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Characterization of resistant HIV variants generated by in vitro passage with lopinavir/ritonavir

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Abstract

Lopinavir (LPV, formerly ABT-378) is an HIV protease inhibitor (PI) that is co-administered with a small amount of ritonavir (RTV), which greatly increases and sustains the plasma levels of LPV. Lopinavir/ritonavir (LPV/r) has shown potent antiviral activity in both therapy-naïve and PI-experienced patients. To assess the effect of pharmacologically relevant ratios of LPV/RTV (LPV/r) on the emergence of resistant HIV in vitro, HIV-1 pNL4-3 was passaged in the presence of increasing concentrations of LPV alone and LPV/r. Passages with fixed 5/1 and 15/1 concentration ratios of LPV/r initially selected I84V and I50V/M46I mutants, respectively. Selection with LPV alone also generated the same initial mutants (I50V/M46I) as the 15/1 LPV/r passage. Further passage produced other mutations previously found to be associated with PI-resistance. Phenotypic susceptibility to both LPV and RTV decreased with successive passages, irrespective of whether RTV was present in the selection experiment. Furthermore, in the two selection experiments that included RTV (at either 5/1 or 15/1 LPV/r ratio), the IC_{50} of RTV at each passage evaluated was at least five-fold higher than the concentration of RTV employed at that passage, while the IC_{50} of LPV toward the passaged virus was similar to the concentration of LPV used at that passage, indicating that the selective pressure was attributable to LPV and not RTV.

Keywords: LPV; RTV; HIV resistance

1. Introduction

Lopinavir (LPV, formerly ABT-378) is a novel HIV protease inhibitor (PI) with approximately 10-fold greater potency in the presence of 50% human serum than ritonavir (RTV) in vitro (Sham et al., 1998; Molla et al., 1998). Pharmacokinetic studies in animals have demonstrated unremarkable bioavailability of LPV when dosed alone. In contrast, co-administration of LPV with RTV achieved mean trough plasma concentrations of LPV >75-fold in excess of its in vitro IC_{50} against wild-type viruses in the presence of 50% human serum (Bertz et al., 1999). These pharmacokinetic properties are attributable to the rapid CYP3A-mediated metabolism of LPV and its inhibition by RTV (Kempf et al., 1997). Lopinavir/ritonavir (LPV/r) in combination with reverse transcriptase (RT) inhibitors has displayed significant antiviral activity in both treatment-naïve and PI-experienced patients (Benson et al., 2002; Murphy et al., 2001). In a double-blind phase III study comparing therapy with LPV/r plus d4T/3TC to nelfinavir (NFV) plus d4T/3TC in antiretroviral therapy-naïve subjects, plasma HIV RNA <400 copies/ml after 48 weeks, was observed in 93% and 82% of LPV/r- and NFV-treated subjects, respectively (Walmsley et al., 2002). Notably, in contrast to NFV-treated subjects, no resistance to LPV was observed in isolates from LPV/r-treated subjects with plasma HIV RNA >400 copies/ml on therapy (Walmsley et al., 2002).

Previously, we have reported the in vitro selection of resistant HIV-1 by passaging the pNL4-3 strain in the presence of increasing concentrations of LPV alone (Carrillo et al., 1998). Because LPV is co-dosed with small amount of RTV, which also inhibits HIV protease, the effect of low, subtherapeutic levels of RTV on the course of resistance development is of interest. To gauge this effect, we performed parallel in vitro selection experiments with LPV alone and two different constant ratios of LPV and RTV that represent the plasma concentration ratios of LPV/r following co-dosing. The results of this study suggest that the

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Table 1
In vitro selection of HIV-1 to LPV alone or LPV/r

