LONG-TERM ANALYSIS OF THE RESISTANCE DEVELOPMENT IN HIV-1 POSITIVE PATIENTS TREATED WITH PROTEASE AND REVERSE TRANSCRIPTASE INHIBITORS: CORRELATION OF THE GENOTYPE AND DISEASE PROGRESSION

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Summary. – In this study, 27 HIV-1-positive patients on long-term highly active antiretroviral therapy (HAART) in the Czech Republic were followed for a period of up to 7 years. Variability of the HIV-1 protease (PR) sequence common in the Czech Republic was observed. Under the pressure of inhibitors of protease (PRIs) and reverse transcriptase (RTIs) mutations in PR were detected. Development of resistance to PRIs was followed by a decrease in CD4 count and increase in viral load. The dynamics of viral load closely corresponded to the accumulation of specific primary mutations in PR and RT. Out of 27 patients 18 developed resistance to PRIs and the prolonged therapy led to the accumulation of a higher number of amino acid changes associated with the resistance and, consequently, cross-resistance to several PRIs was observed. These multi-resistant variants of HIV-1 with mutations in PR could not be inhibited sufficiently with PRIs that are currently available in clinical practice. Efficient yet temporary suppression of viral replication was achieved by a lopinavir (LPV) treatment.

Key words: drug resistance; HAART; HIV-1; inhibitors; protease; reverse transcriptase; sequence variability

Introduction

Rapid development of resistance to inhibitors of HIV-1 currently used in clinical practice represents a major problem in the multi-pronged HAART. It is caused by extensive variability of HIV-1 genome determined by multiple factors including rapid viral turnover (Ho, 1995), high frequency of nucleotide coding errors in reverse transcription and RNA synthesis, and high rate of recombination (Ji *et al.*, 1996).

Drug pressure leads to the selection of drug-resistant virus mutants. The changes in the sequence of PR gene leading to changes in the sequence of PR and finally resulting in PRI resistance include mutations in and around the substrate/inhibitor binding cleft in PR that are called primary mutations. Amino acid exchanges occur also in regions that might influence PRI binding indirectly or have compensatory effect on the substrate cleavage (Wlodawer and Vondrasek, 1998): these mutations are called secondary or compensatory. There is a limited number of primary mutations that are characteristic for the resistance to specific

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Abbreviations: 3TC = lamivudin; ABC = abacavir; AZT = azidothymidin (zidovudin); d4T = stavudin; ddC = dideoxycytidin (zalcitabin); ddI = dideoxyinosin (didanosin); EFV = efavirenz; env = genes for viral envelope proteins; gag = genes for viral structural proteins; HAART = highly active antiretroviral therapy; IDV = indinavir; LPV = lopinavir; LPV/r = combination of LPV and RTV; NFV = nelfinavir; NVP = nevirapin; PBMC = peripheral blood mononuclear cells; PRI = inhibitor of PR; PR = protease; RT = reverse transcriptase; RTI = inhibitor of RT; RTV = ritonavir; SQV = saquinavir

