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Quantification of the impact of HIV-1 reverse transcriptase and protease mutations on the efficacy of rescue HAART

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Abstract

The reduction in the efficacy of rescue treatment (administered on a clinical basis) due to drug resistance was retrospectively quantified in 55 human immunodeficiency virus type 1 (HIV-1)-infected patients failing highly active antiretroviral therapy (HAART) by using a novel score calculation system based upon HIV-1 reverse transcriptase (RT) and protease (PR) mutations. Patients were all naive for nelfinavir (NFV) and efavirenz (EFV) and were assigned to one of the following rescue therapy schedules: (i) 17 patients received NFV + EFV + stavudine (d4T) (group A); (ii) 14 patients received NFV + saquinavir (SQV) + lamivudine (3TC) + d4T/zidovudine (AZT) (group B); (iii) 19 patients received NFV + d4T + didanosine (ddI)/3TC/zalcitabine (ddC) (group C); (iv) five patients received miscellaneous treatments including NFV (group D). Responders were considered patients showing a drop in HIV-1 RNA level $> 0.5 \log_{10}$ after 3 months of therapy. Forty-eight (28 responders and 20 non-responders) out of 55 patients completed the first 3 months of rescue therapy and reduction in HIV-1 viral load was found to be significantly higher in group A compared to groups B and C (81.2% responders vs. 38.5 and 40.0%, respectively). At baseline, no patient carried EFV- or d4T-resistant HIV-1 strains, despite prolonged administration of d4T, while 41/48 (87.2%) patients had mutations conferring resistance to NFV in the absence of previous treatment with this drug. A significant inverse correlation between reduction in viral load and reduction in therapy efficacy due to drug resistance, as determined by the score calculation system, was found ($r = 0.62$). A cut-off value of 36% reduction in therapy efficacy showed a positive predictive value (capacity to detect failure of rescue treatment) of 81.2% and a

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negative predictive value (ability to detect successful treatment) of 75.8%. In addition, 45 out of 48 patients completed also the 9–12 month period of rescue therapy and 10/28 responders had a rebound in HIV-1 viral load level detected after the first 3 months of rescue therapy. Of these, 5/7 (71.4%) showed a further reduction in rescue therapy efficacy due to the emergence of new mutations.

Keywords: Reverse transcriptase; Protease; Drug combinations; Resistance

1. Introduction

Recently, several antiviral drugs have become available for treatment of individuals infected by human immunodeficiency virus type 1 (HIV-1). Therapy with multiple drug combinations (highly active antiretroviral therapy, HAART), that include protease (PR) and reverse transcriptase (RT) inhibitors, can reduce viral load in plasma to undetectable levels, lead to a significant increase in the number of $CD4^+$ cells, and provide a substantial clinical benefit. Nevertheless, HIV-1 strains with reduced drug susceptibility have been described (Schinazi et al., 1996a, 1997). The molecular basis of drug resistance has been identified in specific mutations in the RT and PR gene products (Patick et al., 1997; Eastman et al., 1998; Hertogs et al., 1998; Hirsch et al., 1998; Winters et al., 1998; De Jong et al., 1999). Recently, many groups have reported the in vivo emergence of drug-resistant HIV-1 variants associated to administration of a single class of RT or PR inhibitors or to a double combination therapy (Boucher, 1996; Ho and Webber, 1996; Lech et al., 1996; Ercoli et al., 1997; Lorenzi et al., 1997; Eastman et al., 1998; Winters et al., 1998). However, a limited number of reports are available on the appearance of mutations in the HIV-1 RT and PR genes in patients failing HAART (Gunthard et al., 1998; Lorenzi et al., 1999). The emergence of viral strains with mutations in these genes may be one of the major factors limiting the efficacy and duration of benefit of the antiretroviral treatment.

In the present study, a simplified score calculation system was developed to quantitate the potential clinical and virologic impact of RT and PR mutations on the efficacy of the adopted rescue therapy in patients failing HAART.

2. Materials and methods

2.1. Patients and study design

Fifty-five HIV-1-infected patients (43 males and 12 females) with clinical and virologic failure of HAART were enrolled in four Infectious Disease clinical centers located in Northern Italy (Pavia, Brescia, Busto Arsizio, Cremona). The frequency of RT and PR mutations and their impact on the efficacy of the adopted rescue treatments were retrospectively evaluated in this patient population. The eligibility criteria were: (i) ongoing HAART treatment; (ii) treatment failure after >3 months of treatment defined as viral rebound ($\geq 0.5 \log_{10}$ over the nadir HIV-RNA values), or stable level of viral load, associated with decreased or stable level of $CD4^+$ T-cell counts. None of the 55 patients enrolled had received nelfinavir (NFV) in the past. Thus, all of the rescue therapy schedules were based on the administration of antiretroviral combinations including NFV. According to clinical evaluation, the 55 patients were assigned to one of the following treatment schedules: (i) 17 (30.9%) patients were treated with NFV + efavirenz (EFV) + stavudine (d4T) (group A); (ii) 14 (25.4%) patients received NFV + saquinavir (SQV) + lamivudine (3TC) + d4T/zidovudine (AZT) (group B); (iii) 19 (34.5%) patients were given NFV + d4T + didanosine (ddI)/3TC/zalcitabine (ddC) (group C); and (iv) the remaining five patients were treated with different drug combinations (NFV + SQV + ddI, two patients; NFV + SQV + d4T, two patients; NFV + SQV + d4T + ddI, one patient) (group D). With respect to rescue HAART, patients were classified as 'responders' when showing a decrease in HIV-1 RNA plasma levels $\geq 0.5 \log_{10}$ (Saag et al., 1996; Andreoni et al., 1997) after 3 months of therapy. Virological mon-

itoring was carried out up to 9–12 months in 45 patients, and subjects not showing an increase $\geq 0.5 \log_{10}$ in HIV-1 RNA levels above the nadir reached after 3 months of therapy were defined as 'sustained responders'.

2.2. Samples

EDTA-treated blood samples were drawn from each patient prior to adoption of the rescue antiretroviral therapy (baseline) and 3 months afterwards. Plasma was separated from blood cells by centrifugation at 1800 rpm for 10 min at room temperature, and immediately stored at −80°C.

2.3. Immunological and virological monitoring of rescue HAART by HIV-1 RNA quantification and CD4$^+$ T-cell counting

HIV-1 RNA in plasma was quantified every 3 months for up to 12 months follow-up by using two commercially available assays: (i) Quantiplex (Chiron Corporation, Emeryville, CA), with a detection limit of 500 HIV-1 RNA copies/ml; and (ii) Monitor Amplicor (Roche Diagnostic Systems, Branchburg, NJ) with a detection limit of 200 copies/ml. During the study an improved version of these commercial assays was made commercially available with a detection limit of 50 and 20 copies per millilitre, respectively. CD4$^+$ T-cell counts were determined by flow-cytometry analysis.