Passage virus (pNL4-3)	LPV/r concentrations used in selection (nM)		
	A: LPV/r = 5/1	B: LPV/r = 15/1	C: LPV alone
p1	1/0.2	1/0.067	1
p2	5/1	5/0.33	5
p3	30/6	30/2	30
p4	45/9	45/3	45
p5	60/12	60/4	60
p6	90/18	90/6	90
p7	90/18	90/6	90
p8	120/24	120/8	120
p9	180/36	180/12	180
p10	360/72	360/24	360
p11	720/144	720/48	720
p12	750/150	750/50	750
p13	750/150	750/50	750
p14	1000/200	1125/75	1125
p15	1200/240	1200/80	1200
p16	1350/270	1350/90	1350
p17	1500/300	1500/100	1650
p24	3500/700	3500/233.3	3500
p25	4000/800	3750/250	ND
p26	ND	4000/266	ND

ND: not done.

contribution of RTV to antiviral activity in vivo is likely to be negligible, and that the outcome of in vitro passage may be in part dependent on the stochastic nature of the process and/or the culture conditions.

2. Materials and methods

2.1. Generation of HIV-1 resistant to LPV/r by in vitro passage

HIV-1 variants resistant to LPV/r were selected as described previously (Carrillo et al., 1998). Briefly, MT4 cells (2×10^6) were infected with the pNL4-3 strain of HIV-1 at an MOI of 0.003 for 2 h, washed, then cultured in the presence of LPV. In two selection experiments (A and B), fixed ratios of LPV and RTV concentrations (A: 5/1, B: 15/1) were used. In parallel, pNL4-3 was passaged in presence of LPV alone (experiment C) for comparison. Viral replication was monitored by determination of p24 antigen levels in the culture supernatant (Abbott Laboratories, Chicago, IL), as well as by observation for any cytopathic effect (CPE) present in the cultures. When p24 antigen levels were positive, the viral supernatants were filtered and frozen at −80 °C for subsequent analysis. Infected cells were washed, lysed, and then stored at −20 °C for subsequent analysis. Virus was serially passaged using one aliquot of viral supernatant from the proceeding passage to infect fresh MT4 cells. The concentration of LPV used in the initial passage was 1 nM for all three selection experiments (A, B, and C) (Table 1). In passage 1 of selection A (5/1, LPV/r) and B (15/1, LPV/r), 0.2 and 0.067 nM of RTV, respectively, was added. Viruses derived from passage 1 of the selection experiments A, B and C were designated A1, B1 and C1, respectively. Following each passage, the drug concentrations in the subsequent passage were gradually increased. After 5 months of selection, the concentration of LPV was 3.5-4 μM in the final passage (A25, B26 and C24, respectively).

2.2. Determination of activity against the passaged variants in MT4 cells

MT4 cells were infected with MOI 0.003 of HIV-1 at 1×10^6 cells/ml for 1 h, washed twice to remove unadsorbed virus, resuspended to 1×10^5 cells/ml of medium, seeded in a 96-well plate at 100 μl per well, and treated with an equal volume of 1% dimethylsulfoxide (DMSO) solution of inhibitor in a series of half log dilutions in media. All experiments were performed in triplicate. The virus control culture was treated in an identical manner except no inhibitor was added to the medium. The cell control was incubated in the absence of inhibitor or virus. Plates were incubated for 5 days in a CO_2 incubator at 37 °C. On day 5, stock solution of MTT (4 mg/ml in PBS, Sigma cat. # M 2128) was added to each well at 25 μl per well. Plates were further incubated for 4 h, treated with 20% SDS plus 0.02N HCl at 50 μl per well to lyse the cells. After an overnight incubation, optical density was measured by reading the plates at 570/650 nm wavelengths on a BIO-TEK microtitre plate reader (BIO-TEK Instruments, Inc., Winooski, VT). Percent CPE reduction was calculated according to the procedure described previously (Molla et al., 1998).

2.3. Cloning and sequence analysis of the HIV protease coding region from selected passages

DNA from the infected cells was extracted, and a nested PCR protocol was employed to amplify the protease coding region. The amplified products were purified, then blunt-end ligated into the TA cloning vector (Invitrogen, Carlsbad, CA). Mini-prep plasmid DNA from individual colonies was purified, and then sequenced using an ABI-373 DNA sequencer (Applied Biosystems, Foster City, CA).

