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The cumulative occurrence of resistance mutations in the HIV-1 protease gene is associated with failure of salvage therapy with ritonavir and saquinavir in protease inhibitor-experienced patients☆

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Abstract

Salvage therapy with ritonavir (RTV) and saquinavir (SQV) failed to achieve virological and immunological improvement in 24 HIV-infected patients who discontinued triple therapy with RTV or indinavir (IDV) because of failure or intolerance to treatment. Changes in the HIV-1 protease gene sequence were analyzed prospectively in 14 patients. No primary protease mutation was found prior to the use of protease inhibitors. After 7 months of treatment with IDV or RTV, primary resistance mutations at codons *pol* 46 and/or *pol* 82 were observed in 11 of 13 patients. After 16 weeks on RTV-SQV, novel primary mutations related to SQV emerged in 7 of 13 patients, together with an increase in the number of secondary resistance mutations. Our observations indicate that the cumulative occurrence of resistance mutations in the protease gene was associated with failure of antiretroviral therapy. The presence of mutations to a first protease inhibitor may represent a risk factor for the failure of a subsequent treatment with a second line protease inhibitor.

Keywords: Protease inhibitor; Antiviral therapy; Mutations; Protease gene; Resistance; HIV

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1. Introduction

Protease inhibitors are powerful agents for the treatment of HIV infection (Flexner, 1998). There is increasing concern, however, about the risk of occurrence of resistance mutations in the protease gene in patients receiving combined therapy with protease inhibitors (Hirsch et al., 1998). Both ritonavir (RTV) and saquinavir (SQV) have independently been demonstrated to prolong survival and delay disease progression in large controlled clinical trials in protease inhibitor-naive patients (Collier et al., 1996; Schapiro et al., 1996; Cameron et al., 1998). RTV dramatically enhances plasma levels of SQV by reversibly inhibiting cytochrome P-450-mediated clearance of SQV, thereby partly overcoming the poor bioavailability of the drug (Merry et al., 1997). The primary resistance mutations selected by RTV (*pol* V82A/T/F) (Molla et al., 1996) are distinct from those selected by SQV (*pol* G48V and *pol* L90M) (Boucher, 1996).

Encouraging results of a randomized open-label study assessing the efficacy of RTV-SQV combination therapy in protease inhibitor-naive patients with CD4 cells between 100 and 500×10^6/l have been reported (Cameron et al., 1999). In patients who remained on study after 60 weeks, RTV and SQV resulted in a 3 $\log_{10}$ reduction in mean viral load, together with a mean increase in CD4 cells of 200×10^6/l (Cameron et al., 1999) and an improvement in cell-mediated immune function (Angel et al., 1998). Here we report an open study of salvage therapy with RTV and SQV in 24 HIV-infected patients who discontinued triple therapy with RTV or indinavir (IDV) because of virological failure and/or intolerance to treatment. RTV in combination with SQV failed to achieve a sustained decrease in plasma viral load above 1 $\log_{10}$ copies/ml as compared with baseline values. Failure of therapy was associated with the cumulative occurrence of multiple resistance mutations to SQV in addition to mutations to RTV or IDV that had accumulated prior to the switch to RTV and SQV. Our observations document that the cumulative occurrence of resistance mutations in the protease gene is associated with virological and immunological failure of therapy.

2. Methods

2.1. Study design and patients

HIV-seropositive patients exhibiting persistently elevated plasma levels of HIV RNA above 3 $\log_{10}$ copies/ml on a combined regimen of IDV or RTV with nucleosidic reverse transcriptase inhibitors (NRTIs), or who presented severe side effects requiring the interruption of IDV or RTV, were included in the study at the time of initiation of salvage therapy with a combination of RTV and SQV. Patients with prior exposure to SQV were excluded. NRTIs and prophylaxis for opportunistic infections were maintained unchanged as the patients were switched to the RTV-SQV combination. Patients were enrolled in the study between October 1996 and March 1997. A prospective follow-up of 16 weeks was planned.

