CHARACTERISATION OF MUTATED PROTEINASES DERIVED FROM HIV-POSITIVE PATIENTS: ENZYME ACTIVITY, VITALITY AND INHIBITION

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Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

HIV protease (PR) specifically cleaves viral polyproteins to yield infectious progeny virus particles. Inactivation of PR leads to loss of virus infectivity and PR thus became an attractive pharmaceutic target. Indeed, seven protease inhibitors (PI) have been approved for clinical use to date. However, emerging resistant viral variants with reduced sensitivity to PIs become a major obstacle to successful control of viral replication. We have previously reported the design, testing and structural analysis of a pseudopeptide inhibitor, QF34, which efficiently inhibits a wide variety of PR variants. In a clinical study, we have monitored more than 100 HIV-positive patients in the Czech Republic undergoing highly active antiretroviral therapy including PI. In this paper we describe kinetic characterisation of two highly resistant PR species isolated from these patients. The mutated proteases accumulated as much as 14 amino acid exchanges and develop resistance to saquinavir, ritonavir, indinavir and nelfinavir with vitality value up to 150. Kinetic analyses revealed that second-generation PI lopinavir and QF34 retained their subnanomolar potency against both multidrug resistant PR variants. These results suggest a route to the design of PIs capable of inhibiting a variety of resistant PR mutants.

Keywords: Enzyme inhibitors; Enzyme kinetics; HIV protease; Aspartic proteases; Viral resistance; Antiviral drugs; AIDS; Peptidomimetics; Pseudopeptides.

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The protease inhibitors (PI)[+] were introduced into clinical practice in 1995–1996 and, together with the application of highly active antiretroviral therapy (HAART), caused a decreased mortality and prolonged life expectancy of HIV-positive patients. However, the selection pressure of a virostatic leads to rapid selection of viral variants resistant to a specific inhibitor. Mutations rendering the enzyme resistant to PIs have been observed in more than half of the 99 residues of the HIV protease (PR). Such resistant PR variants have developed in HIV-positive patients after treatment with most of the seven protease inhibitors (PIs) approved for clinical use.

The rapid development of the resistant PR variants can be explained by natural variability of the virus and the dynamic viral replication in infected patients[1]. The virus responds to the selection pressure of a PI through accumulation of mutations in the PR binding cleft that directly influence the binding of the PI (primary mutations), mutations at sites distant from the active site. which influence the PI binding indirectly by their influence on subdomain flexibility of the PR molecule[2] (secondary mutations), or mutations outside the PR coding region, which change the amino acid sequences at the processing sites of the Gag-Pol polyprotein and increase thus the capability of the mutated PR to process viral polyprotein at these sites[3,4].

The primary mutations occur predominantly in and around the PR binding cleft. The specificity pockets of the enzyme (denoted S1-Sn and S1′-Sn′ to depict sites accommodating residues N- and C-terminal to the cleaved peptide bond, respectively, while the corresponding substrate residues binding to these subsites are accordingly denoted P1-Pn and P1′-Pn′)[5] are obvious candidates for mutations that could directly influence inhibitor binding[6,7] (for a review, see literature[8]). Although these mutations lead only to minor structural changes, they result in a dramatic decrease in inhibitor binding (for reviews, see literature[2,9–11]). Under prolonged selection pressure of the PI, the virus responds by concerted accumulation of mutations, often as many as 10–14 [6,12], which are located outside the binding cleft of the protease.

\+ ***Abbreviations used***: 3TC, lamivudin; ABC, abacavir; AZT, azidothymidin (zidovudin); d4T, stavudin; ddC, dideoxycytidin (zalcitabin); ddI, dideoxyinosin (didanosin); EFV, efavirenz; FACS, fluorescence-aided cell sorter; HAART, highly active antiretroviral therapy; IDV, indinavir; k_{cat}, catalytic rate constant; K_i, inhibition constant; K_m, Michaelis constant; LPV, lopinavir; LPV/r, combination of lopinavir and ritonavir (Kaletra™); MUT, mutant; NFV, nelfinavir; Nle, norleucin; Nph, 4-nitrophenylalanine; NVP, nevirapin; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; PI, protease inhibitor; PR, protease; RT, reverse transcriptase; RTV, ritonavir; SQV, saquinavir; wt, wild-type.

