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Human Immunodeficiency Virus Type 1 Protease Genotypes and In Vitro Protease Inhibitor Susceptibilities of Isolates from Individuals Who Were Switched to Other Protease Inhibitors after Long-Term Saquinavir Treatment

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An understanding of the mechanisms of virologic cross-resistance between human immunodeficiency virus type 1 protease inhibitors is important for the establishment of effective treatment strategies for patients who no longer respond to their initial protease inhibitor. Protease gene sequencing results from patients treated with saquinavir showed significant increases in the frequency of the G48V protease mutation in patients receiving higher doses of the drug. In addition, all six patients who developed the G48V mutation during saquinavir therapy developed the V82A mutation either on continued saquinavir or after a switch to nelfinavir or indinavir. In vitro susceptibility assays showed that all 13 isolates with reduced susceptibilities to two or more protease inhibitors had either the G48V or L90M mutation, along with an average of six other protease mutations. Reduced susceptibility to nelfinavir was found in 14 isolates, but only 1 possessed the D30N mutation. These results suggest that mutations selected in vivo by initial saquinavir therapy may provide more cross-resistance to the other protease inhibitors than has been previously reported.

Protease inhibitors have become a very important class of drugs for the treatment of human immunodeficiency virus infection (reviewed in references 5 and 13). Significant decreases in plasma virus levels and elevations in CD4 T-cell counts are typically found in patients receiving any of the four currently approved protease inhibitors. This effect is relatively short-lived, however, in patients receiving protease inhibitors as monotherapy, and triple-drug therapy with combinations of protease and reverse transcriptase inhibitors is now recommended.

Mutations in the protease gene have been shown to be involved in conferring reduced susceptibilities of virus isolates to protease inhibitors. Most of these associations have been confirmed by in vitro experiments, and many have been associated with clinical failure in patients and the reduced susceptibilities of isolates from these patients (3, 15, 17). In vitro selection experiments have indicated that several mutations that appear in the protease gene seem to be associated only with certain protease inhibitors. Although some unique mutational patterns appear in vivo when protease inhibitors are administered as monotherapy in protease inhibitor-naive patients, several mutations appear to be associated with all protease inhibitors. For example, the protease mutation L90M has been reported to appear most frequently in saquinavir-treated patients (8) but also is found after treatment with other protease inhibitors (4, 15, 17). In contrast, the G48V mutation, which appears less frequently than L90M, appears to be associated almost exclusively with saquinavir therapy.

In vitro cross-resistance among proteases appears to range from minor to complete, depending on the particular mutation or combination of mutations studied. Little data, however, has been published regarding clinical cross-resistance to protease inhibitors, i.e., information regarding the outcome for patients who fail to maintain viral load suppression on their first protease inhibitor and are switched to another. As clinical use of protease inhibitors increases, a greater number of patients who no longer respond to their initial drug regimens will be seen. Options for a subsequent treatment regimen are currently ill defined.

In this study, protease gene mutations in plasma virus and in vitro susceptibility data from viral isolates from patients who failed to maintain viral load suppression on saquinavir therapy and were subsequently switched to nelfinavir or indinavir were examined. Specimens were obtained from patients involved in three clinical studies at Stanford whose clinical results were presented elsewhere, as follows. (i) In a study of antiretroviral-drug-naive patients who received either 3,600 or 7,200 mg of saquinavir monotherapy per day for 6 months (18), patients who showed benefits from the drug (as defined by elevated CD4 cell counts from baseline and/or suppression of viral load to below baseline) were allowed to continue saquinavir treatment and to receive reverse transcriptase inhibitors. (ii) A subset of patients from the first study were entered into a follow-up study in which they were switched to indinavir monotherapy for 4 weeks and then received zidovudine and 2′,3′-dideoxy-3′-thiacytidine for 20 additional weeks (19). (iii) A third study involved 16 saquinavir (1,800 mg/day)- and reverse transcriptase inhibitor-experienced patients whose protease inhibitor was switched to nelfinavir (12).

