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Association of a Novel Human Immunodeficiency Virus Type 1 Protease Substrate Cleft Mutation, L23I, with Protease Inhibitor Therapy and In Vitro Drug Resistance

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We observed a previously uncharacterized mutation in the protease substrate cleft, L23I, in 31 of 4,303 persons undergoing human immunodeficiency virus type 1 genotypic resistance testing. In combination with V82I, L23I was associated with a sevenfold reduction in nelfinavir susceptibility and a decrease in replication capacity. In combination with other drug resistance mutations, L23I was associated with multidrug resistance and a compensatory increase in replication capacity.

Human immunodeficiency virus type 1 (HIV-1) drug resistance mutations have been identified, for the most part, during the preclinical and early clinical development of a new drug. However, the contribution of some mutations to drug resistance, particularly those that are uncommon or that exert their effect only in combination with other mutations, is often not ascertained until a drug has been used in large numbers of individuals.

In a recent study, we reported that mutations at 45 of the 99 amino acid positions in the protease—including 22 not previously associated with drug resistance—were significantly associated with protease inhibitor treatment (14). One of these mutations, L23I, was observed in 18 of 1,240 individuals receiving one or more protease inhibitors compared with 0 of 1,004 untreated individuals ($P < 0.001$). This mutation was of particular interest because it is highly conserved in untreated individuals and is in the substrate cleft of the protease enzyme.

In the present study, we describe the protease inhibitor treatments that select for L23I, the protease mutations that are associated with L23I, and the effect of L23I on protease inhibitor susceptibility.

Patients and virus isolates. Between 1 July 1997 and 31 December 2003, the Stanford University Hospital Diagnostic Virology Laboratory sequenced 6,425 HIV-1 isolates from 4,303 persons. Forty-three isolates (0.7%) from 31 persons had the protease mutation L23I. L23I was present in pure mutant form in 28 isolates and as part of an electrophoretic mixture with the wild type in 15 isolates. L23I was present in 21 of 26

independent clones (81%) from six isolates that underwent clonal sequencing.

Antiretroviral treatment histories were available for 28 of the 31 patients (Table 1). L23I developed in three persons receiving nelfinavir and in one person receiving saquinavir as their sole protease inhibitor. In patients who had previously been treated with other protease inhibitors, L23I developed during treatment with nelfinavir in one person, ritonavir-boosted saquinavir in five persons, ritonavir-boosted amprenavir in two persons, and nelfinavir followed by ritonavir-boosted saquinavir in one person.

Concomitant protease mutations. Isolates from five patients—including three who had received nelfinavir as their sole protease inhibitor, one who received nelfinavir following an 8-week course of indinavir, and one who had received nelfinavir following a 38-week course of saquinavir—had the protease mutation V82I. V82I, a polymorphism that occurs in about 1 to 2% of untreated and treated persons with subtype B viruses and that confers minimal if any resistance to the available protease inhibitors (1, 7), was present in sequences from 83 of 4,303 (1.9%) persons in this data set. L23I was significantly more common in persons with V82I than without V82I (5 of 83 [6.0%] versus 23 of 4,220 [0.5%]; $P < 0.001$). Phylogenetic analysis of the sequences of these five isolates demonstrated a mean 6.8% divergence (range, 4.3 to 8.1%) and confirmed that this pattern developed independently in these persons rather than resulting from epidemiologic spread or laboratory cross-contamination.