2.2. CD4 cell counts and plasma HIV-1 RNA

Absolute numbers of CD4 T lymphocytes were determined in whole blood by flow cytometry using the FACScan® cytofluorometer and the Cellquest® software (Becton Dickinson, Mountain View, CA). Plasma HIV RNA levels were determined by quantitative polymerase chain reaction (PCR) (HIV-1 Monitor test®, Roche Diagnostic Systems, Inc., Branchburg, NJ) The threshold of the assay was 200 copies/ml. CD4 cell counts and plasma HIV RNA levels were measured at baseline, and every 4 weeks thereafter.

2.3. Endpoints

Progression of HIV disease and adverse events were recorded throughout the study. Clinical failure was defined by the occurrence of an AIDS-defining event according to the revised 1993 CDC classification, or death due to disease progression. Endpoints for toxicity were fatal or life-threatening events, laboratory abnormalities of grade 3 or 4 not related to HIV infection, and adverse events that had resulted in discontinuation of RTV-SQV. Compliance was not assessed.

An immunological response to therapy was defined as an increase in the absolute number of

CD4 cells by at least 50×10^6 cells/l above baseline. A virological response to therapy was defined as a decrease in plasma viral load by at least 1 $\log_{10}$ copies/ml as compared with baseline or achieving a plasma HIV RNA level of less than the threshold of detection in the assay (Piketty et al., 1998).

2.4. Direct sequencing of the HIV-1 gene pol from cell-free plasma virions

Plasma samples were collected prior to the switch to RTV-SQV, and after 16 weeks of therapy with RTV and SQV for the genotypic analysis of the HIV-1 gene *pol* encoding for the aspartyl protease p32. An additional sample collected prior to the first protease inhibitor treatment was obtained in 14 patients for whom plasma samples stored at −80°C were available. Therefore, we concentrated the analysis of the prospective determination of protease mutations on these 14 patients.

RNA was extracted from 140 μl of plasma by selective binding on a silica-based membrane in a spin column (QIAamp Viral RNA Extraction Kit®, Qiagen, Hilden, Germany), according to the Manufacturer's instructions. The reverse transcriptase mixture (under the final reaction volume of 20 μl) contained 4 μl of 5-fold reaction buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$), 0.5 μl of RNase inhibitor (Boehringer, Mannheim, Germany), 200 μM of each dNTP (Pharmacia Biotech, Uppsala, Sweden), 50 IU of Moloney murine leukemia virus reverse transcriptase (Superscript II RNase H reverse transcriptase, Gibco BRL, Gaithersburg, M.D.), 25 pmol of the primer GAG 1 (5′-ATA ATC CAC CTA TCC CAG TAG GAG AAA T-3′), 25 pmol of the primer B′ (5′-GGT GAT CCT TTC CAT CC-3′), and 5 μl of RNA solution. For cDNA synthesis, reaction mixtures were incubated for 60 min at 42°C; reverse transcriptase was then inactivated for 5 min at 95°C. A volume of 1 μg of cDNA was further processed for aspartyl protease p32 gene DNA amplification, by means of a nested PCR using as outer primer sets, GAG1 and B′ (giving a 1512-bp amplicon), and as inner primer set, P13 (5′-AGA AGA GAG CTC CAG GTT TGG -3′) and P18 (5′-AGT CTC AAT AGG ACT AAT GGG-3′) (giving a 400-bp amplicon). The outer PCR consisted of an initial denaturation for 5 min at 94°C, followed by 35 cycles of amplification (94°C, 60 s; 51°C, 60 s; 72°C, 90 s), and a final 15-min elongation (72°C). A volume of 5 μl of the first PCR product were taken for the inner PCR, which consisted of an initial denaturation for 5 min at 94°C, followed by 35 cycles of amplification (94°C, 45 s; 50°C, 45 s; 72°C, 60 s), and a final 15-min elongation (72°C). The mix used for both outer and inner PCR contained 25 pmol of each primer, 1.5 IU of *Taq* DNA polymerase (Phamarcia Biotech), 200 μmol of each dNTP, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, and 50 mM KCl. The 400 bp-PCR products were visualized under UV transillumination by ethidium bromide staining after electrophoresis on the same 2.0% agarose gel. A volume of 40 μl of final PCR products were thereafter purified by the QIAquick PCR purification Kit® (Qiagen). Direct sequencing of purified amplicons was performed using the dideoxynucleotide chain termination method, according to fluorescent-based cycle sequencing with dye dichlororhodamine-labeled terminators (ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit□, Perkin–Elmer, Applied Biosystems, Inc., Foster City, CA) and an automatic sequencer (ABI Prism 310 Genetic Analyzer, Perkin–Elmer). Two opposing strands from each PCR product were obtained by using the 5′ and 3′ inner primers, subsequently aligned with the software Sequencer Navigator 1.0.1 (Applied Biosystems, Inc.).