2.4. HIV-1 RT and PR analysis

Following HIV-1 RNA extraction, RT gene was analyzed using both the LiPA assay (Innogenetics Murex Biotech Limited, Center Road Temple Hill, Dartford, Kent, UK) or sequence analysis, while the PR gene was directly sequenced. Viral RNA was extracted from 100 μl of plasma by the guanidinium–thiocyanate method as previously described (Bagnarelli et al., 1991) and the dry pellet was resuspended in 50 μl of RNAse free water. A volume of 5 μl of resuspended RNA was used in an RT-PCR reaction by using the LiPA HIV-1 RT amplification kit. Then, RT mutations were identified by using the LiPA HIV-1 RT kit according to the Manufacturer's protocol. Since mutations conferring resistance to d4T and non-nucleoside RT inhibitors are not taken into consideration by the LiPA kit, the entire RT from patients shifted to a combination therapy including EFV was sequenced. In addition, a short fragment of RT (spanning codon 75) from patients receiving a rescue therapy including d4T was sequenced. Due to the very low prevalence, RT mutations T69S-SS and Q151M known to confer resistance to multiple NRTI (Hirsch et al., 1998; De Jong et al., 1999) were not routinely evaluated.

RT-PCR was performed to amplify both the RT and PR gene. In detail, the RT reaction was first performed using 10 μl of the resuspended RNA in the presence of 100 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), 200 nM PR2-antisense (5′-ATCCATTCCTGGCTTTAATTTTACT-3′) for PR gene and RT2-antisense (5′-TTGACAGACCAGCTGTCTTTTTCTGGCAG-3′) for RT gene, 0.2 mM deoxynucleoside triphosphates and 20 U of RNasin. After reverse transcription at 37°C for 45 min, the amplification reaction was carried out for 50 cycles by using an automated thermal cycler (model 9600; Perkin Elmer Cetus, Norwalk, CT) in a mixture (final volume 100 μl) containing 1 × PCR buffer (50 mM NaCl, 10 mM Tris–HCl [pH 8.3], 1.5 mM $MgCl_2$), 2.5 U of Taq DNA polymerase, and a final concentration of 500 nM each of PR1-sense (5′-GATAGACAAGGAACTGTATCCTTTA-3′), PR2-antisense, RT1-sense (5′-GGACCTACACCTGTCAACATAATTGGAAGAAA-3′) and RT2-antisense primers, for PR and RT genes, respectively. The amplification profile was as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s.

PCR products were directly submitted to sequence analysis using an automatic sequencer (model 377 Applied Biosystems-Perkin Elmer, Foster City, CA) and the Dye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS, (Perkin Elmer Cetus, Norwalk, CT) according to the recommended conditions. Primers used in sequence reactions were as

follows: RT6-sense (5′-GACAGAAGAAAAAA-TAAAAGCATT-3′) and RT4-antisense (5′-TCT-TTTTCTGGCAGCACTATAGGC-3′); PR3-sense (5′-CTTCCCTCAGGTCACTCTTT-3′) and PR4-antisense (5′-TGGCTTTAATTTTACTG-GTA-3′). These primers span codons 41–236 of RT and the entire PR gene.

2.5. Evaluation of the rescue therapy efficacy with respect to the presence of multidrug resistant HIV-1 strains

The potential reduction in activity of the rescue therapy with respect to the presence of RT and PR mutations was retrospectively quantitated in 45/48 (93.7%) patients who completed a 3-month treatment, in the three other patients the analysis could not be performed because of lack of RT or PR amplification. The reduction in treatment efficacy was correlated with the change in HIV-1 viral load after 3 months of therapy.

In order to quantitate the level of reduction in treatment efficacy a score calculation system was developed. Each antiretroviral drug was assigned an activity score of 10, expressing the full (100%) antiviral activity. Specific mutations known to confer different levels of resistance to RT and PR inhibitors in vitro (Schinazi et al., 1997), were divided into two groups: (i) primary mutations conferring a high level of resistance (> 10-fold increase in ID_{50} which was considered to correspond to a complete or nearly complete inhibition of the drug activity) were assigned a resistance score of 10; (ii) secondary mutations conferring a moderate level of resistance (< 10-fold increase in ID_{50} which was arbitrarily considered to give a 30% reduction in drug efficacy) were assigned a resistance score of 3 (Tables 1 and 2) (Larder, 1996; Markowitz and Ho, 1996; Schinazi et al., 1996b; Hirsch et al., 1998). As an example, a PR L90M mutation decreased SQV activity by 10-fold and I84V decreased NFV activity by 3-fold. Similarly, RT T215Y decreased AZT activity by 10-fold, and M184V decreased ddI activity by 3-fold and 3TC activity by 10-fold.

Thus, the residual activity for each drug could be calculated as follows:

$$\frac{\text{activity score}(10) - \text{resistance score}}{\text{activity score}} \times 100$$

Table 1
Sequential changes in median values of plasma human immunodeficiency virus type 1 (HIV-1) RNA load and $CD4^+$ cell counts after 9–12 months of rescue antiretroviral therapy decided on a clinical basis in patients with history of highly active antiretroviral therapy (HAART) failure

Patients	HIV-1 RNA values and $CD4^+$ cell counts							
	Baseline		3 months		9 months		12 months	
	Viral load[a]	$CD4^+$	Viral load	$CD4^+$	Viral load	$CD4^+$	Viral load	$CD4^+$
Non-responders (n = 20):								
Number	20	20	20	20	16	16	15	15
Median values (log_{10})	97 920 (4.99)	161	89 080 (4.94)	204.5	35 870 (4.55)	161	50 000 (4.69)	149
Range	30 150 –1 000 000	19–489	7620 –1 000 000	13–364	11 400 –1 000 000	5–430	299 –1 000 000	5–420
Responders (n = 28):								
Number	28	28	28	28	25	25	23	23
Median values (log_{10})	99 380 (4.99)	118	499 (2.69)	210	299 (2.47)	240	299 (2.47)	279.5
Range	3332–800 000	30–530	270–28 630	48–660	49 –1 000 000	29–830	49–549 000	72–627

[a] HIV-1 RNA copy number per millilitre of plasma.