Table 1. Characteristics of the patients and their treatment

Patient code	Sex	Subtype	Treatment with PRIs (months)	Resistance developed	Treatment with RTIs	Resistance developed
CZ1	M	B	SQV (29), IDV (35)	N	AZT, DDC, d4T, 3TC	Y
CZ13	M	B	IDV (38), LPV/r (17)	Y	AZT, DDC, d4T, 3TC, ABC, DDI, EFV	Y
CZ15	M	B	SQV (9), RTV (2), IDV (25), NFV (3), RTV (4), LPV/r (11)	Y	AZT, DDC, d4T, 3TC, ABC, DDI, EFV, NVP	Y
CZ16	M	B	SQV (17), RTV (6), IDV (18)	Y	AZT, DDC, d4T, 3TC, DDI, NVP	Y
CZ18	M	B	SQV (14), RTV (7,5), IDV (20), NFV (8), LPV/r (4)	Y	AZT, DDC, d4T, 3TC, ABC, DDI, EFV, NVP	Y
CZ20	M	B	SQV (8), IDV (6), SQV(7), NFV (31)	Y	AZT, DDC, d4T, 3TC, NVP	NA
CZ22	M	B	SQV (22), IDV (24)	N	AZT, d4T, 3TC	Y
CZ24	M	B	SQV (39), IDV (22)	Y	AZT, DDC, d4T, 3TC, EFV	Y
CZ25	M	B	SQV (30), IDV (31)	Y	AZT, DDC, d4T, 3TC, DDI, EFV	Y
CZ26	M	B	SQV (14), RTV (6), IDV (13,5), LPV/r (11)	Y	AZT, DDC, d4T, 3TC, ABC, NVP	Y
CZ28	M	B	SQV (12), IDV (9), RTV(9), NFV (26,5)	Y	AZT, d4T, 3TC, DDI, EFV	Y
CZ29	M	B	SQV (9)	Y	AZT, DDC, d4T, 3TC, EFV	NA
CZ30	F	C	SQV (31), RTV(12), IDV (11), NFV (6,5), RTV (7,5), LPV/r (13)	Y	AZT, DDC, d4T, 3TC, ABC, DDI, EFV, NVP	Y
CZ39	M	B	SQV (55), LPV/r (9)	Y	AZT, DDC, d4T, 3TC, ABC, EFV	Y
CZ42	M	B	SQV (21), IDV (50)	N	AZT, DDC, 3TC	Y
CZ45	M	B	SQV (12), IDV (3), NFV (10)	N	AZT, DDC, d4T, 3TC,DDI	Y
CZ49	M	B	SQV (36), RTV (30)	N	AZT, DDC, d4T, 3TC, EFV	NA
CZ50	M	B	SQV (37), IDV (17), NFV (33)	Y	AZT, DDC, d4T, 3TC, EFV	Y
CZ52	M	B	SQV (46), NFV (3), NFV + RTV (7), LPV/r (13)	Y	AZT, DDC, d4T, 3TC, ABC, EFV, NVP	Y
CZ58	M	B	SQV (36)	N	AZT, DDC	NA
CZ67	M	B	SQV (25), IDV (23)	N	AZT, d4T, 3TC	Y
CZ68	M	B	SQV (35), IDV (7), IDV + RTV (15)	Y	AZT, DDC, d4T, DDI	Y
CZ70	F	B	SQV (25)	Y	AZT, DDC	Y
CZ91	M	B	RTV (48)	Y	AZT, DDC, d4T, 3TC, DDI, EFV	Y
CZ97	M	B	SQV (41), IDV (22)	N	AZT, DDC, d4T, 3TC	Y
CZ99	M	B	SQV (46), NFV (11)	N	AZT, DDC, d4T, 3TC	NA
CZ101	M	B	SQV (25), IDV (22), NFV (12)	Y	AZT, DDC, d4T, 3TC, ABC, DDI, EFV	Y

In the patients CZ52 and CZ68 RTV was used as a pharmaco-kinetic booster (2 x 100 mg per day). Y, N = yes, no. NA = not analyzed.

drug(s) (Boden and Markowitz, 1998). They involve mutations at positions 30, 46, 48, 50, 54, 71, 82, 84 and 90. However, amino acid changes at other positions (10, 20, 36, 63, 71, 77, and 93) also contribute to the resistance (Muzammil *et al*., 2003). Thus, HIV-1 may evolve along many mutational pathways in developing a resistance to a given drug or drug combination. Therefore, in this study we (i) analyzed in detail the resistance in HIV-1-positive patients over a prolonged time period, (ii) attempted to correlate the virus genotype with the surrogate markers of the disease progression, and (iii) made a phenotype analysis of selected drug-resistant mutants with the aim to understand the antiviral resistance at molecular level.

Materials and Methods

Patients. Twenty-seven HIV-1 positive patients admitted at the Clinic of Infectious Diseases, Faculty Hospital Bulovka, Prague, have been closely followed for up to 7 years during 1995–2002. As shown in Table 1, all but one patient harbored HIV-1 of subtype B. The single patient (the patient 35513 in Reinis *et al*., 2001, the patient CZ30 in this study) harbored HIV-1 of subtype C, as confirmed by phylogenetic analysis of the *env* and *gag* genes. All the patients were treated with PRIs in combination with various RTIs. The patients experienced on average 5 different RTIs during treatment. Details of the treatment of individual patients with RTIs and PRIs are given in Table 1. In regular regime, 2 x 600 mg of RTV was given per day with exception of the patients CZ52 and CZ68, who were given 2 x 100 mg per day. The reasons for changes in treatment were drug side effects, development of resistance or subjective feeling of patients.

HIV-1 RNA plasma level was determined by a quantitative RT-PCR (Amplicor HIV-1 Monitor version 1.5).

$CD4^+$ cell count was determined by FACS analysis using CaliburE2771.

Resistance to RTI was determined by hybridization techniques (INNO-LiPA HIV-1 RT, Bayer Diagnostics) as described by Hirsch *et al*. (2000).