The sequences were proofread manually and aligned with the 1997–1998 HIV-1 subtype B consensus sequence from the Los Alamos HIV sequence database, as a consensus sequence for comparison. Primary and secondary mutations known to be associated with drug resistance (Schinazi et al., 1997) were considered. Other mutations, which are considered to be associated with the natural polymorphism of the protease gene (Kozal et al., 1996; Lech et al., 1996) or previously undescribed, were also recorded.

2.5. Statistical analysis

For statistical analysis of the data, CD4 cell counts, plasma HIV RNA and numbers of relevant mutations were expressed as mean $\pm$ SD. Comparison of the data during the prospective follow up for patients on therapy was performed by using the non-parametric Kruskal–Wallis analysis of variance. Changes in mean values for patients on therapy were further performed using the Wilcoxon's signed rank test. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Baseline characteristics of the patients

Twenty-four HIV-seropositive patients were included in the study at the time of initiation of salvage therapy with a combination of RTV and SQV. The characteristics of the patients at initiation of RTV-SQV therapy are summarized in Table 1. The mean CD4 cell count was $108 \pm 108 \times 10^6$ cells/l (ranging between 3 and 342×10^6 cells/l) and the mean plasma HIV viral load was 5.24 ± 0.54 $\log_{10}$ copies/ml (ranging from 3.70 to 6.0 $\log_{10}$ copies/ml). No patient exhibited plasma levels of HIV RNA below the threshold of detection. Eighty-three percent of the patients had experienced at least one AIDS-defining event. The mean duration of previous NRTIs was 43 ± 20 months. Eighteen of 24 patients had received IDV and six had taken RTV for a mean duration of 7.33 ± 1.9 months (ranging between 5 and 12 months). The patients were changed to the RTV-SQV combination because of virological failure (21/24) or occurrence of side-effects (recurrent nephrolithiasis in two and severe diarrhea in one). Daily doses of RTV and SQV were chosen by each patient's physician, according to previous studies (Merry et al., 1997; Kaufmann et al., 1998; Rhone et al., 1998; Cameron et al., 1999). RTV was given at 800 mg (18 of 24 patients) or 1200 mg (6/24) per day. SQV was given at 1200 mg (14/24), 800 mg (8/24) or 1800 mg (2/24) per day. NRTIs were stavudine or zidovudine associated with lamivudine in 22 of the patients. Two patients did not receive NRTIs because of the previous occurrence of side effects to these drugs.

Table 1
Characteristics of the patients at initiation of RTV and SQV treatment

Age (mean $\pm$ SD)	38 ± 5
Gender	
Female: *n* (%)	6 (25)
Male: *n* (%)	18 (75)
Mode of transmission	
Homo/bisexual: *n* (%)	17 (71)
Injecting drug use: *n* (%)	3 (12)
Hetersexual: *n* (%)	2 (8.5)
Other: *n* (%)	2 (8.5)
CD4 cells $\times 10^6$*/l*	
Mean $\pm$ SD	108 ± 108
Median (range)	5.34 (3.70 – 6.0)
Disease category (1993 CDC classification)	
A: *n* (%)	3 (12.5)
B: *n* (%)	1 (4)
C: *n* (%)	20 (83.5)
Duration pf previous NRTIs (months)	
Mean $\pm$ SD	43 ± 20
Median (range)	48 (2–95)
Duration of previous IDV or RTV (months)	
Mean $\pm$ SD	7.3 ± 1.9
Mean (range)	7 (5–12)