Table 2
Prevalence of reverse transcriptase (RT) gene product mutations responsible for drug resistance in 48 patients failing highly active antiretroviral therapy (HAART)[a]

RT mutations	Prevalence ($n = 46$)	Score showing level of resistance to					
		AZT	ddC	DdI	3TC	d4T	EFV
M41L	24 (52.1%)	3	0	0	0	0	0
K70R	15 (32.6%)	3	0	0	0	0	0
T215Y	30 (65.2%)	10	0	0	0	0	0
T69D	7 (15.2%)	0	3	0	0	0	0
M184V	32 (69.5%)	0	3	3	10	0	0
L74V	0 (0.0%)	0	3	10	0	0	0
V75T	0 (0.0%)	0	0	0	0	10	0
S48T	0 (0.0%)	0	0	0	0	0	3
L100I	0 (0.0%)	0	0	0	0	0	3
K101E	0 (0.0%)	0	0	0	0	0	3
K103N	0 (0.0%)	0	0	0	0	0	10
V106A	0 (0.0%)	0	0	0	0	0	3
V108I	0 (0.0%)	0	0	0	0	0	3
Y181C	0 (0.0%)	0	0	0	0	0	3
Y188C	0 (0.0%)	0	0	0	0	0	3
G190A	0 (0.0%)	0	0	0	0	0	3
P236L	0 (0.0%)	0	0	0	0	0	3

[a] Scores were assigned to each mutations as follows: 10 was assigned to mutations conferring high degree (>10-fold increase in ID_{50} values) resistance, while mutations responsible for low degree (≤10-fold increase in ID_{50} values) resistance were assigned a score of 3 (Schinazi et al., 1996a; Markowitz and Ho, 1996; Larder, 1996). A score of 0 indicates no resistance. AZT, zidovudine; ddC, zalcitabine; ddI, didanosine; 3TC, lamivudine; d4T, stavudine; EFV, efavirenz.

As an example, the residual activity of a given drug in the presence of a single mutation conferring moderate resistance was considered to be:

$$\frac{10-3}{10}\times 100 = 70\%\ \text{residual activity}$$

The emergence of multiple mutations conferring resistance to one specific drug and reaching a resistance score greater than 10, was considered to abolish the activity of that drug. Thus, the highest resistance score for each drug was 10. For example, the cumulative effect of three mutations each conferring a low-grade resistance in the PR gene gave a 90% reduction in PR inhibitor efficacy (Boucher, 1996; Newton et al., 1998), and, therefore, the resistance score was 9.

Similarly, the reduction in treatment efficacy of a combination therapy was determined by calculating the sum of each drug residual activity:

$$\left[1-\frac{\text{sum of activity scores } (10\times \text{no. drugs}) - \text{sum of resistance scores}}{\text{sum of activity scores}}\right]\times 100$$

For example, in a three-drug combination therapy where a high resistance to one drug and a moderate resistance to a second one was found, the reduction in treatment efficacy was:

$$\left[1-\frac{(10+10+10)-(0+10+3)}{(10+10+10)}\right]\times 100$$
$$= 43.3\%$$

2.6. Statistical analysis

Wilcoxon test for paired data was used to compare HIV-1 RNA reduction and $CD4^+$ T-cell count increase after 3 months of rescue HAART in responders and non responders. Non-parametric Mann–Whitney *U*-test, with a correction factor (Bonferroni), and Kruskal–Wallis analysis of variance (ANOVA) test were used to compare

HIV-1 viral load and CD4$^+$ cell counts changes in the three different antiviral treatment schedules. In addition, χ^2-test and Fisher's exact test, were used to correlate viral load changes and therapy efficacy reduction in the same groups of patients. All tests were two-sided. Analyses were performed using a Windows Software (StatSoft, Tulsa, OK). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics at baseline

In 35 of the 55 enrolled patients (63.6%) the previous treatment schedule had consisted initially of a combination of two RT inhibitors to which a PR inhibitor was added later, whereas in the remaining 20 patients (36.3%) two RT and one PR inhibitor had been administered since the beginning of treatment. However, all patients had changed their drug regimen at least once during treatment due to one of the following reasons: intolerance, toxicity or lack of virologic response. In particular, 31 patients (56.4%) had been reported to have suffered from compliance problems or side-effects in the past, causing multiple treatment shifts. On the whole, the median duration of HAART treatment had been 12 months (range 1–18 months). In more detail, patients of group A, B, C and D received HAART for a median time of 15, 11, 12 and 13 months (range 6–17, 9–15, 10–16 and 10–15 months), respectively. On the whole, patients had been exposed to PR inhibitors for a median time of 11 months (range 0–2 years), while the median exposure time to RT inhibitors had been 3.9 years (range 1–6 years). At baseline the median plasma RNA level in patients was 117 3000 (5.06 $\log_{10}$) copies/ml (range, 3000– >1 milion) and the median CD4$^+$ T-cell count was 130/μl (range, 12–530). In detail, the median baseline plasma RNA level in patients of group A, B, C, and D was 139 000 (5.14 $\log_{10}$), 52 715 (4.72 $\log_{10}$), 146 000 (5.16 $\log_{10}$), and 9719 (3.98 $\log_{10}$) copies per millilitre (range, 9646–800 000, 8840–750 000, 3000– >1 million and 4600–136 800), respectively. Baseline median CD4$^+$ T-cell count in patients of group A, B, C and D was 130, 175, 103 and 98 (range, 30–489, 20–530, 12–489 and 67–171), respectively.

3.2. Viral load and CD4$^+$ T-cell counts after rescue therapy

Forty-eight out of 55 (87.2%) patients completed the first 3-month treatment period showing good compliance to the rescue therapies, and only a minority of them occasionally reported minor intolerance episodes. In contrast, 7/55 (12.7%) patients discontinued the rescue treatment because of major drug intolerance and were excluded from the study. In detail, adverse events causing treatment discontinuation were reported in 1/17 (5.8%) patients of group A, 1/14 (7.0%) patients of group B, 4/19 (21.0%) patients of group C, and one patient receiving NFV + SQV + ddI in group D. On the whole, a median decrease in viral load of 1.17 $\log_{10}$ compared to the baseline value was observed. In fact, after 3 months of therapy the median viral RNA level in the 48 patients analyzed dropped from 99 380 (4.99 $\log_{10}$, range 3332–1 million) to 6620 (3.8 $\log_{10}$) copies/ml (range, $<$ 200 to >1 million). CD4$^+$ T median cell counts at the same time-point raised from 126/μl (range, 19–530) to 208/μl, (range, 13–660), with a median increase of 83 cells/μl compared to baseline.