RT-PCR and nested PCR. Patients' PBMCs or plasma were assayed for HIV-1 PR gene by RT PCR and nested PCR. External primers JP4Uex (5'-CAGAGCCAACAGCCCCAGCAG-3') and JP3Dex (5'-CTTTTGGGCCATCCATTCCTGGC-3') for PCR and internal primers JP1bUin (5'-TAGAATTCATATGAGAGACA ACTCCCCCT-3') and JP2Din (5'-GGGGATCCTTACTATGGTA CAGTCTCAATAGG-3') for nested PCR were employed (Weber *et al*., 2002). A different pair of internal primers was employed for

amplification of the PR gene of HIV-1 of subtype C, namely C-JP1bUin (5'-TAGAATTCATATGCGAGGAAGCAACACCTTCT-3') and C-JP2Din (5'-GGGGATCCTTACTATGGTACAGTTTCAATGGG-3'). Plasma samples were assayed under following conditions: the reverse transcription step proceeded at 42°C for 45 mins, PCR consisted of 35 cycles of denaturing at 95°C for 60 secs, annealing at 55°C for 60 secs and extension at 72°C for 2 mins. Nested PCR consisted of 35 cycles of denaturing at 95°C for 30 secs, annealing at 55°C for 30 secs and extension at 72°C for 2 mins. PBMCs were assayed by PCR and nested PCR under the same conditions: 35 cycles of denaturing at 95°C for 30 secs, annealing at 55°C for 30 secs and extension at 72°C for 2 mins.

Nucleotide sequencing. RT-PCR products were sequenced directly using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 310 DNA sequencer (Perkin-Elmer) or were cleaved by the restriction enzymes *Eco*RI, *Bam*HI, and *Nde*I and inserted into pUC19 or pET24a plasmid. For each PCR product, 10 clones were isolated and sequenced as written above or using the ThermoSequenase Kit and an A.L.F. DNA sequencer (Amersham Pharmacia Biotech).

Amino acid sequence analysis. The retrieved nucleotide sequences of the PR gene were used for deduction of corresponding amino acid sequences of PR. For detection of mutations, the latter sequences were compared to the consensus sequence of PR gene of HIV-1 of B subtype. When a mixture of mutated and wild-type sequence was identified in a sample, the mutated sequence was taken into account and further analyzed.

Results and Discussion

Genetic variability of HIV-1 PR from PRI-naive patients

In order to differentiate between naturally occurring mutations and those developed under the selective pressure of PRIs, the HIV-1 B-subtype PR sequence commonly occurring in the Czech Republic was determined (Table 2). In 1996–1999, at the beginning of this study, PR sequences were obtained from 25 PRI-naive patients. Four of these subjects did not experience a PRI therapy and thus were excluded from the study. For 6 patients (including the patient harboring the C-subtype virus) the sequences prior to treatment were not available.

Thirty-four different amino acid substitutions were found at 24 positions in PR (Table 2). The mean number of substitutions was 3.7 per patient with a range from 0 to 8; the most frequent changes (over 20%) were located at positions 10, 35, 37, 62, 63, 77, and 93. The highest polymorphism (3 and more substitutions) was observed at positions 37 (N37S, D, E, T, Y), 63 (L63P, S, A), and 64 (I64V, L, M). The most common mutation was L63P with a prevalence of 52%. No primary mutations were present at positions 30, 48, 82, 84 and 90 prior to the treatment. The M46L substitution, which is also considered a primary mutation for the indinavir resistance (Hirsch *et al*., 2000), was observed in three patients. A sequence identical with the consensus B-subtype one was found only in one individual.

The HIV-1 PR polymorphism pattern observed for the group of Czech patients in this study is similar to that published earlier (Shafer, 1999). However, in our study, rare mutations were observed more frequently, probably due to the smaller total number of compared sequences. The frequency of more common mutations correlated with that observed worldwide. Since most of the patients in this study had been infected before PRIs were applied, the amino acid substitutions indicating transmission of a PRI-resistant virus strain were not observed.

Evolution of resistance

A group of 27 patients was monitored for up to 7 years. All of them were undergoing HAART including PRIs, usually in combination with two RTIs. The patients and their treatment are described in detail in Table 1. Resistance to PRIs was detected in 18 patients and mutations in amino acid sequences of PR found in these patients are shown in Table 3. The resistance to one or more PRIs was defined as occurrence of at least single primary mutation at any position of 30, 48, 50, 82, 84, and 90. Total number of observed primary mutations was 27.