Besides V82I, L23I was accompanied by a wide variety of other protease inhibitor-resistance mutations, including L90M in 20 persons, M46I/L in 14 persons, I54V/L in 12 persons, V82A in 9 persons, F53L/Y in 9 persons, and I84V in 8 persons (Table 1). Among the six persons with viruses containing L23I alone (isolate 6496), L23I plus V82I (isolates 1809, 9752, and

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TABLE 1. Protease inhibitors and concomitant protease mutations in 28 HIV-1 isolates with the substrate cleft mutation L23I

Identification	Protease inhibitors received ^a	Mutation(s) at drug resistance positions	Other mutations
1809	NFV*85	82I	13V, 14R, 15V, 20T, 35G, 41K, 64V, 77I, 93L
9429	NFV*202	46I, 82I, 90M	60E, 62V, 63N, 64V, 72T, 77I, 93L
9752	NFV*108	82I	35D, 41K, 58EQ, 63P, 77I, 93L
8022	SQV*325	54V, 88D, 90M	10I, 13V, 37D, 63P, 71V, 72V, 74S
1277	IDV*8, NFV*64	82I	37T, 63P, 74S, 77I, 93L
2251	IDV*56, NFV*208	46I	10I, 13V, 35D, 37D, 61N, 62V, 63P, 64V, 76E, 77I
5774	IDV*112, NFV*203	46I, 90M	13V, 35D, 37P, 63A, 71V, 77I, 93IM
6496	NFV*20, SQV*112		35D, 37S, 41K, 63T
1800	RTV*78, rSQV*38	46L, 54V, 82A, 84V, 90M	10I, 41RK, 57K, 63P, 71V
1263	IDV*59, rSQV*376	53L, 73S, 90M	10I, 62V, 63P, 69HY, 71T, 77I, 85V
5204	NFV*49, rSQV*82	84V, 88D, 90M	16A, 20R, 36I, 57K, 61H, 63P, 69Q, 71I, 93M
5750	IDV*74, rSQV*85	46I, 53L, 84V, 90M	10I, 35D, 37D, 60E, 63P, 64V, 71V, 76V, 93L
6497	NFV*148, rSQV*156	46L, 48V, 53L, 54V, 82A, 84V, 90M	10IV, 36I, 41K, 62V, 63P, 71V, 74S, 93L
14345	SQV*12, IDV*220, NFV*82	88S	13V, 33F, 37S, 39S, 60E, 71V, 77I
6348	SQV*38, NFV*143	73S, 82I, 88S, 90M	13V, 33I, 35D, 36IL, 57K, 58E, 62V, 63P, 69Y, 71V, 72T, 75I, 93L
608	IDV*97, NFV*24, rSQV*91	46L, 84V, 88D, 90M	10I, 13V, 33M, 63P, 71V, 72M, 89LV
2190	IDV*43, NFV*8, rSQV*108	46I, 53Y, 90M	10I, 63P, 72V, 77I, 89T, 93L
3910	SQV*119, NFV*30, rSQV*65	46L, 54V, 82A, 84V, 90M	10I, 39A, 63P, 71V, 77I
4485	RTV*111, NFV*13, rSQV*89	46L, 53L, 54V, 84V, 90M	10I, 20R, 35D, 36I, 60E, 63P, 71V, 93L
1158	IDV*40, NFV*36, rSQV*21	53L, 54V, 82A, 90M	10I, 35D, 36V, 57K, 60E, 62V, 63P, 71V, 193L
1435	IDV*30, NFV*30, rSQV*58	32I, 46I, 53L, 82A, 90M	10V, 12S, 19Q, 36L, 37S, 62V, 63P, 71V
1458	IDV*62, NFV*68, rSQV*70	46L, 48VA, 54V, 82A, 84V, 90M	10I, 41K, 60E, 62V, 63S, 64V, 69R
5540	NFV*52, IDV*102, rAPV*176	54L, 73C, 90M	10LI, 33F, 62V, 63P, 64V, 66V, 77I, 193L
17599	RTV*104, IDV*130, rLPV*38	46L, 54V, 82A	10I, 15V, 162V, 63V, 69R, 71V, 72K, 76V, 89M, 93L
2095	SQV*73, RTV*48, IDV*31, rSQV*57	53L, 54V, 82AT	10I, 20R, 35D, 36I, 37S, 41K, 60E, 61H, 62IV, 63P, 71V, 87G
8355	IDV, RTV, rSQV*156, SQV/APV*52	32I, 46I, 47V, 53L, 54V, 73C, 90M	10I, 33I, 35D, 36I, 62V, 63P, 64V, 66L, 71AIT, 72MV, 93L
1498	SQV*86, IDV*42, NFV*42, SQV*102	54L, 73S, 90M	10I, 13V, 62IV, 63P, 71V, 72L, 77I
628	SQV*43, IDV*47, rSQV*36, rAPV*24	46L, 82A, 84V, 90M	10I, 58E, 60E, 61EK, 63P, 71T, 72T