3.2. Outcome of RTV-SQV therapy

Five patients discontinued the study between week 3 and week 16. Four patients discontinued RTV-SQV because of side-effects: two patients experienced a severe diarrhea, one patient presented elevated plasma concentrations of liver transaminases and another patient presented with endophtalmy presumably attributable to RTV. All these adverse events resolved after discontinuation of RTV and SQV. One patient discontinued therapy based on a personal decision. Hence, the mean follow-up on RTV-SQV was 14 ± 4 weeks.

CD4 cell counts were 100 ± 76, 133 ± 120, 124 ± 103 and $137 \pm 134 \times 10^6$ cells/l at weeks 4, 8, 12 and 16 of treatment, respectively. These values did not differ significantly from those measured at initiation of RTV-SQV. Five of 19 patients, however, exhibited an increase in CD4 cells above 50×10^6/l at 4 months.

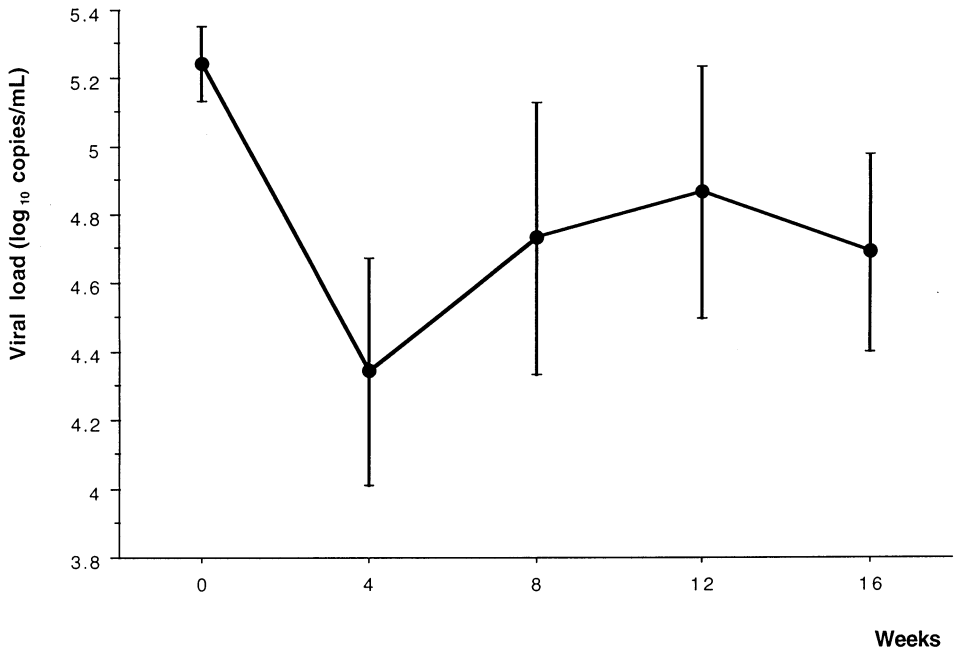

Fig. 1. Changes in plasma HIV RNA levels (mean ± SD) during salvage therapy with RTV and SQV. Twenty-four protease inhibitor-experienced patients were followed up for a mean of 14 weeks.