The most prevalent primary mutation was L90M. It was found in 16 out of 18 cases exhibiting PRI resistance. The high incidence of this particular mutation typical for the

Table 2. HIV-1 protease polymorphism

L 10	T 12	I 13	K 14	I 15	G 16	L 19	L 33	E 35	M 36	N 37	P 39	R 41	M 46	I 62	L 63	I 64	C 67	H 69	A 71	I 72	V 77	I 93	F 99
I-28	A-8 E-4	V-12	R-8	V-12	A-4	I-8	V-4	D-24	I-4	S-8 D-4 E-4 T-4 Y-4	S-4	K-16	L-12	V-28	P-52 S-12 A-4	V-8 L-4 M-4	E-4 S-4	Q-4	T-8	V-8	I-28	L-24	L-4

HIV-1 protease polymorphism (as compared to the consensus sequence of HIV-1 B subtype) found in isolates retrieved from 25 PR-naive patients from Czech Republic in 1995–1999. Frequency of amino acid substitutions is given in %.

Table 3. Resistance development during HAART in selected patients

Patient code	Treatment at the time of genotyping (months)	Resistance-associated mutations in HIV-1 PR[a]																
		Primary				Secondary and compensatory												
		48	82	84	90	10	20	24	36	46	54	60	63	71	72	73	74	77	93
	–												P						
CZ13	IDV (21)		A					I		L	V		P						
	IDV (38) LPV/r (17)		A			I		I		L	V		P	V					
	–												P						
CZ15	SQV (7)				M	I							P						
	SQV (9) RTV (2) IDV (25) NFV (3) RTV (4) LPV/r (11)		A		M	I	I M		I		V		P	V		S			
	–					I							P					I	L
CZ16	SQV (8)				M	I			I				P	V		S		I	L
	SQV (17) RTV (6) IDV (18)			V	M	IV	M			L			P	V		S		I	L
	–					I							P	T					L
CZ18	SQV(8)				M	I							P	IVT				I	L
	SQV (14) RTV (7,5) IDV (20) NFV (8) LPV/r (4)		A		M	V	R		I		V		P	V	M				
	–												A					I	
CZ20	SQV (7) IDV (6) SQV (7) NFV (8)				M														
	SQV (7) IDV (6) SQV (7) NFV (31)				M														
	–					I							S						
CZ24	SQV (39)				M	I	M		I				T	T					
	SQV (39) IDV (22)				M	I	M		I				T	T					
	not available	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CZ25	SQV (21)				M				I				S						
	SQV (30) IDV (22)								I				S						
	–									L			P						
CZ26	SQV (12)				M					L			P	V					L
	SQV (14) RTV (6) IDV (13,5) LPV/r (7)		A		M	I				L	V	E	P	V	V				L
	–					I							P	T	V			I	
CZ28	SQV (9)			V		I			I			E	P	T	V			I	
	SQV (12) IDV (9) RTV (9) NFV (26,5)			V	M	I							P	T	V			I	
	–									L			P						L
CZ29	SQV (6)				M					L			P						L
	SQV (9)				M					L			P						L
	not available	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CZ30*	SQV (12)				M				I					V			S		L
	SQV (31) RTV (12) IDV (11) NFV (6,5) RTV (7,5) LPV/r (13)			V	M	F	R		I		V		P	V			P		L
	not available	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CZ39	SQV (31)				M								P	V			P	I	L
	SQV (52)				M								P	V		S	A	I	L
	not available	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CZ50	SQV (16)		A		M		R		I				P	V					L
	SQV (37) IDV (17) NFV (33)				M					I		E	P	V	M			I	L
	–																A	I	
CZ52	SQV (25)				M								P					I	
	SQV (46) NFV (3) NFV+RTV (7)				M								P					I	
	–																		
CZ68	SQV (33)				M								P					I	
	SQV (35) IDV (12) IDV+RTV (15)				M								P						
	–												P						
CZ70	SQV (10)				M								P						
	SQV (25)				M								P						
	–												P						L
CZ91	RTV(7)		A										P						L
	RTV(48)		A		M		R		I				P	V					L
	–																		
CZ101	SQV (25) IDV (16)	V	A								T			V			S	I	
	SQV (25) IDV (22) NFV (5)	V	A			I					T			V			S	I	

*Substitutions at positions 36 and 93 are considered natural for C-subtype patients; in the case of patients CZ52 and CZ68, RTV was used as a pharmacokinetic booster (2 x 100 mg per day). Amino acid changes in PR associated with the resistance to PRIs were found by comparison to the consensus B sequence. Patients in the study who did not develop resistance to PRIs are not included.