Different PIs select different patterns of mutations. The resistance to saquinavir develops in 50% of treated patients within the first year of therapy[13] and is mostly associated with mutations G48V and L90M [14]. Treatment with ritonavir usually leads to multiple mutations[7] with dominant amino acid exchanges in positions 82 and 84. Indinavir typically selects resistant mutations relatively rapidly[6] (12–24 weeks), although the resistance is usually conferred by multiple exchanges in a number of positions, typically V82A, M46I, accompanied by mutations in positions 10, 20, 24, 63, 71 and 90 [4,15]. Nelfinavir selects the unusual mutation D30N and, less frequently, exchange in positions 35, 36, 46, and 88 [16].

The PIs currently used in clinics are derived from pseudopeptide substrate analogs and were designed to fit into the binding subsites of the HIV PR active-site cleft. Therefore, mere 4–5 amino acid exchanges can lead to significant cross resistance[6]. Some amino acid exchanges seem to be more relevant for the cross resistance than others (e.g. V32I, M46I,L,F, I47V, G48V, V82A,I,T, I84V, L90M [17]).

The molecular mechanism of the resistance development was analysed by kinetic analysis of recombinant mutated PR species and their X-ray structure in complexes with inhibitors[19–25]. Based on this structural information, several laboratories designed second-generation PIs, capable of potently inhibiting multi-resistant HIV-1 PR species[26,27]. X-ray structure determinations, theoretical calculations and thermodynamic analyses of the binding of PIs to the mutated PR species seem to suggest that the flexibility of the groups occupying individual binding sites together with proper balance between hydrophobic and polar interactions are keys for the wide specificity of the inhibitor[17].

In our previous analyses[18,28,29], we have used HIV-2 PR as a model for PI-resistant PR species and developed a wide-specificity compound capable of inhibiting various HIV PR species, denoted QF34. In this paper we present data confirming wide specificity of the inhibitor, using two highly resistant PR species derived from HIV-positive patients undergoing prolonged treatment with a series of protease inhibitors.

EXPERIMENTAL

Patients

Within a long-term epidemiological study, HIV-positive patients receiving HAART at the Faculty Clinic Bulovka in Prague have been closely followed for the presence of resistant HIV species[18]. Selection of the patients for this study (patients CZ101 and CZ15) was based on the length of the PI treatment and clinical markers suggesting resistance development.

The HIV-1 RNA copy number was determined by a quantitative reverse trascriptase-polymerase chain reaction (RT-PCR) (Amplicor HIV-1 Monitor version 1.5) and the $CD4^+$ cell count was determined by fluorescence-aided cell sorter (FACS) analysis using CaliburE2771. Both analysed virus species belonged to the B clade of HIV-1.

DNA Amplification

The PR coding region from HIV-1 was isolated and amplified from patient plasma and/or peripheral blood mononuclear cells (PBMC) by RT-PCR/PCR. A detailed description will be given elsewhere. Briefly, for DNA amplification, the mononuclear cells were isolated by centrifugation (Ficoll–Paque Plus, Amersham Pharmacia Biotech AB, Uppsala, Sweden), lysed with proteinase K at 56 °C and the cell lysate served directly as a template for amplification. The DNA amplification was performed by nested PCR in a PCR Mini Cycler (MJ Research, Inc. Waltham, MA, U.S.A.) using two sets of primers. The external set was JP4Uex (5′-CAGAGCCAACAGCCCCAGCAG-3′) and JP3Dex (5′-CTTTTGGGCCATCCATTCCTGGC-3′), the internal primers were JP1bUin (5′-TAGAATTCATATGAGAGACAACAACTCCCCCT-3′) including *Eco*RI and *Nde*I sites at the 5′-end, and JP2Din (5′-GGGGATCCTTACTATGGTACA-GTCTCAATAGG-3′) including a *Bam*HI restriction site at its 5′-terminus. For the RT-PCR, the viral plasma RNA was isolated from 200 μl of patient plasma using the High Pure Viral RNA Kit (Roche Molecular Biochemicals), according to the manufacturer's recommendations. Isolated RNA served directly as a template for RT-PCR using the RNA PCR Core Kit (Perkin–Elmer Corporation, Norwalk, CT, U.S.A.) in a thermocycler GeneAmp PCR System 2400 (Perkin–Elmer).