In more detail, of the 48 patients examined, 20 (41.6%) were classified as 'non-responders', because the level of HIV-1 viral RNA did not decrease more than 0.5 $\log_{10}$ after 3 months of therapy (Table 1). In this group, the median decrease in viral load was 0.05 $\log_{10}$ (range, $-1-+$ 0.49). In contrast, at the same time-point, all 28 (58.3%) patients classified as 'responders' showed a significant drop (Wilcoxon test, $P = 0.001$) in the median viral load value of 2.3 $\log_{10}$ (range 0.57–3.39) (Table 1). In addition, responders had a median increase in CD4$^+$ T-cell counts ($+92$/μl; range, $-53-+440$) which was significantly higher (Wilcoxon test, $P = 0.002$) than that observed within the non-responder group ($+43.5$/μl; range, $-125-+160$) (Table 1).

Follow-up data up to 9–12 months were available for 27/28 (96.4%) responders. In this group, although median HIV-RNA values did not increase compared to nadir values after 3 months of treatment, ten patients (37.0%) showed an increase in HIV-1 RNA levels of $>0.5\ \log_{10}$. In addition, median CD4$^+$ cell counts raised to 240 and 279.5, respectively (Table 1). On the other hand, follow-up data were available at 9–12 months of rescue therapy for 18/20 (90.0%) non-responders. In this group, 16 (88.8%) patients did not show any decrease in HIV-1 RNA levels, while median CD4$^+$ cell counts did not increase (Table 1).

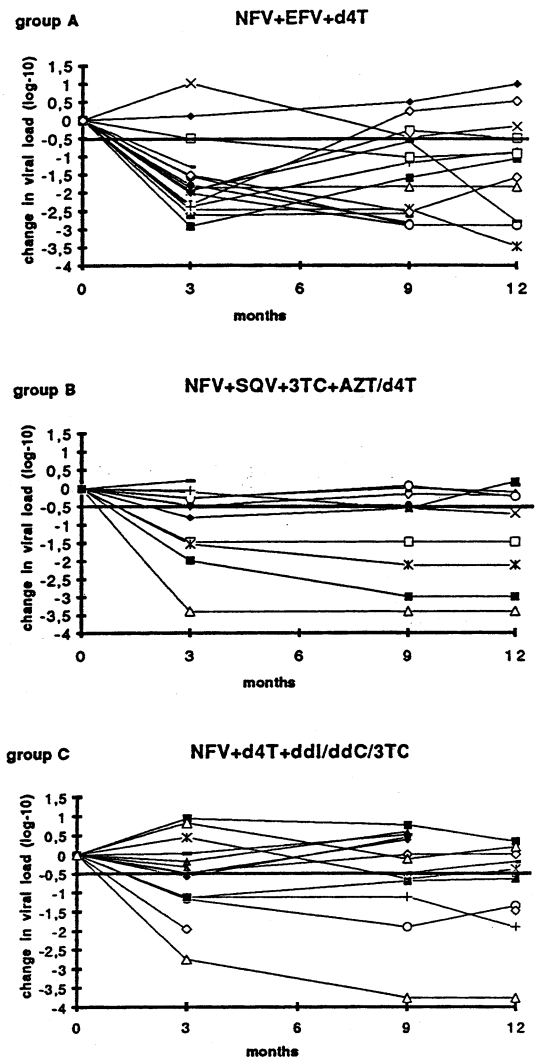

Fig. 1. Changes in median values of plasma human immunodeficiency virus type 1 (HIV-1) RNA load in groups A, B and C of patients treated with different rescue combination therapy schedules following previous highly active antiretroviral therapy (HAART) failure.

3.3. Evaluation of the response to different combination therapy schedules

After 3 months of treatment, 13/16 patients (81.2%) in group A, 5/13 (38.5%) patients in group B and 6/15 (40%) patients in group C were responders (Fig. 1). Finally, all of the four patients of group D were responders showing a median decrease in viral load of $1.12\ \log_{10}$. Median viral load values between these three groups were statistically different (Kruskal–Wallis ANOVA test, $P=0.018$). The drop in HIV-1 load was significantly different between group A and groups B and C (Mann–Whitney U-test, $P=0.025$ and $P=0.0095$, respectively). In detail, median HIV-1 RNA level dropped to 1536 ($3.32\ \log_{10}$, range, 280–370 000) copies/ml in patients of group A, 16 030 ($4.20\ \log_{10}$, range, <200–138 960) copies/ml in patients of group B and 40 000 ($4.60\ \log_{10}$, range, <200– >1 million) copies/ml in patients of group C, corresponding to a median decrease in viral load of $1.93\ \log_{10}$, $0.45\ \log_{10}$ and $0.47\ \log_{10}$, respectively. No significant difference was observed between the last two groups.

After 9–12 months of treatment, a rebound of HIV-1 viral load level observed after 3 months of therapy was detected: (i) in 6/13 (46.1%) of group A, while one patient did not complete the follow-up period; (ii) in no patient of group B, while one patient did not complete the follow-up; (iii) in 2/6 (33.3%) patients of group C (Fig. 1); (iv) and finally, in 2/4 (50%) patients of group D (data not shown).

3.4. Analysis of RT and PR mutations at baseline

Retrospective analysis of the RT and PR genes at baseline showed that all of the 48 patients

(100%) harbored HIV-1 strains resistant to RT and/or PR inhibitors because of mutations which were related to the drug regimens previously experienced. In two patients (4.1%) it was not possible to analyze the RT gene and in one patient (2.0%) the PR gene, due to lack of amplification. Four patients (8.3%) did not show mutations in the RT gene, but showed mutations in PR only, and four patients (8.3%) did not show mutations in the PR gene, but had mutations in RT only. However, the majority of patients (37/48, 77.0%) showed the presence of drug resistance-related mutations in both genes. The eight patients showing mutations in RT or PR only, did not present any apparent difference in terms of treatment compliance or tolerance with respect to the majority of patients, and all of them had been treated in the past with two or more RT and PR inhibitors for a comparable period of time. As for mutations found in the RT gene, 44/46 patients (95.6%) showed one or more amino acid change known to induce resistance to nucleotide analogs in vitro. In particular, 39/46 patients (84.7%) showed one or more mutations known to be related to AZT resistance, and 34 patients (73.9%) showed one or more mutations related to ddC resistance. The M184V mutation conferring cross-resistance to ddC, ddI and 3TC, was found in 32 patients (69.5%). Details of prevalence of single mutations and the relevant assigned scores are reported in Table 2. Interestingly, no patient showed the mutation V75T which has been reported to confer resistance to d4T (Lacey and Larder, 1994; Schinazi et al., 1996a). In addition, no mutations known to confer resistance to EFV or other non-nucleoside analog RT inhibitors (Jeffrey et al., 1998) were found in these patients (Table 2).