[a]Upper line: the mutations found prior to the therapy. Middle line: the mutations found at the time point when the first primary mutation was identified. Lower line: the last detected mutations during the therapy.

resistance SQV (Boden and Markowitz, 1998) could be explained by the fact that all but two of these patients were treated with SQV for a prolonged period of time (Table 3). Other frequent mutations included V82A, I84V and G48V. They were accompanied by accessory and/or secondary mutations that had emerged under the selection pressure of PRIs. Since the patients that had been treated with amprenavir or nelfinavir (NFV) as first-choice inhibitors were not included in our study, the mutations D30N and I50V, that represent typical "signature" resistance mutations for these two compounds, were not observed. The patients treated with SQV (Invirase, hard gel formulation) first developed a primary mutation on average after 15 months (in the range from 6 to 39 months) of treatment. The L90M mutation was detected in 14 patients, while G48V was observed in one subject only. This fact corresponded to the low incidence of the G48V mutation associated with the Invirase treatment (Jacobsen *et al*., 1996). The V82A mutation was accompanied by L90M in one subject and by G48V in another one. The I84V mutation was found in one patient.

After the treatment with SQV for various periods of time, 14 out of 16 subjects who had developed resistance to SQV switched to a different PI, usually IDV (7 patients), RTV (5 patients), NFV (1 patient) or LPV/r (1 patient). Ten patients then continued with HAART including more than two PRIs. Under selective pressure of the latter treatment, more primary mutations developed in already PRI-resistant subjects (Table 3). There developed the V82A mutation in 8 patients and the mutations I84V plus L90M in 2 patients.

Besides these primary mutations also various secondary mutations, as compared to the virus sequences from untreated patients, were observed: L10IV (6x), M36I (8x), M46IL (3x), I54VT (5x). The mean number per subject was 4.6 secondary mutations (in the range from 0 to 8).

The patients treated with RTV and IDV developed the V82A mutation in 21 and 7 months, respectively. After a prolonged treatment with RTV (48 months) another primary mutation, L90M appeared together with M36I and A71V. After a prolonged treatment with IDV (38 months) and LPV/r (17 months) no other primary mutation emerged, only secondary ones, L10I and A71V.

A special attention should be paid to the patient CZ30 harboring HIV-1 subtype C. The respective PR sequence prior to the therapy was not available. After 4 months of treatment with SQV, as much as 8 substitutions were detected (Fig. 1). Only 2 of those mutations (M36I and I93L) occurred more frequently in patients treated with PRIs. However, they were reported to occur very often in HIV-1 C-subtype PI-naive patients (Pieniazek *et al*., 2000; LosAlamos HIV databases: http://www.hiv.lanl.gov). In comparison to the C-subtype PR consensus sequence, only the mutations S12T and E35D were identified.

The first primary L90M mutation was found after 9 months of the therapy. As a consequence of 10 months lasting treatment with SQV and RTV, the I84V substitution emerged. During the further 20 months of therapy (IDV, NFV, and RTV) a few more secondary mutations accumulated. After 12 months of the treatment with LPV, the F53L mutation was detected. It has been shown to be associated with a reduced susceptibility to LPV (Kempf *et al*., 2001). Interestingly, the L10I mutation was replaced by a rather unusual L10F. In summary, during 65 months of HAART the total of 18 amino acid substitutions accumulated in PR when compared to consensus B-subtype sequence.

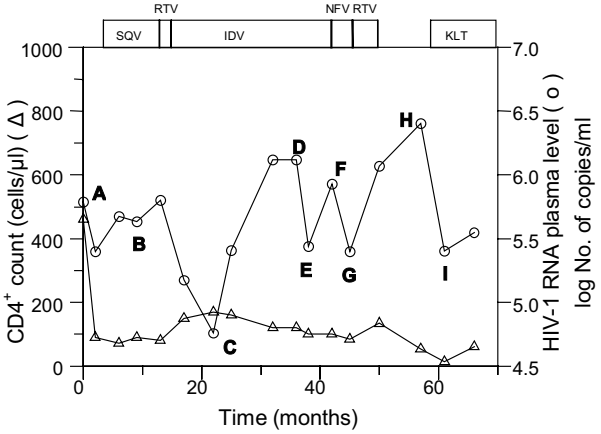
In spite of a prolonged treatment with PIs, PR sequences from 9 patients did not show any sign of primary mutation. All of them were exposed to the selection pressure of SQV followed by other PIs on average for 30 months (in the range from 8 to 42 months). In two patients no changes in the PR sequence were observed during the treatment. In 7 subjects 1–4 mutations were detected. The most prevalent one was the substitution at position 63 (L63P or L63S), while others (I15V, A60G and T94S) were random and their occurrence was observed more frequently in the patients treated with PRIs (data not shown). The adherence to treatment plays an important role in the development of resistance to PRIs. A poor adherence (a low selection pressure) and the opposite, strict adherence together with infection by HIV-1 variants more susceptible to HAART (such as a CXCR4-specific strain (Philpott *et al*., 2001)) may cause a slower selection of primary mutations.