^a Protease inhibitors shown in bold represent courses of treatment for which a pretherapy isolate was wild type at position 23 and a posttherapy isolate had the protease mutation L23I. The numbers following the asterisks indicate the number of weeks that the protease inhibitor was administered. APV, amprenavir; IDV, indinavir; rLPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; rSQV, low-dose ritonavir in combination with saquinavir; rAPV, low-dose ritonavir in combination with amprenavir.

1277), or L23I plus a single known drug resistance mutation (isolates 2251 and 14345), the development of L23I was associated with rebounding plasma HIV-1 RNA levels and subsequent virologic suppression with a new regimen containing either a nonnucleoside reverse transcriptase inhibitor or a

boosted protease inhibitor. Among the 22 persons with two or more known drug resistance mutations in addition to L23I, there was no single virologic response pattern.

In vitro drug susceptibility. To study the phenotypic effects of molecular infectious clones with L23I, four pairs of isogenic

TABLE 2. Drug susceptibility of HIV-1 isolates containing the substrate cleft mutation L23I

Identification no.	Description ^a	Codon 23 ^b	Mutation(s) at drug resistance positions	Other protease mutation(s)	Reduction in drug susceptibility (fold) ^c						Replication capacity ^d (%)
					APV	IDV	LPV	NFV	RTV	SQV	
NL43	NL43	WT	None	37S	1.0	1.0	1.0	1.0	1.0	1.0	100
NL43+L23I	NL43+L23I	I	None	37S	1.0	0.6	0.8	1.3	0.8	0.6	36
1277	1277 ^e 23wt	WT	82I	20R, 37T, 63P, 74S, 77I, 93L	1.0	0.8	0.6	3.2	1.2	0.8	62
1277	1277	I	82I	20R, 37T, 63P, 74S, 77I, 93L	0.9	0.9	0.8	6.7	1.7	1.3	10
4485	4485 clone 3	WT	46I, 53L, 54V, 84V, 90M	10I, 20R, 35D, 36I, 60E, 63P, 71V, 93L	6	12	24	37	139	132	35
4485	4485 clone 6	I	46I, 53L, 54V, 84V, 90M	10I, 20R, 35D, 36I, 60E, 63P, 71V, 93L	9	9	28	56	187	306	59
4733	4733 clone 3	WT	46L, 54V, 82A, 84V, 90M	10V, 15V, 36I, 62V, 63P, 71V, 93L	7	11	35	20	63	25	6
4733	4733 clone 1	I	46L, 54V, 82A, 84V, 90M	10V, 36I, 62V, 63P, 71V, 93L	6	6	26	18	63	41	39
9752	SUH Lab ^f	I	82I	35D, 41K, 58QE, 63P, 77I, 93L	1.8	0.9	1.0	7.0	2.9	1.4	NA
8622	SUH Lab	I	54V, 88D, 90M	10I, 13V, 36IM, 37DN, 63P, 71V, 72, 74S	1.3	5.0	NA ^g	34	22	60	NA
1800	SUH Lab	I	53L, 54V, 82A, 90M	10V, 57K, 63P, 71V	2.3	6.3	26	15	65	20	NA
11637	Colonna ^f	I	46I, 84V, 88S	10I, 13V, 35D, 37Y, 41K, 71T, 77I, 89M	0.3	12	NA	66	3.6	12	NA
11644	Colonna	I	24F, 46I, 53L, 73T, 84V, 90M	10I, 13V, 15V, 16A, 35D, 62V, 64V, 77I, 93L	4.3	24	NA	56	19	24	NA
11643	Colonna	I	30N, 88D	10F, 13V, 37S, 41K, 62V, 63P, 70R, 71T, 93L	1.4	2.3	NA	142	0.3	NA	NA