2.4. Determination of activity against mutant HIV molecular clones

Proviral mutant clones were constructed by cloning the *Apa*I-*Sma*I fragment from PCR products derived from viral supernatant into a pNL4-3-s shuttle vector. The complete sequence of the protease gene from the mutant viral population was confirmed by DNA sequencing. The mutant molecular clones were used to transfect 293T cells. Forty-eight hour post-transfection, the supernatants were assayed for p24 expression, and where positive, used to infect MT4 cells. For drug susceptibility assays, each virus at MOI of 0.003 was used to infect 10^6 MT4 cells in a volume of 2 ml. After

incubation for 1 h, infected cells were washed, resuspended in fresh RPMI medium to a density of 1×10^5/ml and plated in duplicate into 96-well plates in the presence of two-fold dilution of the appropriate drug. Five days after infection, cell-free culture supernatant was collected and the levels of p24 were determined by HIV-1 p24 ELISA (Abbott Laboratories). The IC_{50} for each compound was determined by the reduction of p24 antigen.

2.5. Molecular modeling

The crystal structure of LPV bound to HIV-1 protease (Stoll et al., 2002) was analyzed using the Insight II molecular modeling software program (Accelrys, San Diego, CA). A Connolly surface was calculated for protein residues Asp30, Val32, Val82 and Ile84 sidechains to highlight the outline of the active site region. Both symmetrical partner residues of the protein were considered for the analysis. A van der Waals surface was calculated for LPV to show the spatial occupation of the inhibitor.

3. Results

3.1. Selection of LPV-resistant HIV-1 by in vitro passage with LPV/r or LPV alone

Administration of two investigational doses of LPV/r (200/100 and 400/100 mg BID) provides plasma ratios of LPV and RTV of 5 to 1 and 15 to 1, respectively (Lal et al., 1998). Consequently, to select HIV variants resistant to LPV/r, pNL4-3 was serially passaged in MT4 cells in the presence of increasing fixed concentration ratios of LPV/r (experiment A: 5/1 and experiment B: 15/1, respectively). Concurrently, selection experiments were performed using LPV alone (Table 1). In all three selection arms, virus was initially grown in the presence of 1 nM of LPV, and the concentration of LPV was incrementally increased to 3500–4000 nM (passage p24–26), keeping constant ratios of LPV/r (5/1 = A and 15/1 = B) in selection experiments A and B. During the course of the 5-month selection procedure, viruses in each of the three selection arms were simultaneously passaged such that the concentrations of LPV increased at a nearly identical rate in each arm. The viral stocks from selected passages were titered, and the susceptibility to LPV and RTV was determined (Table 2). The IC_{50} values for LPV and RTV against passage A11 variant (0.31 and 0.97 μM, respectively) were 10- and 12-fold higher than against the pNL4-3 wild-type strain. Viruses selected from passage A17 and A25 were 54- and 100-fold resistant to LPV. These viruses (A17 and A25) also displayed 38–50-fold reduced susceptibility to RTV (Table 2). Viruses selected from passage B11 and C11 were 19- and 13-fold resistant to LPV, respectively, and further passages (B17, B26, and C24) produced highly resistant (≥31-fold) populations of variants. The phenotypic susceptibility to RTV also decreased with successive passages irrespective of whether RTV was present in the passage experiment (Tables 1 and 2). In the two selection experiments that included RTV (A, 5/1 and B, 15/1), the IC_{50} of RTV against each passaged virus was at least five-fold higher than the concentration of RTV used at each corresponding passage for selection (Tables 1 and 2). By contrast, the IC_{50} of LPV against the passaged variants closely mirrored the concentration of LPV present at each respective passage. Furthermore, the concentrations and IC_{50} of LPV increased at a similar rate in all three passage arms. Taken together, these results indicate that the presence of RTV provided no additional selective pressure over that of LPV in these in vitro passage experiments.