Forty-four out of 47 patients (93.6%) showed amino acid changes in the PR, which are known to confer different degrees of resistance to PR inhibitors (Schmit et al., 1996; Eastman et al., 1998; Hertogs et al., 1998). In more detail, 18 patients (38.2%) had one or more mutations related to SQV resistance, 37 patients (78.7%) mutations involved in ritonavir (RTV) resistance, and, finally, 35 patients (74.4%) mutations conferring resistance to indinavir (IDV). Details of prevalence of single mutations and the relevant assigned scores are reported in Table 3.

Although none of these patients had been previously treated with NFV, 41 patients (87.2%) presented changes known to decrease the activity of NFV in vitro to a different degree (Ho and Webber, 1996; Schinazi et al., 1996b; Perry and Benfield, 1997; Winters et al., 1998) (Table 3). It should be stressed that all mutations responsible for NFV resistance (with exception of D30N, V77I and N88D) (Schinazi et al., 1996a) are responsible for cross-resistance to several PR inhibitors and are likely to have been induced by previous treatments with other compounds of the same group. However, surprisingly, 15 patients (31.9%) carried the V77I mutation, which has been reported in the past only in patients exposed to NFV.

3.5. Evaluation of the impact of RT and PR mutations on the reduction in efficacy of the rescue combination therapy

Since RT and PR mutations profile was missing in three patients, the reduction in efficacy of the different rescue combination therapy schedules was retrospectively determined in 45 (81.8%) patients (25 responders and 20 non-responders) with respect to the HIV-1 viral load by using the above reported score calculation system. The median of the drug efficacy reduction in all the 45 patients was 33% (range 0–95%) with a median HIV-1 RNA decrease of $0.82 \log_{10}$ (Fig. 2A). In more detail, responders showed a median reduction in treatment efficacy of 20% (range 0–57.5%) and a median drop in viral load of $1.8 \log_{10}$ (Fig. 2B), whereas non-responders showed a median reduction in treatment efficacy of 43.3% (range 0–95%) and a median drop in viral load of $0.13 \log_{10}$ (Fig. 2C). Statistical analysis of the reduction in treatment efficacy between the two groups of patients showed a significant difference (Fisher's exact test, $P = 0.0001$). Furthermore, a direct correlation was found between the level of treatment efficacy reduction and the lack of viral load drop ($r = 0.62$; $P = 0.00006$). In addition, by setting a cut-off at 36% of treatment efficacy reduction, most responders could be clearly differentiated from most non-responders (Fig. 2B,C). In fact, 22/25 (88.0%) patients with a viral drop $> 0.5 \log_{10}$

Table 3
Prevalence of protease (PR) gene product mutations responsible for drug resistance in 48 patients failing highly active antiretroviral therapy (HAART)[a]

PR mutations	Prevalence ($n = 47$)	Score showing level of resistance to			
		SQV	RTV	IDV	NFV
G48V	6 (12.7%)	10	0	0	3
I54V	8 (17%)	3	3	3	0
I84V	6 (12.7%)	3	3	3	3
L90M	13 (27.6%)	10	3	3	3
K20M/R	6 (12.7%)	0	3	3	0
D30N	0 (0%)	0	0	0	10
L33F	3 (6.3%)	0	3	0	0
M36I	13 (27.6%)	0	3	0	3
M46I/L	6 (12.7%)	0	3	10	3
A71V/T	14 (29.7%)	3	3	3	3
V82A/F	13 (27.6%)	3	10	10	3
L10I	6 (12.7%)	0	0	3	0
L24I	4 (8.5%)	0	0	3	0
L63P	27 (57.4%)	3	3	3	3
V77I	15 (31.9%)	0	0	0	3
N88D	0 (0%)	0	0	0	3

[a] Scores were assigned to each mutations as follows: 10 was assigned to mutations conferring high degree (>10-fold increase in ID_{50} values) resistance, while mutations responsible for low degree (≤ 10-fold increase in ID_{50} values) resistance were assigned a score of 3 (Schinazi et al., 1996a; Markowitz and Ho, 1996; Larder, 1996). A score of 0 indicates no resistance. SQV, saquinavir; RTV, ritonavir; IDV, indinavir; NFV, nelfinavir.

showed a treatment efficacy reduction of $< 36\%$ (Fig. 2B). The same trend could be observed in the non-responders group. In fact, 13/20 (65.0%) patients with a viral drop $< 0.5 \log_{10}$ had a treatment efficacy reduction of $> 36\%$, while 7/20 (35%) showed a reduction of $< 36\%$ (Fig. 2C). Additional factors might have hampered the treatment efficacy in the latter seven non-responders, because two patients reported occasional non-compliance problems and three had multiple diarrhea episodes. When using 36% drug activity reduction as a cut-off for differentiating non-responders from responders, a positive predictive value (PPV, i.e. ability to detect failure of rescue treatment) of 81.2% and a negative predictive value (NPV, i.e. ability to detect successful treatment) of 75.8% were found. In addition, using the same cut-off, sensitivity of the score system in detecting non-responders was 88.0%, while specificity (ability to detect responders) was 65.0%.

3.6. Failure of rescue combination therapy in the responders group after 9–12 months of rescue therapy

RT and PR mutations were analyzed in seven of ten patients (three from group A, two from group B and two from group D) showing a rebound in HIV-1 RNA levels after 9–12 months of rescue HAART, while in 3/10 patients samples were not available for testing (Table 4). Interestingly, 5/7 (71.4%) patients had an increase in the resistance score with respect to baseline, whereas two patients (nos. 1 and 5) did not. In detail, in patients no. 2, 3 and 4 (from group A) the change K103N in RT (conferring resistance to EFV) emerged in association with other mutations (baseline resistance scores were 0, 10, and 33%, whereas follow-up resistance scores were 66.6, 66.6 and 66.6%, respectively), while in patients no. 9 and 10 (from group D) additional mutations in

Table 4
Drug resistance profile in patients showing a rebound in human immunodeficiency virus type 1 (HIV-1) RNA levels after 9–12 months of rescue therapy