^a NL43 is the control used in the PhenoSense assay, and the fold resistance to this isolate for each drug is 1.0.
^b WT, wild type.
^c APV, amprenavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir. Boldface indicates values that represent decreased drug susceptibility.
^d Because NL43 is the control, the percent replication capacity for this isolate is 100%.
^e SUH, Stanford University Hospital.
^f R. J. Colonna, K. Hertogs, B. Larder, K. Limoli, G. Heilik-Snyder, and N. Parkin, 4th Int. Workshop HIV Drug Resist. Treatment Strategies, abstr. 8.
^g NA, not applicable.

TABLE 3. Sequences of NC/P1 and P1/P6 protease cleavage sites in clones with and without L23I

Identification	Description	Position 23 ^a	Drug resistance mutation(s)	Sequence change(s) ^b	
				NC/p1 (RQANFLGK)	p1/p6 (PGNFLQSR)
1277	Clone with L23I	I	82I	RQANFLGK	PGNFLQSR
4485	Clone 3	WT	46I, 53L, 54V, 84V, 90M	RQANFLGR	PGNFVQNR
4485	Clone 6	I	46I, 53L, 54V, 84V, 90M	RQANFLGR	PGNFVQNR
4733	Clone 3	WT	46L, 54V, 82A, 84V, 90M	RQVNFLGK	PGNFLQNR
4733	Clone 1	I	46L, 54V, 82A, 84V, 90M	RQVNFLGK	PGNFLQNR

^a WT, wild type.

^b Boldface indicates differences from the consensus subtype B sequence.

viruses were created containing either a wild-type or mutant residue at position 23 (Table 2). Two pairs of recombinant molecular infectious clones were created from plasma samples that, by direct PCR sequencing, had an electrophoretic mixture at position 23 indicating the presence of a quasispecies containing a mixture of wild-type and mutant viruses. The third pair of isogenic viruses consisted of the common laboratory strain NL43 and a site-directed mutant, NL43+L23I. The fourth pair of isogenic viruses consisted of a recombinant molecular infec-

tious clone (isolate 1277) and a modified version of that clone in which L23I was removed by site-directed mutagenesis, restoring the wild-type residue at this position (1277_23wt).

To create recombinant molecular infectious clones, the 3' part of *gag* and the complete protease gene amplified from samples were ligated into deleted pNL43 vector with 3'-*gag*/protease deleted (pNL43 digested with *Apa*I and *Msc*I) (5). Competent *Escherichia coli* cells were transformed with vectors containing infectious HIV-1 recombinants, and colonies were