DNA Sequencing

The DNA coding region for HIV PR was sequenced directly after PCR amplification or after subcloning into the bacterial plasmid pUC19 using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer). The sequencing reaction products were analysed on an ABI Prism 310 DNA sequencer (Perkin–Elmer).

Expression and Purification of Recombinant PRs

The DNA coding regions for PR species from patients CZ15 and CZ101 were obtained from the second round of PCR from clinical samples using an internal set of PCR primers. The PCR products were cleaved by *Nde*I and *Bam*HI and ligated into the pET24a plasmid vector for bacterial expression. The host strain *E. coli* BL21 (DE3) (Novagen) was used to overexpress the PR precursors. Proteases were purified from inclusion bodies by solubilisation in urea, followed by chromatography on QAE-Sephadex and Mono S-Sepharose (Amersham Pharmacia Biotech), as described previously[18,29,30]. The concentrations of the enzymes used in this study was determined by active-site titration using lopinavir as a tight-binding inhibitor.

Activity and Inhibition Assay

Inhibition constants were determined by a spectrophotometric assay with the chromogenic peptide substrate KARVNle*NphEANle-NH_2. Typically, 8 pmol of PR was added to 1 ml 0.1 M sodium acetate buffer (pH 4.7), containing 0.3 M NaCl, 4 mM EDTA, 20 nmol of substrate

and various concentrations of an inhibitor dissolved in DMSO [30]. The final concentrations of DMSO were kept below 2.5%. The substrate hydrolysis was followed as a decrease in absorbance at 305 nm using an Aminco Bowman DW2000 spectrophotometer.

The data obtained were analysed using the equation for competitive inhibition according to Williams and Morrison[31]. All the K_i values are the averages of at least two determinations.

RESULTS AND DISCUSSION

PR Variants Derived from HIV-Infected Patients with Highly Resistant Viral Strains

Prolonged treatment of HIV-positive patients with PIs leads to the development of PR sequences with the mutation pattern that is too complex to enable reliable prediction of the sensitivity of individual PIs to the mutated virus species. The primary and secondary mutations are not independent and can modulate the enzyme activity in a way that is difficult, if not impossible, to predict. Therefore, in order to test the ability of an inhibitor to block resistant mutants of HIV PR, it is necessary to analyse its activity against PR species isolated from patients that developed a resistant phenotype during PI treatment.

Of a group of 138 patients monitored for HIV drug resistance within a long-term study analysing PI resistance development in the Czech Republic, twenty-seven were followed for the development of changes in the HIV PR coding sequence for a period of up to 7 years. Two patients were selected from this study for complete characterisation of their mutated PR species in order to expand the repertoire of available PR variants for the testing of broad-specificity compounds (Table I). Both of them have a long history of highly active antiretroviral treatment (HAART; see Fig. 1). PR sample CZ101

TABLE I
Characteristics of the HIV-positive patients involved in the study (patients CZ101 and CZ15) and their treatment

Patient	Sex	Date of birth	Seropositive since	PI treatment (months)	Mutations in HIV-1 PR (compared with B consensus)
CZ101	male	1938	1989	SQV(25), IDV(23), NFV(4)	L10I, I13V, R41K, G48V, I54T, I64V, A71V, T74S, V77I, V82A, I93M
CZ15	male	1953	1988	SQV(9), RTV(2), IDV(25), NFV(4), RTV(4), LPV/r(2)	L10I, I15V, K20I, E35D, M36I, N37S, R41K, I54V, I62V, L63P, A71V, G73S, V82A, L90M

