	*-0.1*	*-0.6*	*0.2*	*0.1*	*-0.4*	0.7
	175848_2 (I47V)	*P*	*NA*	*0.1*	*1.3*	*0.2*	*0*	1.6	20.7
	175848_3 (I47A)	**P**	**>95.8**	**0.2**	**2.3**	**0.3**	**0**	**2.7**	**121**
SQV	164917_3 (I47A)	R	0.3	*NA*	*NA*	*NA*	*NA*	-0.6	0.5
	165220_3 (I47A)	S	0.2	*NA*	*NA*	*NA*	*NA*	-0.3	0.6
	166701_3 (I47A)	R	0.3	*NA*	*NA*	*NA*	*NA*	-0.4	0.6
	175236_3 (I47A)	R	0.1	*NA*	*NA*	*NA*	*NA*	-1.1	0.2
	175848_3 (I47A)	R	0.7	*NA*	*NA*	*NA*	*NA*	-0.6	0.4
APV	164917_3 (I47A)	S	13.3	*NA*	*NA*	*NA*	*NA*	1.9	6.1
	165220_3 (I47A)	S	21.2	*NA*	*NA*	*NA*	*NA*	1.4	3.7
	166701_3 (I47A)	S	26.4	*NA*	*NA*	*NA*	*NA*	1.7	4.9
	175236_3 (I47A)	S	7.7	*NA*	*NA*	*NA*	*NA*	0.9	2.2
	175848_3 (I47A)	S	>47.3	*NA*	*NA*	*NA*	*NA*	2.1	7.5

Table 2: The affect of PR codon 47 substitutions on the predicted binding energy changes for LPV, SQV and APV. To assess the affect of the codon 47 substitutions on LPV binding,the variants were modeled with I47 and V47 as well as A47. ΔE_{bind} = ΔE_{el} + ΔE_{vw} + ΔE_{hb} + ΔE_{ts}; ΔE_{el}: electrostatic term; ΔE_{vw}: van der Waals term ΔE_{hb}: hydrogen bonding term; ΔE_{ts}: side chain entropy term; SP fold: fold resistance change calculated from ΔE_{bind}. [2]NA: Not Available.

Fig. 4a: SQV bound to superimposed I47 (WT) and A47 (MUT) PR Variants

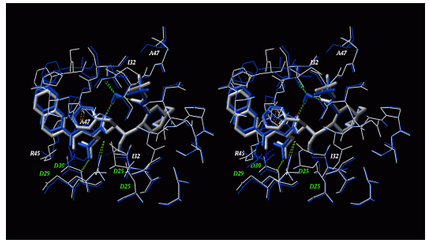

Fig. 4a. Superimposed stereo views of Saquinavir complexes with wild type HIV-PR (model based on the crystal structure; blue) and with the mutant PR variant 17536_47A (white). The ligands and flap water molecules are shown as stick models. Mutated residues close to the ligand are labeled in white; some other important PR residues are labeled in green. Ligand-protein hydrogen bonds are shown colored by donor-acceptor distance (from blue for the shortest H-bonds to green to red for the longer ones). Note the tighter packing of PR flap residues around SQV, and two additional ligand-protein hydrogen bonds in the mutant complex.

Fig. 4b: SQV bound to superimposed I47 and A47 PR Variants

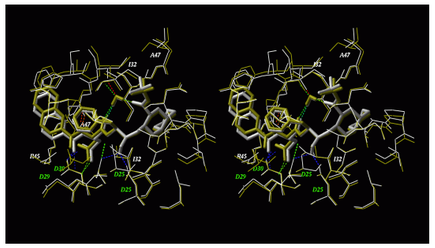

Fig. 4b: Superimposed stereo views of Saquinavir complexes with the mutant PR variants 17536_47A (white) and 17536_47I (yellow). See caption to Fig. 4a for details. Structural changes in the 17536_47I mutant complex are less pronounced than in 17536_47A mutant, and only one additional hydrogen bond is formed.

Discussion

➢ We have identified a PI resistance mutation emerging in the clinical population that had only been reported previously during in vitro passaging experiments and, in two cases, in a clinical database. This I47A substitution confers high level resistance to LPV and, although infrequent, is positively correlated with increased LPV utilization. These data are in agreement with recent studies proposing an expanded LPV mutation index, including PR residue 47 (Isaacson et al 2002, Parkin et al 2003).

➢ Most LPV-resistance associated mutations may be selected by one or more of the other five PIs in clinical use, complicating the identification of mutations specifically selected by LPV in patients with prior PI experience. Surveillance of large clinical mutation databases may facilitate the identification of patterns more specifically associated with the utilization of a particular drug.

➢ Structural phenotypic methods enable the rapid computational assessment of the significance of new mutational patterns. The loss of VDW interactions between the PR and the LPV ligand as a result of substitutions at residue 47 causes a significant loss of binding affinity for LPV. An opposing affect is seen for SQV binding to these variants. A tighter packing of the protease flap around the SQV ligand and additional hydrogen bonding between the mutant protease and the ligand result in a gain of binding affinity. The consequent increased susceptibility of these variants to SQV may have clinical implications for continued PI therapeutic avenues in PI-experienced patients harboring virus with this mutation.

➢ The alanine substitution at PR residue 47 may emerge in a stepwise fashion. The appearance of the I47V substitution may increase the likelihood of developing high level LPV resistance through the subsequent emergence of I47A. The negative correlation between I47A and mutations at residue 54, a mutation frequently associated with LPV resistance (10 + 54 + 82 combination), suggest the existence of a distinct mutational pathway to high level LPV resistance. Clinical investigations with detailed patient histories may further assist in characterizing this pathway.