The mean plasma level of HIV RNA was 4.34 ± 1.32, 4.73 ± 1.19, 4.86 ± 1.16 and 4.69 ± 1.15 $\log_{10}$ copies/ml, at weeks 4, 8, 12 and 16, respectively (Fig. 1). At week 4 of therapy, the mean plasma viral load was significantly decreased as compared with baseline ($P = 0.007$). At weeks 8, 12 and 16, the mean levels of HIV RNA were below those measured at baseline, although the difference did not reach significance. At 4 months of follow up, five of 19 patients exhibited plasma levels of HIV RNA that were reduced by more than 1 $\log_{10}$ copies/ml as compared with baseline. No patient, however, exhibited plasma levels of HIV RNA below the threshold of 200 copies/ml. A combined immunological and virological response was observed in two of 19 patients at week 16. One patient whose CD4 cells did not increase on therapy, experienced a new AIDS-defining event (microsporidiosis) during follow up. No death occurred among the study patients.

3.3. Sequential analysis of protease pol gene mutations

We investigated the presence of protease resistance mutations by sequencing the protease gene in 14 patients: (I) prior to initial protease inhibitor therapy; (II) at the time of switch to RTV-SQV; (III) after 16 weeks of RTV-SQV combination treatment. A significant increase in the total number of mutations occurred between time points II and I, and between time points III and II (Fig. 2A).

No primary protease mutation was found in any of the patients prior to the initial use of protease inhibitors. By the time of switch to RTV-SQV, i.e. after a mean period of 7 months of treatment with IDV or RTV, the selection of primary resistance mutations was observed in 11 of 13 patients (85%). The latter mutations involved codons *pol* 46 ($n = 7$) and/or *pol* 82 ($n = 9$) (Table 2). One patient on IDV harbored the *pol*

D30N mutation, although he had not been exposed to nelfinavir. One patient on RTV harbored the *pol* L90M mutation. After 16 weeks on RTV-SQV, the occurrence of novel primary mutations related to SQV were observed in seven of 13 patients (Table 2): the G→V mutation at codon *pol* 48 occurred in five patients; the L→M mutation at codon *pol* 90 was seen in three patients. Noticeably, reversion of primary protease mutations at codons *pol* 46 and *pol* 82 was observed in three and four patients, respectively. The number of primary protease resistance mutations did not vary significantly during RTV-SQV therapy (Fig. 2B), due to simultaneous occurrence of novel primary mutations and reversion of preexisting mutations. Hence, ten of 13 patients harbored primary protease resistance mutations after 4 months on RTV-SQV combination. The three remaining patients, who did not harbor primary protease mutations, were among the non-responder patients.

Secondary resistance mutations in the protease gene were observed in seven of 11, 11 of 13 and 12 of 13 patients at time points I, II and III, respectively. The mean number of secondary mutations significantly increased between time points II and I, and between time points III and II (Fig. 2C). The pattern of secondary mutations (Table 3), i.e. *pol* L10V/I, K20R, L24I, M36I, I54V, L63P, A71T, V77I, was similar prior to protease inhibitor therapy, at the time of switch to RTV-SQV and after 4 months of RTV-SQV. In addition to the primary and secondary protease mutations as defined by Schinazi et al. (1997), we observed a high number of other mutations in all patients' samples, either related to the natural polymorphism of the HIV-1 protease gene or that had not previously been described (Table 4). The mean number of these mutations did not differ significantly between time points I (3.9 ± 1.6), II (3.9 ± 1.4) and III (4.5 ± 1.8) (Fig. 2D).