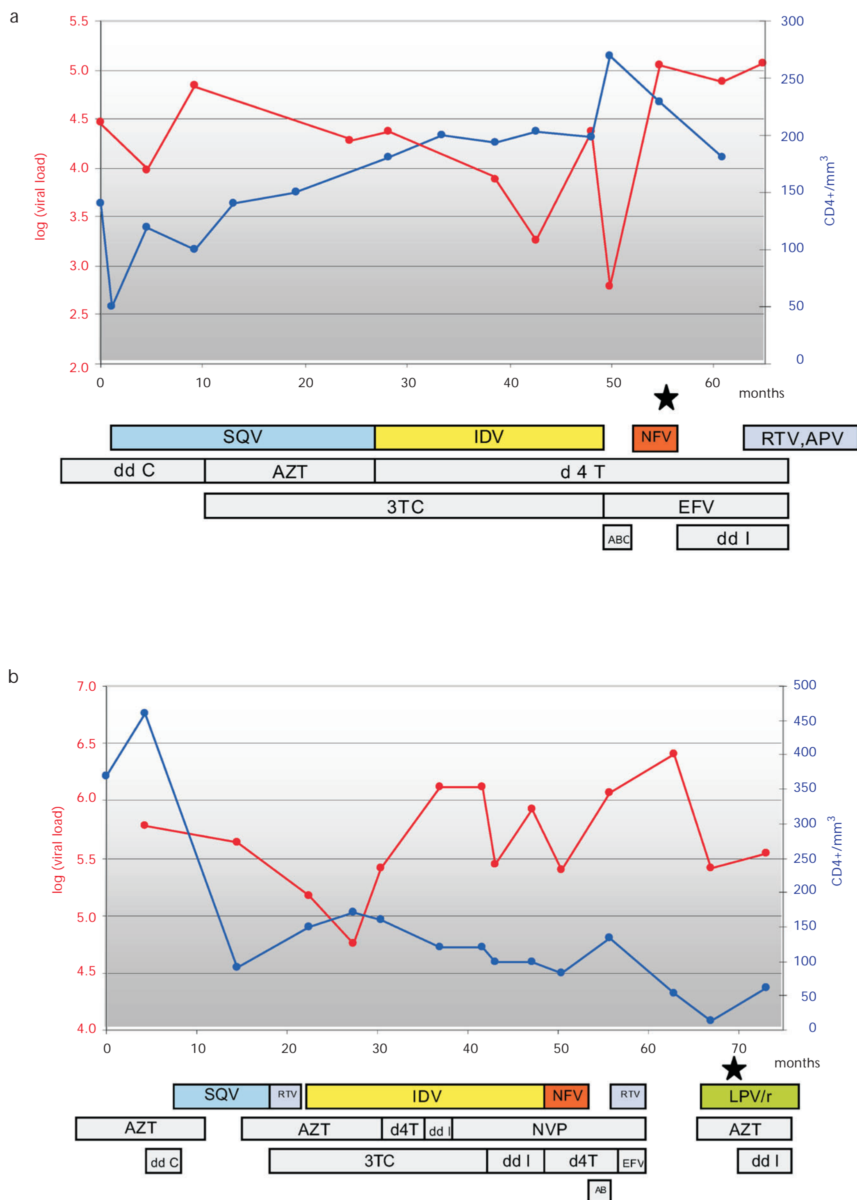

FIG. 1
Correlation of viral load ($\log_{10}$ copies of HIV-1 RNA/ml of plasma) and CD 4 count response (cells/mm^3) with the amino acid changes in the PR sequence for patients CZ101 (a) and CZ15 (b) undergoing long-term treatment with PIs

corresponds to an HIV-positive patient treated consecutively with saquinavir, indinavir and, at the time of the sampling, with nelfinavir. As much as 7 RT inhibitors were also administered to this individual in the period between December 1996 and April 2002. Figure 1a shows the values of the viral load and CD4+ cell count over the period of 6 years. The asterisk depicts the time point when the sequence PR CZ101 was obtained. This time point corresponds to a sharp increase in the viral load, accompanied by a decrease in CD4 count, which suggests resistance development. Indeed, the sequence comprises as much as 11 primary and secondary mutations when compared with consensus B-sequence. The important primary mutations involve substitution G48V typical for the saquinavir resistance and V82A associated with ritonavir and indinavir resistance.