3.2. Identification of mutations in the protease coding region responsible for phenotypic changes

PCR amplification and sequencing of the protease gene indicated that the I84V mutation was present in LPV/r (5:1 ratio) treated virus populations by passage 6 (A6) (Fig. 1). This mutation has previously been observed when HIV was passaged in the presence of LPV alone (Carrillo et al., 1998). Following this change, a sequential accumulation of mutations at the following order of I84V/L10F/M46I/V32I/I47V/Q58E was observed from passages A9 to A25 (Fig. 1).

In contrast, the I50V/M46I double mutant was observed in selection experiments B and C (15/1 LPV/r and LPV alone, respectively) at passage 8 (B8) and passage 7 (C7), respectively (Fig. 1). These two mutations were retained in the subsequent passages of both selection B (B9–B26) and C (C9–C24). However, the additional mutations that appeared in subsequent passages were different (Fig. 1). The quadruple mutant I50V/M46I/A71V/V82F was present at passage B17, and subsequently three additional mutations (K45I, I54V, and L33F) were selected by passage B26. In passage C, two additional mutations (L10F and I47V) were selected on top of I50V and M46I by passage C17. After further passage (C24) 4 new mutations (V32I, E34Q, Q61H and E65Q) appeared.

We have previously identified mutations in the HIV-1 *gag* p7/p1 and p1/p6 proteolytic cleavage sites during in vitro selection with LPV alone (Carrillo et al., 1998). To see if similar mutations occurred during in vitro selection with LPV/r combinations, we sequenced the p7/p1 and p1/p6 junctions from several passages of selection A, B and C (data not shown). The p1/p6 cleavage site was altered from F/L to F/F (P'_1 residue Leu to Phe) in the passage A25 virus (LPV/r, 5/1), a mutation is identical to that reported previously (Carrillo et al., 1998). This alternation was absent in passaged viruses from both selections B and C (data not shown). In contrast to earlier findings (Carrillo et al., 1998), the p7/p1 cleavage site was not changed in passaged viruses from all three selection experiments (A, B and C).

Table 2
Susceptibility of the passaged variants to LPV and RTV

Virus	Concentration of compound used in selection (μM)		Mean IC_{50} (μM)[a]		Fold resistance[b]	
	LPV	RTV	LPV	RTV	LPV	RTV
pNL4-3 (WT)	NA	NA	0.03	0.08	1	1
A11	0.72	0.144	0.31	0.97	10	12
A17	1.5	0.3	1.62	3.07	54	38
A25	4.0	0.8	3.0	4.0	100	50
B11	0.72	0.048	0.57	1.75	19	22
B17	1.5	0.1	0.92	2.09	31	26
B26	4.0	0.267	3.20	3.23	107	40
C11	0.72	0	0.39	0.79	13	10
C24	4.0	0	1.79	1.70	60	21

[a] The values shown are representative of three separate experiments.
[b] Fold resistance relative to wild-type pNL4-3 IC_{50}. WT: wild-type; NA: not applicable.

3.3. Susceptibility of mutant clones to protease inhibitors

To determine the effect of genotypic changes of the passaged variants on the susceptibility to LPV and cross-resistance to other PIs, mutant clones were constructed with the specific mutations observed during selections A–C. The susceptibilities of the constructed mutants to LPV and other PIs are provided in Table 3. Clone c11-c1 (clone number 1 of the clones that derived from passage 11 of selection C), containing the I50V/M46I/E21K mutant genotype, displayed low-level (three-fold) reduced susceptibility to LPV. As expected, reduced susceptibility to amprenavir (APV, six-fold) was also noted. In contrast, the mutant clones representing the final passages in each selection (clones a25-c2, b26-c12 and c24-c1) were highly resistant to LPV, RTV and APV (range 22–177-fold, median 98-fold). Clone a25-c2, with key mutations at positions 10, 32, 46, 47, and 84 together the p1/p6 cleavage site mutation displayed >10-fold resistance to indinavir (IDV), NFV, and atazanavir (ATV), as well as seven-fold resistance to tipranavir (TPV). Similarly, the clone b26-c12, which contained L10I, L33F, K45I, M46I, I50V, I54V, A71V and V82F, was 23- and 31-fold resistant to IDV and NFV, respectively. In contrast, clone c24-c1, with key mutations at positions 10, 32, 46, 47 and 50, exhibited only modestly decreased susceptibility to IDV (three-fold) and NFV (five-fold). Notably, both b26-c12 and c24-c1 were susceptible to ATV and TPV, and all mutant clones displayed wild-type susceptibility to SQV.