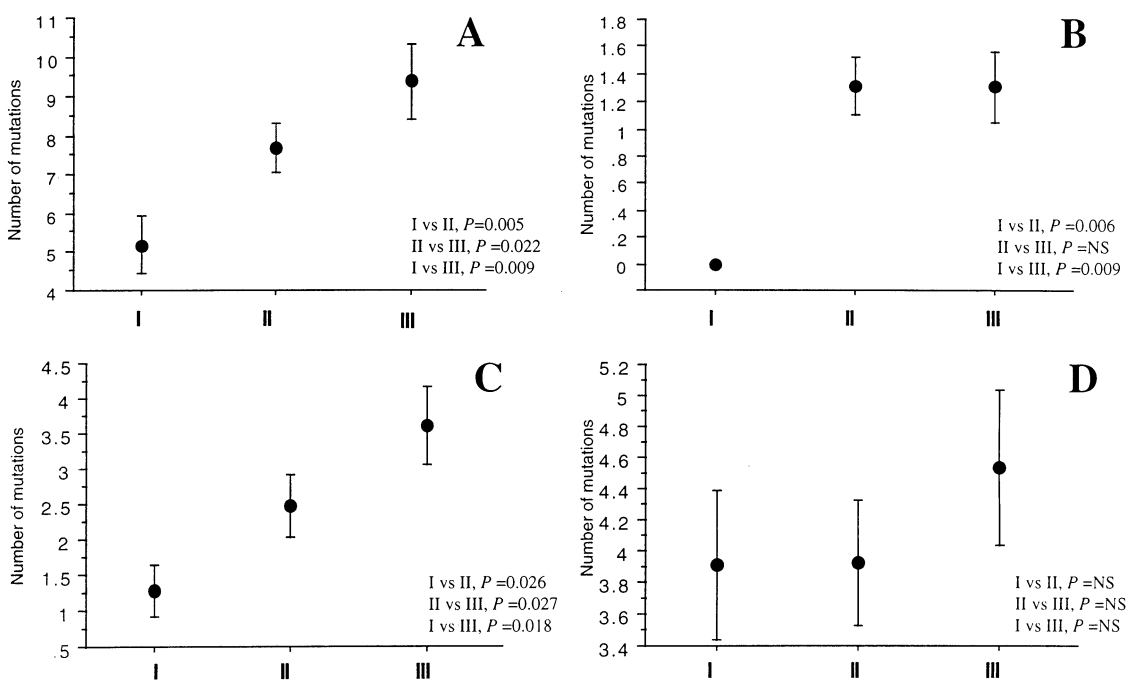

Fig. 2. Occurrence of mutations in the HIV protease *pol* gene during protease inhibitor therapy. The figure depicts the number (mean ± SD) of protease mutations as assessed prospectively prior to exposure to any protease inhibitor (I), after first line treatment with IDV or RTV (II) and after 4 months of salvage therapy with RTV and SQV (III). Panel 2A: total number of protease mutations. Panel 2B: primary protease resistance mutations. Panel 2C: secondary protease resistance mutations. Panel 2D: other mutations in the protease gene. Primary and secondary resistance mutations were recorded according to Schinazi et al. (1997).

Table 2
Occurrence of primary protease resistance mutations during protease inhibitor therapy[a]

Patients	(I): prior to protease inhibitor	(II): after first line protease inhibitor (IDV or RTV)	(III): after 4 months on RTV-SQV
1	0	0	G48V, L90M
2	0	M46I, V82A	G48V, V82A
3	0	0	L90M
4	0	M46L, V82A	G48V, V82A
5	0	M46I, V82A	M46I
6	0	M46I	*NA*
7	0	V82F	G48V
8	0	M46I, V82A	M46I
9	0	D30N	0
10	0	M46I, V82F	0
11	0	M46L, V82A	M46L, V82A
12	ND[b]	*ND*	0
13	NA[c]	V82F	V82F-L90M
14	NA[c]	V82A, L90M	G48V, V82A, L90M

[a] The presence of protease mutations was determined by sequencing the protease gene in 14 patients. Primary mutations were recorded according to Schinazi et al. (1997). Primary resistance mutations selected in the presence of IDV were *pol* M46I/L and *pol* V82A/T/F; the primary mutation selected by RTV was *pol* V82A/T/F; primary mutations selected by SQV were *pol* G48V and *pol* L90M.

[b] NA: sample not available.

[c] ND: not done because of the presence of a PCR inhibitor.