Correlation between patients' virological profile and mutations in PR

Viral load, $CD4^+$ count and mutations in PR were monitored during the treatment of 11 patients. Fig.1 demonstrates the data for 3 selected patients. After the start of the treatment with PIs, a 20-fold decrease in viral load was observed during the first year in the patients CZ15 and CZ26. The patient CZ30 harboring C-subtype of HIV-1, did not respond to treatment with the first PI, SQV, but it did to the second PI, RTV.

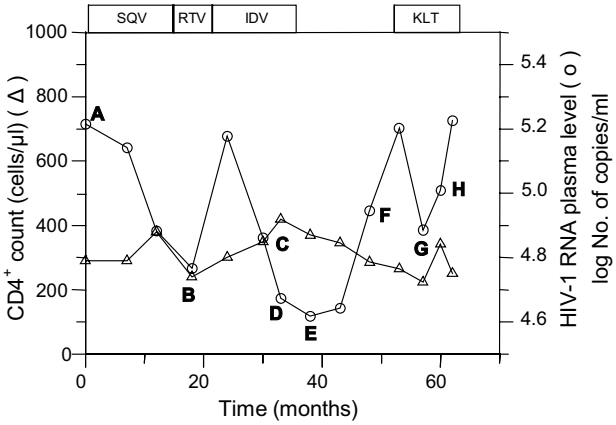
A marked rebound in viral load was connected generally with the development of primary mutation (L90M, V82A, I84V, and G48V) followed by the development of secondary and accessory mutations if the selection pressure of PRIs persisted. The acquisition of resistance to one PRI often led to cross-resistance to other PIs. Therefore, the virological response to the first PI used was usually more pronounced than that to the PRIs used subsequently. The decrease of viral load was usually followed by appearance of additional mutations and consequently by virological failure. This fact led to accumulation of multiple mutations in PR that generated multi-resistant HIV-1.

CZ15



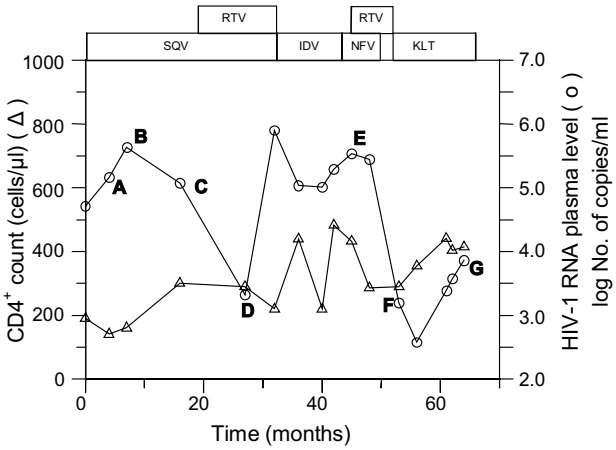
A	I15V, E35D, N37S, R41K, **L63P**
B	**L10I**, I15V, E35D, N37S, R41K, **L63P, L90M**
C	**L10I**, I15V, E35D, **M36I**, N37S, R41K, **L63P, G73S, L90M**
D	**L10I**, I15V, **K20M**, E35D, **M36I**, N37S, R41K, **L63P, A71V, G73S, L90M**
E	**L10I**, I15V, E35D, N37S, R41K, **L63P, A71T, L90M**
F	**L10I**, I15V, E35D, N37S, R41K, **L63P, A71V, G73S, L90M**
G	**L10I**, I15V, E35D, N37S, R41K, I62V, **L63P, A71V, G73S, L90M**
H	**L10I**, I15V, **K20I**, E35D, **M36I**, N37S, R41K, **I54V**, I62V, **L63P, A71V, G73S, V82A, L90M**
I	**L10I**, I15V, **K20IM**, E35D, **M36I**, N37S, R41K, **I54V**, I62V, **L63P, A71V, G73S, V82A, L90M**