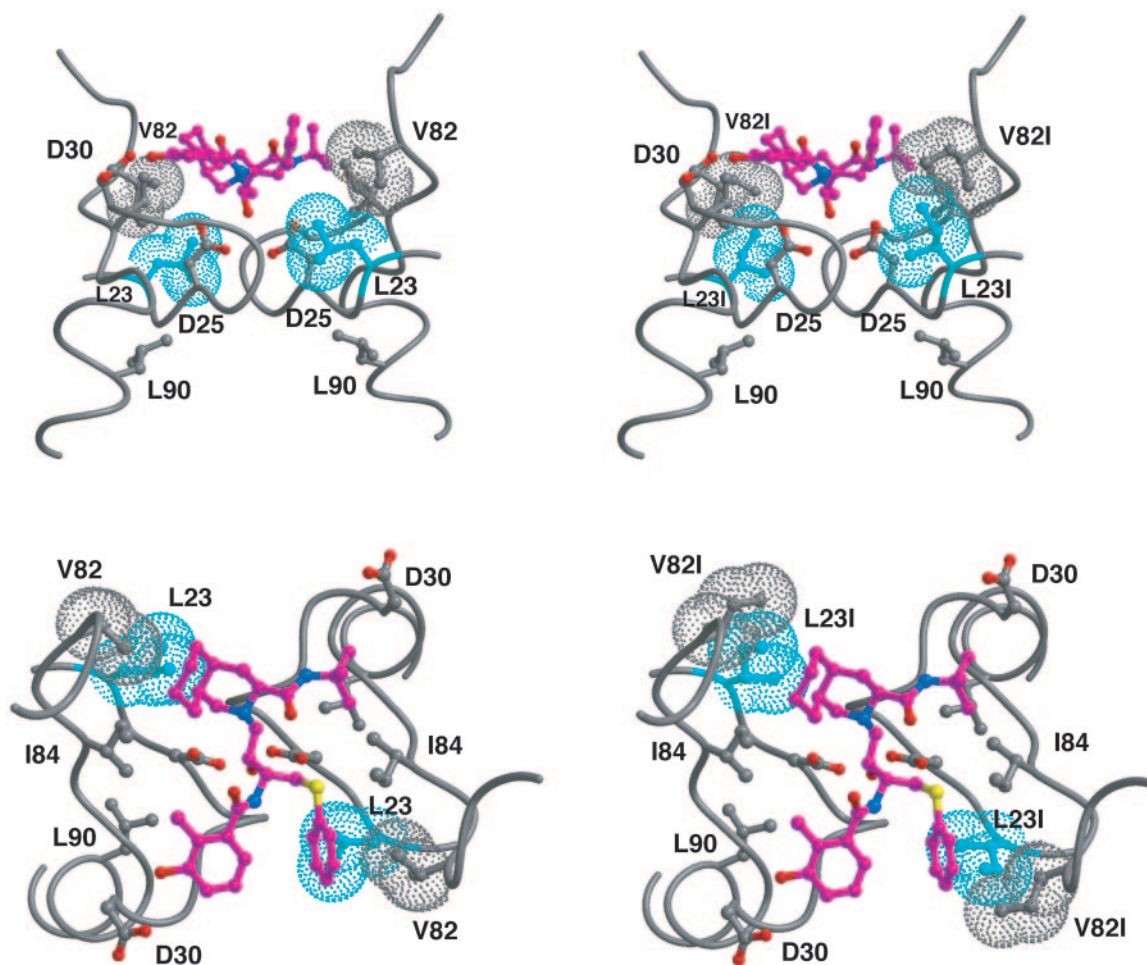


FIG. 1. Packing of residue 23 within the crystal structure of HIV-1 protease bound to nelfinavir (1OHR) (6). Shown are two views of the active site separated by 90°. The images on the left are the crystal structure, and those on the right are with side chains of L23 and V82, each replaced with isoleucine within the graphics program MIDAS (4). Residue 23 is colored cyan; the other atoms are colored by atom type, with the carbons in nelfinavir colored magenta. van der Waals surfaces are shown for the side chains of residues 23 and 82.

selected for confirmatory sequencing and transfection into CEM cells to create infectious virus stock. Recombinant isolates were tested with the PhenoSense assay (ViroLogic, South San Francisco, Calif.), an assay that has a coefficient of variation for protease inhibitors between 14 and 17% for both wild-type and mutant HIV-1 isolates (9).

Table 2 shows the drug susceptibility results (PhenoSense, ViroLogic) of the four pairs of isolates described above and of one uncloned clinical isolate. By itself, L23I had no effect on drug susceptibility when placed in the wild-type NL43 vector. However, in clone 1277, which also contained V82I, L23I contributed to nelfinavir resistance, raising the level of resistance with V82I alone from 3.2-fold to 6.7-fold—a level similar to that observed in the uncloned clinical isolate that also had L23I and V82I (isolate 9752). In both of these comparisons, L23I was associated with decreased replication capacity (from 100% to 36% in the NL43 backbone and from 62% to 10% in clone 1277).