Patients	Rescue therapy	Baseline		Mutations		%Resistance score[a]	3 months		12 months		Mutations		%Resistance score
		VL[b]	CD4$^+$	RT	PR		VL	CD4$^+$	VL	CD4$^+$	RT	PR	
1	NFV+EFV+d4T	185 260	240	184V,215Y	48V,54V,63P,82A	20	2090	214	58 800	340	215Y	46I,63P,82F,90M	20
2	NFV+EFV+d4T	167 460	130	41L,69D,215Y	No mutation	0	800	240	549 000	380	41L,100I,103N,215Y	30N,63P,77I,88D	66.6
3	NFV+EFV+d4T	117 300	155	184V,215Y	10I,48V,63P,71V,77I,82A	33	499	244	15 632	298	103N,108I,215Y	10I,48V,63P,71V,77I,82A	66.6
4	NFV+EFV+d4T	800 000	96	215Y	63P	10	9272	130	200 957	240	41L,103N	46I,63P,90M	66.6
5	NFV+EFV+d4T	800 000	30	No mutation	63P	10	983	208	69 135	248	70R	90M	10
6	NFV+EFV+d4T	16 560	115	No mutation	No mutation	0	499	305	4440	300	ND	ND	ND
7	NFV+d4T+ddC	4400	77	184V,215Y	63P	20	299	48	1950	72	ND	ND	ND
8	NFV+d4T+3TC	53 760	228	41L,70R,184V	63P,77I	53	14 240	250	153 000	266	ND	ND	ND
9	NFV+SQV+d4T+ddI	9719	171	70R,184V,215Y	36I,63P	30	499	216	5860	456	41L,67N,70R,215Y	10I,36I,46L,48V,63P,82A90M	50
10	NFV+d4T+SQV	89 300	67	41L,184V,215Y	63P,71T,77I	30	3400	120	22 849	107	41L	10I,46L,63P,77I,84V,90M	66.6
11[c]	AZT+3TC+SQV+NFV	181 000	120	41L,184V	63P	40	18 630	140	15 800	160	41L,215Y	63P	32.5
12[c]	NFV+EFV+d4T	139 000	45	No mutation	77I	10	499	153	49	251	41L	77I	10

[a] Resistance score, reduction of rescue treatment efficacy as calculated using the resistance score system.
[b] VL, viral load expressed as HIV RNA copy number millilitre.
[c] Two representative cases of sustained responders.

PR were shown (baseline resistance scores were 30 and 30%, while follow-up resistance scores raised to 50 and 66.6%, respectively). In addition, Table 4 reports sustained response in patients no. 11 and 12 in whom appearance of new mutations was not associated with an increase of the resistance score. In fact, in patient no. 11 the appearance of 215Y change in RT (conferring high level of AZT resistance) was compensated by disappearance of 184V (associated with resistance to 3TC), while in patient no. 12 the additional mutation was not associated with resistance to drugs included in the relevant treatment schedule.

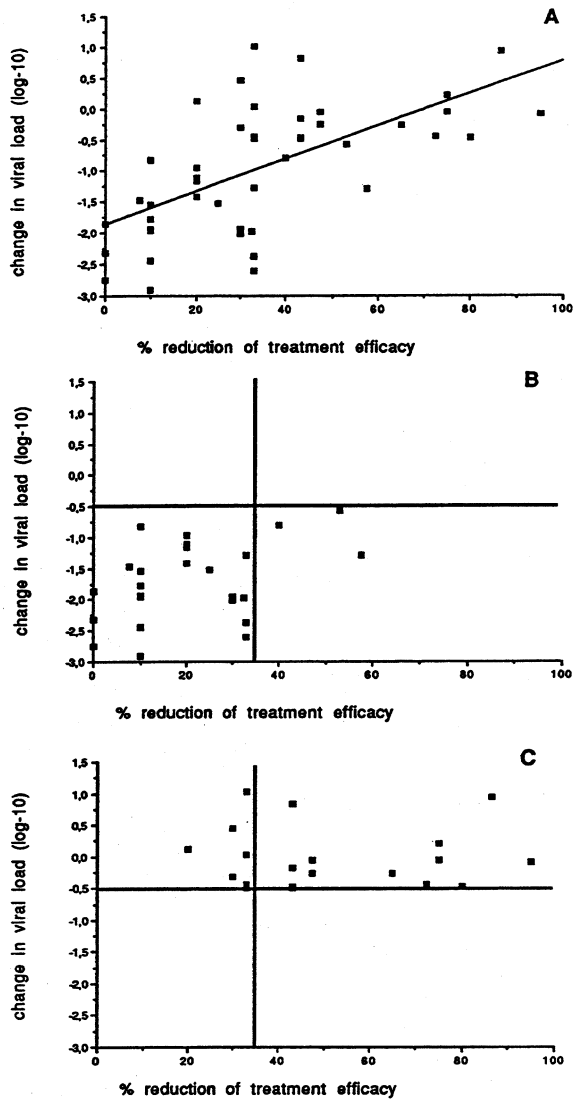

Fig. 2. Correlation of human immunodeficiency virus type 1 (HIV-1) viral load change and treatment efficacy reduction of the rescue therapy. (A) All patients; (B) responders; (C) non-responders. Median drop of viral load (log_{10}): A, −0.82; B, −1.80; C, −0.13. Median reduction in treatment efficacy (%): A, 33.0; B, 20.0; C, 43.3.

4. Discussion

Recently, several groups have reported the emergence of HIV-1 drug-resistant mutants in patients treated with HAART. However, most of these studies evaluated only a limited number of patients (Boucher, 1996; Cimoch et al., 1998; Gunthard et al., 1998; Shafer et al., 1998).

In this study, 48 patients with virologically proven lack of response to HAART were examined retrospectively to study the presence of multidrug-resistant HIV-1 strains and the mutation patterns detected in the HIV-1 RT and PR genes products. In addition, the possibility of correlating the RT and PR changes to the virologic response observed after the adoption of the rescue therapy was investigated. Multiple mutations recognized to be responsible for antiretroviral drug resistance in vitro were found in HIV-1 RT and/or PR genes in plasma of all patients analyzed at baseline (prior to starting rescue therapy). Most patients displayed mutations in both genes, while a minority of subjects harbored HIV-1 strains with changes in the RT or PR only. The kinetics of the emergence of resistant viral variants was not investigated, but it seems conceivable that the selection of multidrug resistant strains is a multistep phenomenon which might have sequentially involved the two genes. In fact, all patients analyzed faced multiple treatment changes because of intolerance, non-compliance to treatment or lack of virologic efficacy. Although we did not correlate the effect of single treatment schedules and the emergence of resistant variants, it appears of interest that no patient treated with d4T developed resistance to the drug. In addition, no pretherapy EFV-resistant strains were detected.

The finding of multidrug-resistant HIV-1 strains during HAART failure confirms the involvement of these mutations in the clinical setting (Eberle et al., 1995; Schinazi et al., 1996b;

Arts et al., 1998; Edelstein et al., 1998), including the clinical course of the disease and the failure of the rescue therapy. This result is in agreement with previous studies (Durant et al., 1998; Hertogs et al., 1998; Izopet et al., 1998; Lorenzi et al., 1999) reporting the utility of determining HIV-1 drug resistance profiles before changing therapy, and could represent the basis for the selection of the most efficient rescue drug combinations. In this respect, it appears clinically useful the finding that in 41/48 (85.4%) patients PR mutations known to confer resistance to NFV were detected, although no patient had been previously treated with this drug. This finding may be explained by the cross-resistance conferred to HIV by exposure to other PR inhibitors during previous treatments (Ho and Webber, 1996; Patick et al., 1997; Schinazi et al., 1997).