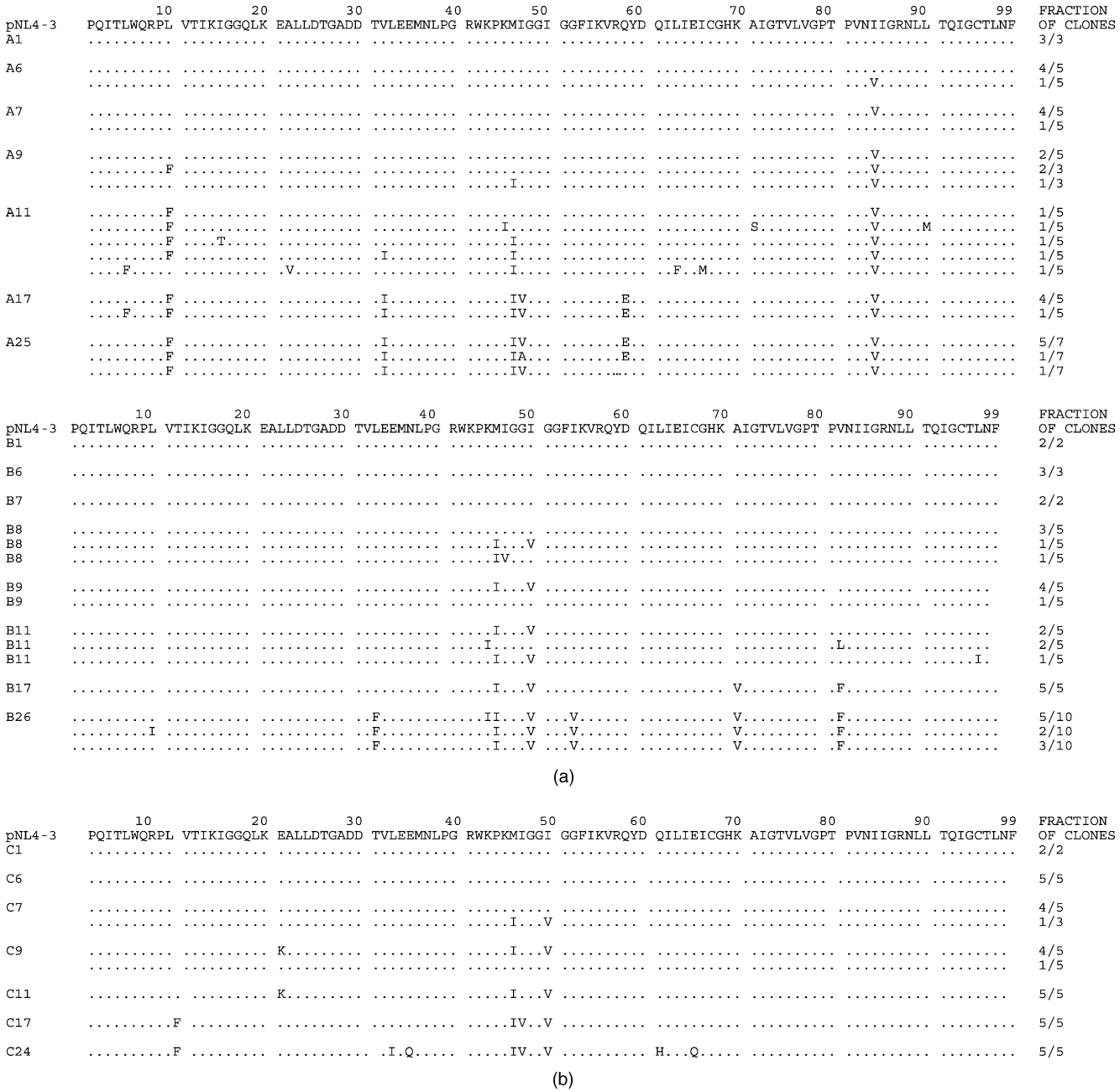
3.4. Molecular modeling of interaction of I50V and I84V mutations and LPV