Protease gene sequences from the plasma of patients were determined by reverse transcription and PCR methods previously described (21), except that the reverse transcription and first-round PCR primers were MAW-26 (TTG GAA ATG TGG AAA GGA AGG AC) and RT21 (16). Second-round PCR primers were PRO1 (19) and RT20 (16). Sequencing of the second-round product was performed by using dye-labelled dideoxy-terminator kits (Applied Biosystems, Foster City, Calif.) with primers proseq (AAG AGA GCT TCA GGT TTG G) and PSR2 (ATG CCT TTA TTT TTT CTT CTG TC).

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TABLE 1. Frequencies of protease mutations in saquinavir-treated patients

Mutation	% of patients with mutation at saquinavir dose (mg/day) of:	
	1,800[a]	3,600 or 7,200[b]
L10I or L10V	40	41
M46I	0	0
G48V	5	35[c]
L63P	73	70
A71V	56	47
V82A	5	18
I84V	3	6
L90M	40	41

[a] Data from reference 8; 37 to 55 patients treated for a mean of 46 weeks (range, 32 to 60 weeks).
[b] 17 patients treated for a mean of 77 weeks (range, 40 to 124 weeks).
[c] $P = 0.0012$ (by Fisher's exact test).

To rule out laboratory contamination of sequencing results, the uniqueness of each sequence result was confirmed by analyzing nucleotide sequence divergence among all sequences generated in the laboratory. Genotypic mixtures were reported when the minority population was at least 30% of the total.

Reports describing protease mutations in patients treated with 1,800 mg of saquinavir per day have indicated that the L90M mutation occurs with the highest frequency (2, 8). This mutation is also seen in patients treated with all other protease inhibitors (4, 5, 17, 22). The G48V mutation has also been shown to appear with saquinavir therapy, although at a substantially lower frequency than L90M (8), and appears to be relatively unique to saquinavir. In this study, protease gene sequences were obtained from 16 patients who received either 3,600 or 7,200 mg of saquinavir monotherapy per day for 63 ± 28 weeks (mean ± standard deviation) (range, 24 to 104 weeks) and had an average overall saquinavir exposure (with or without reverse transcriptase inhibitors) of 77 ± 28 weeks (range, 40 to 124 weeks). Results shown in Table 1 indicate that there was a significant increase in the frequency of the G48V mutation in patients who had received the higher doses of saquinavir (3,600 or 7,200 mg/day), compared to published data from similar patients who had received the standard (1,800 mg/day) dose of saquinavir for a mean of 46 weeks (8). There was also an increase in the frequency of the V82A mutation, although this was not statistically significant. The frequencies of other protease inhibitor mutations did not differ between saquinavir doses. Because there was a slight difference in treatment duration between the patients in the low- and high-dose saquinavir studies, it is difficult to completely separate the impact of dose and/or total drug exposure from the impact of duration of treatment. Nevertheless, since higher doses of saquinavir provide greater plasma drug levels and greater viral load suppression (18), these higher doses of saquinavir resulted in greater selective pressure on the virus, which elicited the G48V mutation with greater frequency.

All patients who developed the G48V mutation on saquinavir therapy eventually developed the V82A mutation either on continued saquinavir therapy (three of six patients) or after a switch from saquinavir to nelfinavir (two of six) or indinavir (one of six). Two recent reports have also identified this dual genotype emerging from saquinavir-treated patients (7, 20). For two patients for whom the V82A mutation was not evident after saquinavir therapy by population-based sequencing, analysis of 10 to 12 molecular clones derived from PCR amplicons (TA Cloning Kit; Invitrogen, Carlsbad, Calif.) of plasma virus RNA showed the presence of small populations (≤20%) of V82A-containing viruses. These results indicate that the G48V-V82A genotypes that developed during saquinavir therapy were expanded and potentiated during subsequent therapy with either nelfinavir or indinavir. The emergence or persistence of the G48V-V82A genotype after a change in protease inhibitor therapy is consistent with in vitro susceptibility data (Table 2) that shows these isolates to have reduced sensitivities to nelfinavir, saquinavir, and indinavir.