CZ26



A	**M46L,** I62V**, L63P, I93L**
B	**M46L, K55R,** I62V**, L63P, A71V, L90M, I93L**
C	**M46L, L63P, A71V, L90M, I93L**
D	**M46L, I54V, K55R, D60E,** Q61E**,** I62V**, L63P, A71V,** I72V**, V82A, L90M, I93L**
E	**M46IL, I54V, I55R,** I62V**, L63P, A71V,** I72V**, L90M, I93L**
F	**L10I,** I13T**, M46L, I54V, K55R, D60E,** Q61E**,** I62V**, L63P A71V,** I72V**, V82A, L90M, I93L,** C95F
G	**L10I, M46L, I54V, K55R, D60E,** Q61E, I62V**, L63P, A71V,** I72V**, V82A, L90M, I93L**
H	**L10I,** I15V**, M46L, I54V, I55R, D60E,** I62V**, L63P, A71V,** I72V**, V82A, L90M, I93L**

CZ30



A	I15V, L19I, E35D, **M36I**, R41K, H69K, L89M, **I93L**
B	I15V, L19I, E35D, **M36I**, R41K, H69K, **A71V**, **T74S**, L89M, **L90M**, **I93L**
C	I15V, L19I, E35D, **M36I**, R41K, Q61H, **L63P**, H69K, **A71V**, **T74S**, L89M, **L90M, I93L**
D	I15V, L19I, **K20R**, E35D, **M36I**, R41K, **I54V**, **L63P**, H69K, **V82I**, **I84V**, L89M, **L90M, I93L**
E, F	**L10I**, I15V, L19I, **K20R**, E35D, **M36I**, R41K, **I54V**, **L63P**, H69K, **A71V**, T74P, **I84V**, L89M, **L90M, I93L**
G	**L10F**, L19I, **K20R**, L33F, E35D, **M36I**, R41K, **F53L**, **I54V**, **L63P**, H69K, **A71V**, T74P, **I84V**, L89M, **L90M, I93L**

Fig. 1

Time course of CD4⁺ count, HIV-1 RNA plasma level and amino acid substitutions in three selected patients

A-I: amino acid substitutions in the PR gene, as compared to the consensus sequence of PR gene of HIV-1 of B subtype, at given time points. The mutations associated with resistance are in bold. GenBank Acc. Nos. of the sequences are AY352051-AY352073.

The relationship between the CD4 T cell counts and plasma HIV-1 RNA levels is complex, most likely depending on the immunological response of individual patient (DeHovitz *et al.*, 2000).

In several cases we observed suppression of multi-resistant virus replication upon treatment with LPV. However, this effect was only temporary. A rebound of plasma levels during the LPV treatment was accompanied by changes in the PR sequence as in the case of the patient CZ30 (Fig. 1). In this HIV-1 C-subtype, the F53L substitution emerged under the selection pressure of LPV and the mutation L101 was replaced by L10F.

All the patients under study were also treated with various RTIs (Table 1). We attempted to identify the changes in CD4 count or viremia in the patients CZ15, CZ26 and CZ30 that could be attributed to the development of RTI resistance. In the case of the patient CZ15 (Fig. 1), the only suppression of plasma HIV-1 RNA levels caused by RTI alone (not in combination with PRIs) was achieved by ddI after 30 months of treatment. This finding was obtained despite the fact that, in this case, also a resistance to ddI was detected; (the mutations M41L, K70R, T69D and M184V were observed (Fig. 1, time point D). The rebound of viral load was followed by the emergence of the T215F mutation in RT. This mutation together with M184V after the treatment with AZT, ddC, 3TC and d4T was also observed in the patient CZ26 (Fig. 1, time point C). Subsequent treatment with ABC resulted in the emergence of the mutations M41L and L74V and the disappearance of the M184V mutation after 50 months of therapy (Fig. 1). In the case of the HIV-1 C-subtype patient CZ30, only the mutations M184 and T215Y were detected after treatment with AZT, DDC, d4T, 3TC, ABC, DDI, EFV, and NVP. It should be noted, however, that hybridization techniques (such as LiPA) may not be accurate enough for HIV-1 subtypes other than B. None of the 3 patients depicted in Fig. 1 responded to the treatment by non-nucleoside RTIs NNRTI, NVP and EFV.