To understand the potential impact of the I50V and I84V mutations observed during the selection experiments reported here, we analyzed the crystal structure of the LPV/HIV protease cocrystal structure. Residues 50 and 84 are located within the active site and make hydrophobic surface contact with central portions of LPV (Fig. 2A and B). For each change from the wild-type Ile to the mutant Val, the contact surface between protein and inhibitor decreased consistently with the decreased susceptibility of viruses containing these mutations. Specifically, the *ortho*-carbon of the p1 benzylic group of LPV makes van der Waals

Table 3
Susceptibility of mutant clones to protease inhibitors

Mutant virus[a]	Mutations	Fold resistance[b]							
		LPV	RTV	APV	IDV	NFV	SQV	ATV	TPV
pNL4-3 (IC_{50} nM)[c]	WT	1 (18)	1 (60)	1 (24)	1 (29)	1 (18)	1 (15)	1 (3)	1 (120)
a25-c2	L10F/V32I/M46I/I47V/Q58E/I84V + F/F in p1/p6 cleavage site	62	44	99	18	13	2	19	7
b26-c12	L10I/L33F/K45I/M46I/I50V/I54V/A71V/V82F	103	177	141	23	31	1	1	3
c11-c1	E21K/M46I/I50V	3	2	6	2	1	1	1	2
c24-c1	L10F/L23I/V32I/E34Q/M46I/I47V/I50V	91	22	98	3	5	1	2	1

[a] Mutant viruses were named to reflect the corresponding passages and the clone numbers in which that particular protease and cleavage site sequence were observed.
[b] Fold resistance = IC_{50} of mutant/IC_{50} of wild-type pNL4-3. LPV, lopinavir; RTV, ritonavir; APV, amprenavir; IDV, indinavir; NFV, nelfinavir; SQV, saquinavir; ATV, atazanavir; TPV, tipranavir.
[c] The values in parentheses are wild-type IC_{50} values. The IC_{50} values of mutant viruses (not shown) can be calculated by IC_{50} of wild-type x the fold resistance. WT: wild-type.



(a)

(b)

Fig. 1. Amino acid sequence alignment of the protease coding region from HIV-1 variants selected with LPV/r (a) or LPV alone (b). The amino acid sequence of the protease coding region from clones derived from different passages of three selection experiments (A, B and C) is indicated. The passaged viruses were named to reflect their corresponding selection experiment and the passage number. For example, virus derived from passage 1 of selection experiment A (LPV/r = 5/1) was designed as A1. The top line shows the protease sequence of the wild-type pNL4-3 clone. Identity with this sequence at individual positions is indicated by dots. The fraction of clones containing each unique protease sequence is indicated to the right.

contacts of 3.6 and 4.8 Å, respectively, with the terminal methyl groups of the Ile residues 50 and 84. Upon mutation to valine, these distances expand to 4.8 and 5.8 Å, respectively, reflecting loss of van der Waals contact and individually creating unoccupied volumes within the active site of approximately the size of one water molecule. Because HIV protease is C_2-symmetric, the unoccupied volume is created on both sides of the active site. Given the extreme hydrophobic character of this region with no opportunities for polar or hydrogen bonding interactions, actual occupation of these volumes by water is unlikely. With unoccupied volume, the enthalpy of binding of LPV to the mutant enzymes would be expected to be lower, corresponding to a loss of affinity.