A total of 31 primary viral isolates obtained from 23 patients treated with saquinavir or with saquinavir followed by nelfinavir were evaluated for sensitivities to nelfinavir, saquinavir, and indinavir (Table 2). The 90% inhibitory concentration (IC_{90}) for each isolate was determined by the ACTG/DOD consensus method (1). In addition, the sequences of the viral stocks used for susceptibility testing were determined from viral RNA, as described above for plasma virus. Nucleotide sequences from the viral stocks were compared to the nucleotide sequences of the patients' plasma virus sequences to rule out laboratory contamination of sequencing or susceptibility results. The results showed that seven isolates from five patients had reduced sensitivities to saquinavir, nelfinavir, and indinavir. Four of those isolates were from patients who received only saquinavir therapy, while two were from patients who received nelfinavir after saquinavir. Isolates from six patients had reduced susceptibilities to saquinavir and nelfinavir but maintained sensitivity to indinavir. Four of these isolates were from patients who had received nelfinavir after saquinavir, and two were from patients who had received only saquinavir. Isolates from 17 patients who had failed to maintain viral load suppression on either low- or high-dose saquinavir were sensitive to all three protease inhibitors. There was no significant correlation between duration of saquinavir treatment and reduced sensitivity to any protease inhibitor.

All of the primary patient isolates (Table 2) that had reduced susceptibilities to more than one protease inhibitor possessed either the G48V or L90M mutation, along with an average of 6.4 additional protease gene mutations. The G48V mutation was significantly associated with resistance to two or more protease inhibitors (4 of 13 isolates with resistance versus 0 of 17 isolates without resistance [$P = 0.026$, by Fisher's exact test]). The L90M mutation was not significantly associated with resistance to two or more protease inhibitors, as 6 isolates with the L90M mutation were sensitive to all three protease inhibitors (9 of 13 isolates with resistance versus 6 of 17 isolates without resistance [$P = 0.155$, by Fisher's exact test]). In this and other published studies, the L90M mutation alone has had little measurable impact on susceptibilities to saquinavir (15), nelfinavir (17), ritonavir (15), and indinavir (15). The accumulation of additional protease mutations, more than four in this study, appears to be necessary for significant resistance to protease inhibitors. The identification of the type and nature of the mutations necessary for conferring protease inhibitor resistance will require analysis of a larger number of L90M-containing virus isolates and/or in vitro mutagenesis studies.

With one exception, reduced susceptibilities to nelfinavir were found in all isolates having reduced susceptibilities to saquinavir, including isolates from patients who were treated only with saquinavir. One isolate with the D30N mutation, reported as being unique to nelfinavir (17), was resistant to nelfinavir but sensitive to saquinavir and indinavir. This is consistent with previously published reports on D30N-containing isolates. However, 13 other isolates displayed reduced susceptibilities to nelfinavir in the absence of the D30N mutation (Table 2). All of these isolates had either the G48V or L90M mutation, among others. These results suggest that nelfinavir

TABLE 2. Relationships between protease mutations and in vitro susceptibilities to protease inhibitors in primary patient isolates[a]