In conclusion, 27 patients on long-term HAART therapy involving SQV and other PRIs were closely followed for up to 7 years. Genetic variations of HIV-1 PR were identified. Under the pressure of PIs, HIV-1 mutants are selected and the development of viral resistance could be followed by surrogate markers like CD4 count and viral load. The dynamics of viral load well corresponded to the accumulation of specific primary mutations in the PR and RT. All 27 patients were treated with PRIs and 18 developed resistance to a given drug. Prolonged therapy led to the accumulation of amino acid changes associated with resistance and consequently a cross-resistance was observed. Multi-resistant variants of HIV-1 with mutations in PR are difficult to inhibit sufficiently with the inhibitors that are currently available in clinical practice. Efficient yet temporary suppression of viral replication was achieved by LPV treatment.

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References

Boden D, Markowitz M (1998): Resistance to human immunodeficiency virus type 1 protease inhibitors. *Antimicrob. Agents Chemother.* **42**, 2775–2783.

DeHovitz JA, Kovacs A, Feldman JG, Anastos K, Young M, Cohen M, Gange SJ, Melnick S, Greenblatt RM (2000): The relationship between virus load response to highly active antiretroviral therapy and change in CD4 cell counts: A report from the Women's interagency HIV study. *J. Infect. Dis.* **182**,1527–1530.

Hirsch MS, Brun-Vezinet F, D'Aquila RT, Hammer SM, Johnson VA, Kuritzkes DR, Loveday C, Mellors JW, Clotet B, Conway B, Demeter LM, Vella S, Jacobsen DM, Richman DD (2000): Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. *JAMA* **283**, 2417–2426.

Ho DD (1995): HIV-1 dynamics in vivo. *J. Biol. Regul. Homeost. Agents* **9**, 76–77.

Jacobsen H, Hanggi M, Ott M, Duncan IB, Owen S, Andreoni M, Vella S, Mous J (1996): In vivo resistance to a human immunodeficiency virus type 1 proteinase inhibitor: mutations, kinetics, and frequencies. *J. Infect. Dis.* **173**, 1379–1387.

Ji X, Klarmann GJ, Preston BD (1996): Effect of human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein on HIV-1 reverse transcriptase activity in vitro. *Biochemistry* **35**, 132–143.

Kempf DJ, Isaacson JD, King MS, Brun SC, Sylte J, Richards B, Bernstein B, Rode R, Sun E (2001): Identification of genotypic changes in human immunodeficiency virus protease that correlate with reduced susceptibility to the protease inhibitor lopinavir among viral isolates from protease inhibitor-experienced patients. *J. Virol.* **75**, 7462–7469.

Muzammil S, Ross P, Freire E (2003): A major role for a set of non-active site mutations in the development of HIV-1 protease drug resistance. *Biochemistry* **42**, 631–638.

Philpott S, Weiser B, Anastos K, Kitchen CM, Robison E, Meyer WA 3rd, Sacks HS, Mathur-Wagh U, Brunner C, Burger H (2001): Preferential suppression of CXCR4-specific strains of HIV-1 by antiviral therapy. *J. Clin. Invest.* **107**, 431–438.

Pieniazek D, Rayfield M, Hu DJ, Nkengasong J, Wiktor SZ, Downing R, Biryahwaho B, Mastro T, Tanuri A, Soriano V, Lal R, Dondero T (2000): Protease sequences from HIV-1 group M subtypes A-H reveal distinct amino acid mutation patterns associated with protease resistance in protease inhibitor-naive individuals worldwide. *AIDS* **14**, 1489–1495.

Reinis M, Bruckova M, Graham RR, Vandasova J, Stankova M, Carr JK (2001): Genetic subtypes of HIV type 1 viruses circulating in the Czech Republic. *AIDS Res. Hum. Retroviruses* **17**, 1305–1310.

Shafer RW, Stevenson D, Chan B (1999): Human Immunodeficiency Virus Reverse Transcriptase and Protease Sequence Database. *Nucleic Acids Res.* **27**, 348–352.

Weber J, Mesters JR, Lepsik M, Prejdova J, Svec M, Sponarova J, Mlcochova P, Skalicka K, Strisovsky K, Uhlikova T, Soucek M, Machala L, Stankova M, Vondrasek J, Klimkait T, Kraeusslich HG, Hilgenfeld R, Konvalinka J (2002): Unusual binding mode of an HIV-1 protease inhibitor explains its potency against multi-drug-resistant virus strains. *J. Mol. Biol.* **324**, 739–754.

Wlodawer A, Vondrasek J (1998): Inhibitors of HIV-1 protease: a major success of structure-assisted drug design. *Annu. Rev. Biophys. Biomol. Struct.* **27**, 249–284.