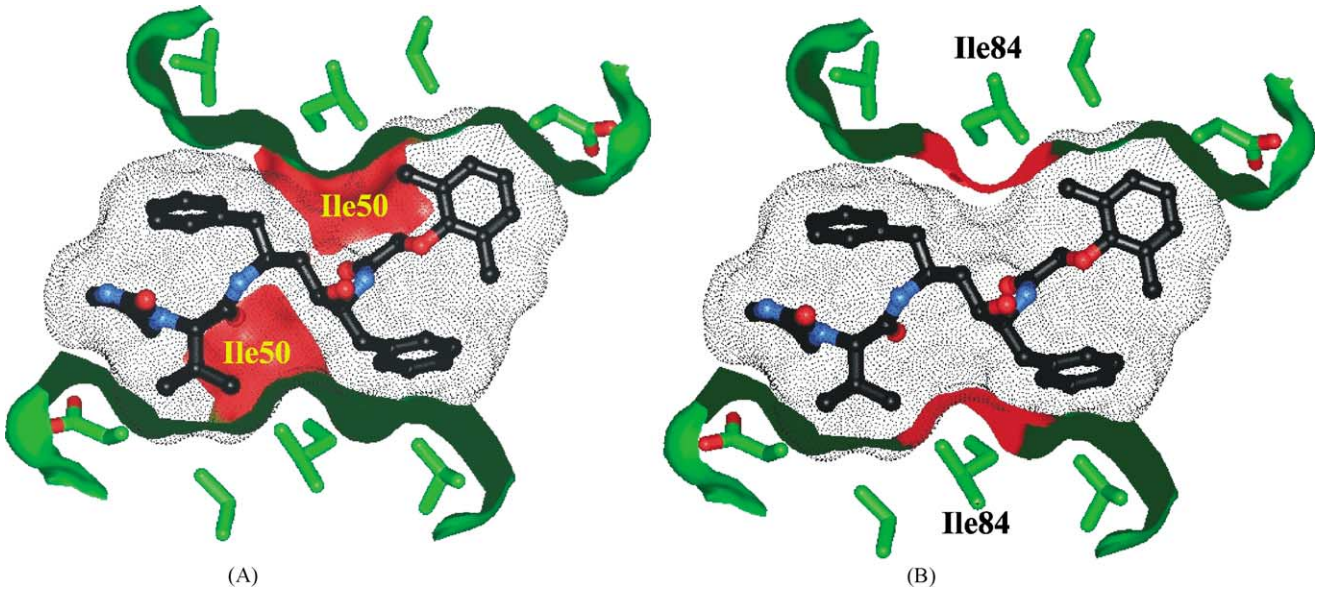


Fig. 2. Crystal structure of LPV/HIV-1 protease. Protein residues Asp30, Val32, Val82 and Ile84 are shown in green with accompanying protein surface in green to outline the active site. The surface of Ile50 residue is shown in red in A (both symmetrical partner residues) and the surface of Ile84 is shown in red in B (both symmetrical partner residues). In both pictures, LPV is shown in ball-and-stick style with carbon atoms in black and the volume of the molecule shown in black dotted surface.

4. Discussion

In this study, we performed independent passage experiments using LPV alone or in combination with RTV at two fixed concentration ratios (5/1 and 15/1) of LPV/r. Passage with 5/1 LPV/r initially selected I84V, whereas I50V/M46I was initially observed with 15/1 LPV/r and LPV alone. Previously, I84V was initially selected after passage pNL4-3 in MT4 cells (same as the present study) with LPV alone (Carrillo et al., 1998), using a similar selection procedure as in the present study. The observation of a similar rate of selection of resistance in the three arms in the current study, with more than one initial mutation pattern regardless of the presence of RTV in the culture, suggests that (1) in vitro selection is in part a stochastic process with several possible outcomes and (2) the low plasma concentrations of RTV following dosing of LPV/r do not significantly contribute to the activity of LPV/r in vivo. At various passages employing LPV/r, the IC_{50} of LPV toward the passaged virus was similar to the concentration of LPV used at that passage, indicating selective pressure by LPV. In contrast, the IC_{50} of RTV toward these passaged viruses was $\geq$five-fold higher than the concentration of RTV employed in the passage. This observation suggests that at concentrations observed after dosing LPV/r, RTV is unlikely to exert selective pressure on HIV in vivo.