Table 3
Occurrence of secondary protease resistance mutations during protease inhibitor therapy[a]

Patients	(I): prior to protease inhibitor	(II): after first line protease inhibitor (IDV or RTV)	(III): after 4 months on RTV-SQV
1	L10V, K20R, M36I	L10V, K20R, M36I	L10V, K20R, M36I
2	L10I, M36I	L10I, L24I, M36I	L10I, M36I, I54V
3	L63P, A71T	L10I, L63P, A71T, V77I	L10I, L63P, A71T, V77I
4	0	0	L10I, L33F, I84V
5	0	L63P, A71V	L10I, L33F, L63P, I84V
6	0	I84V	*NA*
7	L10I, A71T, V77I	L10I, I54V, A71T	L10I, I54V, L63P, A71T, V77I, I84V
8	L10I	L10I, A71V	L10I, L24I, I84V
9	L63P	L63P	L63P
10	0	0	0
11	M36I, L63P	L10I, M36I, I54V, L63P, V77I	L10I, L24I, M36I,I54V, L63P, V77I
12	ND[b]	*ND*	L10I
13	NA[c]	L10I, M36I, I54V, L63P	L10I, M36I, I54V, L63P
14	NA[c]	I54V, L63P, A71V, L90M	L10I, I54V, L63P, A71V, I84V

[a] The presence of protease mutations was determined by sequencing the protease gene in 14 patients. Secondary mutations were recorded according to Schinazi et al. (1997).

[b] NA: sample not available.

[c] ND: not done because of the presence of a PCR inhibitor.

4. Discussion

We report on the lack of virological and immunological efficacy of 'salvage therapy' using RTV and SQV in protease inhibitor-experienced patients. Our study documents that failure of therapy was associated with the sequential and cumulative occurrence of primary and secondary resistance mutations in the HIV protease gene.

After 14 weeks of salvage therapy with RTV and SQV, no significant increase in CD4 cell counts and no sustained reduction in levels of plasma HIV load were observed. A transient decrease in plasma HIV RNA levels of 0.83 $\log_{10}$ copies/ml occurred at four weeks of treatment. No patient achieved plasma HIV RNA levels below the threshold of detection of 200 copies/ml. Similarly, the lack of virological efficacy of RTV and SQV in protease inhibitor-experienced patients has been recently documented (Deeks et al., 1998; Kaufmann et al., 1998; Rhone et al., 1998; Lorenzi et al., 1999), even when NRTIs were switched.

The analysis of the protease gene sequence has brought new insights for the understanding of treatment failure. Prior to the use of any protease inhibitor, no major resistance mutations in the HIV protease gene were found; secondary resistance mutations were encountered in seven of 11 patients; other protease mutations were present in 100% of the samples. The significance of the latter mutations that preexist to therapy is not fully understood. It is often considered that these mutations reflect the natural polymorphism of the HIV protease gene (Kozal et al., 1996; Lech et al., 1996). Following failure of first line protease inhibitor treatment with IDV or RTV, 85% of the patients were found to harbor an HIV strain bearing a major amino acid mutation at codon *pol* 46 and/or *pol* 82, that are known to be associated with high levels of phenotypic resistance to IDV and RTV (Condra et al., 1996; Molla et al., 1996). After switching to RTV and SQV and 16 weeks of therapy with the RTV-SQV combination, we observed the sequential emergence of

Table 4
Mutations in the protease gene other than primary and secondary resistance mutations[a]