In order to evaluate the potential prognostic and therapeutic impact of RT and PR mutations, four groups of patients were analyzed after 3 months of rescue therapy with different treatment protocols. The best virological results were obtained with the combination NFV + EFV + d4T. In this group, patients did not previously receive NFV nor EFV and none of them had mutations known to confer resistance to d4T and EFV. These data indicate that best results are obtained when using drug combinations toward which mutations known to be responsible for resistance during previous treatments were not selected.

A simplified score calculation system was developed in order to quantify the reduction in rescue treatment efficacy and correlate resistance results with clinical outcome. It must be considered that rescue treatment schedules were determined on a clinical basis only, and that the extent of treatment efficacy reduction was retrospectively calculated on stored baseline samples. The level of resistance to a given combination therapy schedule was calculated on the basis of the reported in vitro resistance levels conferred by single RT and PR mutations (Larder, 1996; Markowitz and Ho, 1996). The data demonstrate that responders showed a lower reduction in treatment efficacy due to a lower resistance score, compared to non-responders who showed a higher reduction in treatment efficacy with a higher resistance scores. A cut-off of 36% therapy efficacy reduction could mostly differentiate non-responders from responders with a PPV of 81.2% and a NPV of 75.8%. The score system showed good sensitivity in predicting lack of response to treatment (88.0%), while the relatively low specificity in detecting responders (65.0%) could be due to additional factors interfering with treatment efficacy. In fact, occasional compliance or intolerance problems were retrospectively found to have contributed to reduction in treatment efficacy in 5/7 (71.4%) non-responders with low resistance scores.

However, in this study 10/27 (37.0%) responders had a rebound in viral load after 9–12 months of rescue therapy. This finding indicates that the benefit of the salvage treatments may be limited to the first months of therapy. It is interesting to note that 5/7 patients with viral rebound who could be analyzed for RT and PR mutations showed an increase in the resistance scores, thus confirming the role of new mutations (in particular relevant to EFV resistance) in reducing treatment efficacy. A trend in this respect has been recently reported by Lorenzi et al. (1999). In this paper the level of resistance was calculated on the basis of the number of mutations present in RT and PR. Although the authors found a correlation between the virological lack of response and preexisting mutations they did not give indications for the selection of the most appropriate salvage drug regimen.

In this study, multidrug resistant HIV-1 strains were detected which were likely to be responsible for treatment failure in the study population. In addition, the possibility to predict which treatment will fail and the utility of excluding drugs to which HIV-1 strains were already resistant to were retrospectively demonstrated, in order to select the most effective rescue combination therapy. Although the analysis of a larger series of patients is required to confirm these data, the study emphasizes the importance of determining HIV-1 drug resistance profiles, whenever a new therapy regimen must be adopted. Furthermore, the future development of better mathematical models including compliance, tolerance, toxicity and pharmacodynamic factors might provide the clinician with a better tool for selecting the most effective drug combination schedule.

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References

Andreoni, M., Sarmati, L., Ercoli, L., Nicastri, E., Giannini, G., Galluzzo, C., Pirillo, M.F., Vella, S., 1997. Correlation between changes in plasma HIV RNA levels and in plasma infectivity in response to antiretroviral therapy. AIDS Res. Hum. Retroviruses 13, 555–561.

Arts, E.J., Quinones-Mateu, M.E., Albright, G.L., Marois, J.P., Hough, C., Gu, Z., Wainberg, M.A., 1998. 3′-Azido-3′-deoxythimidine (AZT) mediates cross-resistance to nucleoside analogs in the case of AZT-resistant human immunodeficiency virus type 1 variants. J. Virol. 72, 4858–4865.

Bagnarelli, P., Menzo, A., Manzin, A., Giacca, M., Varaldo, P.E., Clementi, M., 1991. Detection of human immunodeficiency virus type 1 genomic RNA in plasma samples by reverse transcription polymerase chain reaction. J. Med. Virol. 34, 89–95.

Boucher, C., 1996. Rational approaches to resistance: using saquinavir. AIDS 10 (Suppl. 1), S15–S19.

Cimoch, P.J., See, D.M., Pazzani, M.J., Reitar, W.M., Lathrop, R.A., Tilles, J.G., 1998. Application of a genotypic driven rule-based expert artificial intelligence computer system in treatment experienced HIV-infected patients. Immunologic and virologic response. In: 12th World AIDS Conference. Geneva, Abstract no. 32297.

De Jong, J.J., Goudsmit, J., Lukashov, V.V., Hillebrand, M.E., Baan, E., Huismans, R., Danner, S.A., ten Veen, J.H., de Wolf, F., Jurriaans, S., 1999. Insertion of two aminoacids combined with changes in reverse transcriptase containing tyrosine-215 of HIV-1 resistant to multiple nucleoside analogs. AIDS 13, 75–80.

Durant, J., Clevenbergh, P., Halfon, P.H., Delgudic, P., Rahelinirina, V., Sayada, S., Dellamonica, P., 1998. Prevalence of resistance-associated genotypic mutations in plasma HIV of patients failing tritherapy combination. In: 12th World AIDS Conference. Geneva, Abstract no. 32301.

Eastman, P.S., Mittler, J., Kelso, R., Gee, C., Boyer, E., Kolberg, J., Urdea, M., Leonard, J.M., Norbeck, D.W., Mo, H., Markowitz, M., 1998. Genotypic changes in human immunodeficiency virus type 1 associated with loss of suppression of plasma viral RNA levels in subjects treated with ritonavir (norvir) monotherapy. J. Virol. 72, 5154–5164.

Eberle, J., Bechowsky, B., Rose, D., Hauser, U., Von Der Helm, K., Gurtler, L., Nitschko, H., 1995. Resistance of HIV type 1 to proteinase inhibitor Ro 31-8959. AIDS Res. Hum. Retroviruses 11, 671–676.

Edelstein, R.E., Nickerson, D.A., Tobe, V.O., Manns-Arcuino, L.A., Frenkel, L.M., 1998. Oligonucleotide ligation assay for detecting mutations in the human immunodeficiency virus type 1 pol gene that are associated with resistance to zidovudine, didanosine and lamivudine. J. Clin. Microbiol. 36, 569–572.