Both I50V and I84V mutations were the initial mutations that emerged in two separate selection experiments. Analysis of the three-dimensional structure of LPV within the active site of HIV-1 protease indicates that this inhibitor is in close atomic contact with the side chains of both Ile50 and Ile84. The degree of hydrophobic contact between inhibitor and protein would decrease with either mutation, and the resulting affinity of the inhibitor for mutant enzyme would be expected to decrease. Selected mutant viral populations containing either I50V or I84V and two to three additional mutations exhibited 10- to 19-fold reduced susceptibility to LPV (Table 2). However, viral clone c11-c1, with I50V, M46I and E21K, displayed only three-fold reduced susceptibility. More highly mutant clones (a25-c2, b26-c12 and c24-c1) displayed high-level resistance, in accord with strains from passages A25, B26 and C24. Previously, mutations at 11 amino acid positions (10, 20, 24, 46, 53, 54, 63, 71, 82, 84, 90) in HIV protease have been associated with reduced susceptibility to LPV in viral isolates selected in vivo by other PIs (Kempf et al., 2001). The median IC_{50} of LPV against isolates with six to seven mutations of the above 11 mutations was on average 13.5-fold higher than the IC_{50} against wild-type HIV (1.74-fold per mutation) (Kempf et al., 2001). In contrast, the mutant clones, in the present study, with six to eight mutations displayed 62- to 103-fold reduced susceptibility (10- to 13-fold per mutation), presumably due to the fact that the selecting drug was LPV rather than other PIs. Additionally, two out of three passage experiments produced viruses containing I50V, a mutation not included in the above list of 11 due to low prevalence in the data set upon which it was based (Kempf et al., 2000, 2001). Recently, evidence has emerged implicating I50V in producing significantly reduced susceptibility to LPV (Masquelier et al., 2002). The present results are consistent with this observation, and suggest that I50V can be an important contributor to LPV resistance. Notably, the initial studies of LPV/r

in PI-experienced patients (Benson et al., 2002) did not include subjects failing APV, which often selects I50V in vivo (Maguire et al., 2002).

The results of the current study and their implications for the development of resistance should be interpreted in the context of clinical observations. To date, the selection of PI resistance in antiretroviral therapy-naïve subjects with detectable viral load during LPV/r therapy has not been reported (Kempf et al., 2002b). This is presumably due to a pharmacologic barrier to resistance produced by the plasma levels of LPV, which average >75-fold above the serum-adjusted IC_{50} at the trough (Hsu et al., 2002). Indeed, LPV/r retained at least partial activity in subjects whose baseline viruses display up to 40-fold reduced susceptibility to LPV (Kempf et al., 2002a). In vitro, resistance to LPV was selected using constant concentrations that were only partially suppressive. In vivo, concentrations that are selective are unlikely to persist for long during non-adherent periods when LPV concentrations fall to below the IC_{50} (Hsu et al., 2002). Consequently, the outcome of primary selection by LPV/r in vivo is still open to question.

The viral clones representing LPV selection in this study displayed cross-resistance to RTV, APV, IDV and NFV. Some decreased susceptibility to ATV and TPV was also observed. However, susceptibility to SQV was maintained. This profile is generally consistent with studies on LPV/r cross-resistance using either viruses selected by other PIs (Kempf et al., 2000) or isolates from PI-experienced patients who subsequently failed LPV/r therapy with the accumulation of additional mutations (Brun et al., 2001). We have observed a synergistic relationship between LPV and SQV, and additive activity between LPV and either ATV or TPV in vitro (Molla et al., 2002). Taken together, these findings highlight the potential utility of combination therapy with LPV/r and SQV in vivo.

In conclusion, we have generated LPV-resistant HIV through in vitro passages of either LPV alone or LPV/r. The results of this study suggest that the presence of low-dose RTV would not contribute significantly to the activity of LPV/r in vivo. These results provide information on possible pathways to LPV/r resistance, including the I50V mutation; however, their predictability for in vivo selection by LPV is unknown.

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