In combination with other major protease inhibitor resistance mutations, L23I did not have a consistent effect on drug susceptibility. For isolate 4485, L23I was associated with increased levels of resistance to amprenavir, nelfinavir, ritonavir, and saquinavir of about 50 to 100%. For isolate 4733, L23I was associated with decreased resistance to indinavir and lopinavir but increased resistance to saquinavir. In both pairs, L23I was associated with an increase in replication capacity.

NC/p1 and p1/p6 cleavage sites. The NC/p1 and p1/p6 cleavage sites in HIV-1 Gag are preferential sites of mutation in response to the reduction of viral fitness associated with drug resistance mutations in the protease substrate cleft (12). Clones from isolates 4485 and 4733, both of which contained L23I plus multiple drug resistance mutations, had previously described mutations at the NC/p1 or p1/p6 cleavage sites (Table 3). The A-V change at the P2 position of the NC/p1 site, which was observed in the isolate 4733 clones, is one of the most common reported cleavage site mutations (3). The K-R change at the P4' position of the NC/p1 site is a rare change that is not associated with drug therapy (8, 15). The L-V and S-N changes at the P1' and P3' positions of the p1/p6 sites have also been commonly reported (3).

In the two isolates with multiple drug resistance mutations (4485 and 4733), cleavage site mutations were present in the clones with and without L23I and therefore were most likely related to the presence of the mutations other than L23I. The clone from isolate 1277, which did not have drug resistance mutations other than L23I, did not have mutations at either of these cleavage sites.

Structural modeling. Position 23 is located at the base of the active site at the dimerization interface. In crystal structures with substrates and inhibitors, L23 makes direct van der Waals contacts with the P1/P1' sites of the ligand (Fig. 1) (6, 10). Within the HIV-1 protease dimer, the side chain of L23 is tightly packed, making van der Waals contacts with the side chains of R8, P9, I10, V82, and V84 and, across the dimer interface, with G27. Although not quite making direct contact with the catalytic D25, the side chain L23 is a closest neighbor. With such a critical position, it is not surprising that L23 rarely is seen to mutate, as most changes at this site could dramatically impact substrate recognition and protease activity.

The association of L23I with V82I is probably due to the closeness of these two residues within the protease three-di-

mensional structure. When both side chains mutate to isoleucine, they have more freedom to repack the edge of the P1/P1' pocket. This repacking could easily confer resistance to nelfinavir, which packs against both of these residues, as seen in Fig. 1 (6).

Conclusions. Position 23 is highly conserved in HIV-1 and other primate lentiviruses and has been considered an important residue in the enzyme for drug targeting (13). Mutations at this position are uncommon, occurring in 0.7% of patients in this series, 0.2% of 40,000 isolates in a U.S. reference laboratory (2), 0.2% of 25,000 isolates in a Canadian reference laboratory (P. K. Cheung, B. Wynhoven, and P. R. Harrigan, personal communication), and 0.3% of nearly 3,000 patients in a European clinic database (16). Our results show that in combination with V82I, L23I was associated with a six- to sevenfold reduction in nelfinavir susceptibility and a decrease in replication capacity. In combination with other drug resistance mutations, L23I is associated with multidrug resistance and an increase in replication capacity.

Unlike other mutations at position 82 (such as V82A, V82T, V82F, and V82S), the conservative substitution V82I confers minimal or no resistance to currently available protease inhibitors and is selected rarely during protease inhibitor treatment (11). Indeed, it occurs in about 1 to 2% of treated and untreated subtype B isolates, 3% of untreated subtype C isolates, and 75% of untreated subtype G isolates (11).

In conclusion, L23I is a treatment-selected mutation at a highly conserved residue that often occurs with V82I alone and, in this setting, is associated with nelfinavir resistance. It also occurs in combination with other drug resistance mutations, and in this setting, it is associated with multidrug resistance and a compensatory increase in HIV-1 replication capacity.

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