Reduced susceptibility to[b]:	Patient	IC_{90} (μM)[c]			Patient protease inhibitor experience (mo)	Amino acid change in viral isolate protease compared to consensus sequence B at position:																						
		SQV	NFV	IDV		10	14	15	20	30	35	36	37	41	46	48	53	54	57	60	62	63	71	73	74	77	82	84	90	93
SQV, NFV, IDV	397	8.08	9.80	1.57	SQV (10) → NFV (4)	I	R						D		I		L					V	S		I			M	
	1299	6.00	1.91	9.14	SQV (7) → NFV (4)	I					D	I		K						V	P	V	S				V	M	L
	1306	1.18	2.08	0.66	SQV (10) → NFV (4)	I		V				I				V		V		V					A			
	1306	0.92	0.65	0.57	SQV (10)	I		V				I				V		V		V								
	397	0.58	1.78	1.09	SQV (10)		R	V					D				L					V	S					M	
	19	0.58	1.82	0.65	SQV (12)		E					V				V					P	V		S		A			
	39	0.37	0.69	0.72	SQV (6)			V						K							P	T	S					M	
SQV, NFV	34	0.80	0.73	0.35	SQV (6)											V					P			A					
	1294	0.42	2.69	0.43	SQV (22) → NFV (4)				I			I									P	T	S					M	
	1299	0.34	0.50	0.28	SQV (7)	I					D			K						V	P	V	S				V	M	L
	1304	0.21	0.91	0.41	SQV (11) → NFV (4)		R							K							P				I			M	L
	1296	0.20	0.64	0.32	SQV (6) → NFV (4)	I			M												P	T			I			M	L
	1295	0.20	1.13	0.22	SQV (9) → NFV (4)								D						K	E	P	V	S					M	L
NFV	1297	0.03	0.64	0.08	SQV (11) → NFV (4)			V		N	D	I		K							P								
None	28	0.09	0.12	NT	SQV (6)																P			S				M	
	30	0.08	0.21	NT	SQV (6)																P							M	
	1304	0.07	0.24	0.16	SQV (11)		R							K							P				I				L
	1298	0.07	0.25	0.05	SQV (9)	V														V			S					M	
	21	0.07	0.13	NT	SQV (6)																P			A	I				
	1294	0.06	0.27	0.07	SQV (22)																P							M	
	1295	0.06	0.23	0.15	SQV (9)								D								P	V	S					M	L
	1296	0.06	0.20	0.11	SQV (6)	I															P	T			I				L
	1297	0.06	0.15	0.08	SQV (11)			V			D			K							P								
	13	0.06	0.09	NT	SQV (6)		R/K														P								I/L
	8	0.06	0.07	NT	SQV (6)		E														P	V							
	5	0.05	0.05	NT	SQV (6)																P								
	6	0.04	0.01	NT	SQV (6)			V																					
	1300	0.02	0.04	0.02	SQV (8)																P								
	1	0.02	0.08	NT	SQV (6)	I															T	T							
	2	0.02	0.07	NT	SQV (6)																P	V						M	
	18	0.01	0.05	NT	SQV (6)																A								
None[d]		0.03	0.07	0.12	Naive																								

[a] Abbreviations: SQV, saquinavir; NFV, nelfinavir; IDV, indinavir.
[b] Reduced susceptibility is defined as an increase in IC_{90} of at least fourfold over the mean IC_{90} of 16 primary isolates obtained from 16 protease inhibitor-naive patients.
[c] Results are means of two or three tests. NT, not tested.
[d] Isolates from protease inhibitor-naive patients ($n = 16$).

and saquinavir resistance patterns may significantly overlap, similar to what is seen with indinavir and ritonavir resistance patterns (3).

Although one clinical study indicated that nelfinavir did not provide a durable response for a group of highly antiretroviral-drug-experienced patients who had previously taken saquinavir (12), further clinical studies will be needed with different patient populations to ascertain whether nelfinavir, when used with a more potent combination regimen, can be an effective treatment option for patients who fail to maintain viral load suppression on saquinavir. While published data regarding the genotypes of patients who fail to durably respond to saquinavir and switch to indinavir has been lacking, recent reports suggest that patients who possess the L90M mutation and switch to indinavir (6, 19) maintain the L90M mutation and add additional mutations, similar to what has been reported after a switch to nelfinavir (12).

The results presented here suggest that the even greater saquinavir levels achieved by better formulations of saquinavir or combinations of saquinavir and other drugs—for example, ritonavir (10, 14) and nelfinavir (11)—may result in equivalent or greater frequencies of viruses possessing the G48V and G48V-V82A mutations. Because of the reduced susceptibilities of these viruses to currently approved protease inhibitors (Table 2), patients harboring viruses with these genotypes may have few options for subsequent treatment regimens. However, since the high saquinavir levels achieved with protease inhibitor combinations also result in substantially greater reductions of viral load, overall suppression of viral replication by either a combination of protease inhibitors or protease inhibitor plus reverse transcriptase inhibitor combinations may reduce the rate of mutation evolution over the period of effective viral load suppression (9, 18).

The results presented in this report regarding mutation frequencies and in vitro susceptibilities of isolates from patients initiating therapy with saquinavir support a hypothesis by Condra et al. (3). This hypothesis, developed from data on four indinavir-treated patients and a panel of laboratory-developed mutants, suggests that initial therapy with one protease inhibitor may compromise the usefulness of subsequent protease inhibitors. The increased frequency of the G48V mutation in patients treated with higher doses of saquinavir and the eventual emergence of multi-protease-inhibitor-resistant genotypes have implications regarding the effective use of saquinavir. The effective use of both protease and reverse transcriptase inhibitors to suppress viral replication and patient adherence to dosing schedules may be some of the most important factors in slowing the development of mutations that confer drug resistance.

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