Patients	(I): prior to protease inhibitor	(II): after first line protease inhibitor (IDV or RTV)	(III): after 4 months on RTV-SQV
1	I15V, Q18E, E35D, R41K, K43R, R57K, L63V	I15V, Q18E, E35D, R41K, K43R, R57K, V63V/A	I15V, Q18E, E35D, R41K, K43R, R57K, I62V, V63A
2	I15V, N37D, I64V, I93L	I15V, N37D, R41R/S, I64V, I93L	I15V, S41R, K43T, I46L, I62V, I64V, I93L
3	E35D, N37D, V77I, I93L	E35D, N37D, R57K, R87R/K, I93I/L	E35D, N37D, R57K, G73G/S, I93IL
4	T12N, L19I, L63S, I64V	T12N, L19I, F53F/I	T12N, L19I, F53I
5	I15V, E35D, N37S	I15V, E35D, N37S, G51G/A	I15V, E35D, N37S
6	I15V	I15V, I64V	*N*
7	E35D, N37D, L63A, I93L	E35D, N37D, L63A, L77I, I93L	E35D, N37D, I72V, F82A, I93L
8	K14R, L19V, E35D, N37S, R41K, I64V	K14R, L24L/I, R41K, I64V	K14R, E35D, R41K, I64V, T74S
9	E35D, N37S, R41K	E35D, N37S, R41K	T4I
10	T12K, R41K, I64V	T12K, R41K, I64V	T12K, R41K, I64V, I72V, I93L
11	E35D, N37D, I64V, V77I	E35D, N37D, I64V	E35D, N37D, K43T, I64V, T74S
12	ND[b]	ND	L19I, R41K, V75I, I93L
13	NA[c]	I15V, E35D, R41K, I62V, I93L	I15V, E35D, R41K, I62V, I93L
14	NA[c]	L19V, R57K	L19V, F53F/L; R57K

[a] The presence of protease mutations was determined by sequencing the protease gene in 14 patients. The table depicts those mutations that have not been defined as primary or secondary according to Schinazi et al. (1997) (see footnote b to Table 2 and Results).

[b] NA: sample not available.

[c] ND: not done because of the presence of a PCR inhibitor.

mutations related to SQV, i.e. *pol* G48V and/or *pol* L90M, in 54% of the patients, together with an increase in the number of secondary mutations. The pattern of secondary mutations was consistent among the patients. The occurrence of primary mutations to SQV was associated with the reversion of some of the primary mutations related to IDV and/or RTV, suggesting that the resistance pathways towards SQV are different than the ones towards RTV or IDV. In the present study, there was a clear relationship between the accumulation of multiple protease mutations and virological and immunological failure. However, the lack of primary mutation may not be predictive of a virological response to treatment, since the three patients who did not harbor primary protease mutations were non-responder. We cannot rule out that these patients were harboring a minority of resistant virus, since no cloning experiment was performed with virus obtained from these patients. Lorenzi et al. (1997) have reported on the pattern of protease mutations in ten protease inhibitor-naive patients treated with RTV-SQV as first line protease inhibitor therapy. Seven of the patients had preexisting minor mutations at codons *pol* 10, 36 and 63. No new mutations were observed under RTV-SQV treatment in two responder patients, whereas primary and secondary mutations associated with resistance to RTV and SQV were selected during treatment in three patients who failed on therapy.

The acquisition of resistance to protease inhibitors is considered to result from incomplete viral suppression in the presence of the drugs. In the present study, we observed that the occurrence of resistance mutations to a first protease inhibitor was associated with virological failure of first line treatment, and that the subsequent accumulation of primary resistance mutations to SQV, while on the RTV-SQV combination, is associated with failure of salvage therapy. One may speculate that the presence of multiple mutations selected by the first protease inhibitor provides a platform from which other primary and secondary mutations will emerge, with the subsequent occurrence of resistance to a second protease inhibitor in vivo, as it has previously been suggested in vitro (Condra et al., 1995) and recently found in vivo (Lorenzi et al., 1999; Schapiro et al., 1999). Thus, the presence of mutations upon treatment with a first protease inhibitor would represent a risk factor for failure of a second line protease inhibitor treatment.

At present, therapeutic options in patients who failed on first line triple combination therapy with IDV or RTV remain limited. Our observations emphasize the lack of efficacy of salvage therapy with the combination of RTV-SQV in advanced patients and demonstrate that failure of therapy is associated with the sequential and cumulative occurrence of primary and secondary resistance mutations in the HIV protease *pol* gene. Finally, the data suggest that the presence of major mutations in the protease gene can predict failure of protease inhibitors. The interest of determining genotypic and/or phenotypic patterns of resistance to protease inhibitors for the outcome of therapy, however, will require further investigation.

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