Ercoli, L., Sarmati, L., Nicastri, E., Giannini, G., Galluzzo, C., Vella, S., Andreoni, M., 1997. HIV phenotype switching during antiretroviral therapy: emergence of saquinavir-resistant strains with less cytopathogenicity. AIDS 11, 1211–1217.

Gunthard, H.F., Wong, J.K., Ignacio, C.C., Guatelli, J.C., Riggs, N.L., Havlir, D.V., Richman, D.D., 1998. Human immunodeficiency virus replication and genotypic resistance in blood and lymph nodes after a year of potent antiretroviral therapy. J. Virol. 72, 2422–2428.

Hertogs, K., Bethune, M.P., Miller, V., Ivens, T., Schel, P., Cauwenberge, A.V., Van Den Eynde, C., Van Gerwen, V., Azijn, H., Van Houtte, M., Peeters, F., Staszewski, S., Conant, M., Bloor, S., Kemp, S., Larder, B., Pauwels, R., 1998. A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. Antimicrob. Agents Chemother. 42, 269–276.

Hirsch, M.S., Conway, B., D'Aquila, R.T., Johnson, V.A., Brun-Vézinet, F., Clotet, B., Demeter, L.M., Hammer, S.M., Jacobsen, D.M., Kuritzkes, D.R., Loveday, C., Mellors, J.W., Vella, S., Richman, D.D., 1998. Antiretroviral drug resistance testing in adults with HIV infection. J. Am. Med. Assoc. 279, 1984–1991.

Ho, D., Webber, S., 1996. Antiviral and resistance studies of AG1343, an orally bioavailable inhibitor of human immunodeficiency virus protease. Antimicrob. Agents Chemother. 40, 292–297.

Izopet, J., Bicari-See, A., Pasquier, C., Bonnet, E., Marchou, B., Puel, J., Massip, P., 1998. Mutations conferring resistance to zidovudine diminish the virologic response to stavudine plus didanosine therapy. In: 12th World AIDS Conference. Geneva, Abstract no. 32306.

Jeffrey, S., Baker, D., Tritch, R., Rizzo, C., Logue, K., Bacheler, L., 1998. A resistance and cross resistance profile for sustiva (efavirenz, DMP 266). In: 5th conference on retroviruses and opportunistic infections. Chicago, IL, Abstract no. 702.

Lacey, S.F., Larder, B.A., 1994. Novel mutation (V75T) in human immunodeficiency virus type 1 reverse transcriptase confers resistance to 2′,3′-didehydro-2′,3′-dideoxythymidine in cell culture. Antimicrob. Agents Chemother. 38, 1428–1432.

Larder, B.A., 1996. Nucleoside and foscarnet mechanisms. In: Richman, D.D. (Ed.), Antiviral Drug Resistance. Wiley, Chichester, UK, pp. 169–190.

Lech, W.J., Wang, G., Yang, Y.L., Chee, Y., Dorman, K., McCrae, D., Lazzeroni, L.C., Erickson, J.W., Sinsheimer, J.S., Kaplan, A.H., 1996. In vivo sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. J. Virol. 70, 2038–2043.

Lorenzi, P., Yerly, S., Abderrakim, K., Fathi, M., Rutschmann, O.T., Von Overbeck, J., Leduc, D., Perrin, L., Hirschel, B. and Swiss HIV Cohort Study, 1997. Toxicity, efficacy, plasma drug concentrations and protease mutations in patients with advanced HIV infection treated with ritonavir plus saquinavir. AIDS 11, F95–F99.

Lorenzi, P., Opravil, M., Hirschel, B., Chave, J.P., Furrer, H.J., Sax, H., Perneger, T.V., Perrin, L., Kaiser, L., Yerly, S. and the Swiss HIV Cohort Study, 1999. Impact of drug resistance mutations on virologic response to salvage therapy. AIDS 13, F17–F21.

Markowitz, M., Ho, D.D., 1996. Protease inhibitors-mechanisms and clinical aspect. In: Richman, D.D. (Ed.), Antiviral Drug Resistance. Wiley, Chichester, UK, pp. 261–278.

Newton, J.A., Pemberton, A.S., Emmons, W.W., Mayers, D.L., 1998. Genotypic analysis for resistance testing (GART) as a clinical tool: a retrospective analysis. In: 12th World AIDS Conference. Geneva, abstract no. 32292.

Patick, A.K., Kuritzkes, D., Johnson, V.A., Shugarts, D., Bakhtiari, M., Potts, K.E., Farnsworth, A., Anderson, R., Koel, J.L., Hazelwood, J.D., Nail, C.D., Durant, M., Markowitz, M., Ho, D., Richman, D., 1997. Genotypic and phenotypic analyses of HIV-1 variants isolated from patients treated with nelfinavir and other HIV-1 protease inhibitors. International Workshop on HIV Drug Resistance, treatment strategies, and eradication, St. Petersburg, FL, abstract no. 18.

Perry, C.M., Benfield, P., 1997. Nelfinavir. Drugs 54, 81–87.

Saag, M.S., Holodniy, M., Kuritzkes, D.R., O'Brien, W.A., Coombs, R., Poscher, M.E., Jacobsen, D.M., Shaw, G.M., Richman, D.D., Volberding, P.A., 1996. HIV viral load markers in clinical practice. Nat. Med. 2, 625–629.

Schinazi, R.F., Stuyver, L., Wyseur, A., Lloyd, R.M. Jr., Hough, L., Rombout, A., Rossau, R., Rimland, D., 1996. Proviral and virus genotyping using a line probe assay in nucleoside treated HIV infected veterans affairs patients. In: 5th International Workshop on HIV Drug Resistance. Whisler, Canada. Abstract no. 65.

Schinazi, R.F., Larder, B.A., Mellors, J.W., 1996b. Mutations in retroviral genes associated with drug resistance. Int. Antiviral News b4, 95–107.

Schinazi, R.F., Larder, B.A., Mellors, J.W., 1997. Resistance table: mutations in retroviral genes associated with drug resistance. Int. Antiviral News 5, 129–142.

Schmit, J.C., Ruiz, L., Clotet, B., Raventos, A., Tor, J., Leonard, J., Desmyter, J., De Clercq, E., Vandamme, A.M., 1996. Resistance-related mutations in the HIV-1 protease gene of patients treated for 1 year with the protease inhibitor ritonavir (ABT-538). AIDS 10, 995–999.

Shafer, R.W., Winters, M.A., Merigan, T.C., 1998. Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients. Ann. Intern. Med. 128, 906–911.

Winters, M.A., Shapiro, J.M., Lawrence, J., Merigan, T.C., 1998. 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. J. Virol. 72, 5303–5306.