PR sample CZ15 is derived from a patient treated for a prolonged period of time with saquinavir, ritonavir, indinavir and nelfinavir in combination with as much as 10 different RT inhibitors (Fig. 1b). The sample was taken shortly after combination treatment with lopinavir-ritonavir (Kaletra) was started. The later stage of antiretroviral therapy in the time of sampling is mirrored by a higher number of substitutions in the PR region. The primary mutations again involve substitutions V82A and L90M. No primary mutation, typical for the resistance against nelfinavir (D30N, N88D) was observed, probably due to a relatively short period of time when nelfinavir was administered to the patient. On the other hand, primary mutations to nelfinavir also include the L90M. The mutations selected during the treatment with the previous PIs might already provide sufficient cross resistance to nelfinavir.

The localisation of the mutations in the PR molecule is schematically depicted in Fig. 2.

Enzymatic Characterisation of Recombinant Mutated PR Species

Both recombinant proteins were expressed and purified to homogeneity as described above. For kinetic characterisation, chromogenic substrate KARVNle*NphEANle-NH_2, derived from the cleavage site between the capsid and P2 proteins on the HIV polyprotein[32] was used. The values of K_m and k_{cat} are summarised in Table II and are compared with the values for the wild-type (wt) HIV PR. Both mutated proteases show an expected decrease in the catalytic efficiency (4 times decrease for the mutant CZ101 and 2.3 times decrease for CZ15). The catalytic efficiency of the least active PR variant, CZ101, still corresponds to 25% of that of the reference wild-type PR. These clinically isolated variants thus retain the catalytic activity

necessary to support viral replication. The higher activity of mutant CZ15 is conceivably due to secondary mutations that improve the replication of the virus by improving PR activity (probably sites 20, 36 and 63). Interestingly, both PR mutants compensate a rather sharp decrease in k_{cat} values (7.5 and 3 times for CZ101 and CZ15, respectively) by improvement of the substrate binding (K_m decreased twice and 1.4 times, respectively).

Inhibition Analysis and Vitality

Inhibition constants (K_i values) for the clinically used inhibitors saquinavir, indinavir, ritonavir, nelfinavir and lopinavir and for inhibitor QF34 were determined for both PR variants and compared with the values for the

TABLE II
Kinetic analysis of HIV PR mutants isolated from the clinical samples using chromogenic peptide substrate KARVNleNphEANle (for details, see Experimental)

HIV-1 PR	K_m, μmol l^{-1}	k_{cat}, s^{-1}	k_{cat}/K_m, μmol^{-1} l s^{-1}
Wild type	15.1 ± 1.3	30.0 ± 1.8	1.99
CZ101	7.8 ± 0.9	4.0 ± 0.3	0.51
CZ15	11.0 ± 0.7	9.6 ± 0.5	0.87

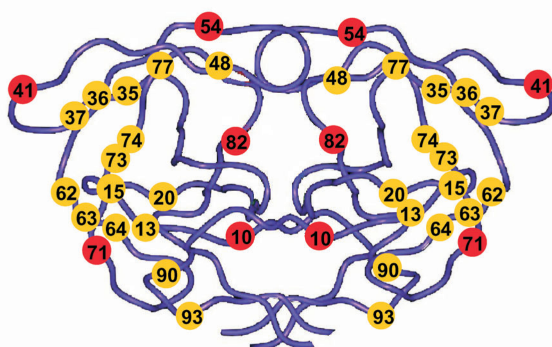

FIG. 2
Schematic representation of the HIV PR structure. Mutated positions are highlighted (red, mutations shared by both patients CZ15 and CZ101; yellow, mutations observed in one patient only)

wild-type PR (for the structures of the inhibitors, see Fig. 3, for the K_i values, see Table III). In order to compare the relative selective advantage of a given PR variant over another one in the presence of an inhibitor, the catalytic efficiency of the PR variant must be included in the relative inhibition calculations. To achieve this, the term "vitality" has been introduced as a measure of resistance. The vitality, *v*, is defined as $v = (K_i k_{cat}/K_m)_{MUT}/(K_i k_{cat}/K_m)_{WT}$ and predicts thus the therapeutic effect of a given PI [33]. Figure 4 presents the vitality values for PR mutants CZ101 and CZ15. Patient CZ101 was treated with saquinavir, indinavir and nelfinavir. Indeed, high resistance (high vitality value) was determined to these inhibitors for the PR mutant corresponding to this patient's virus. The highest vitality value

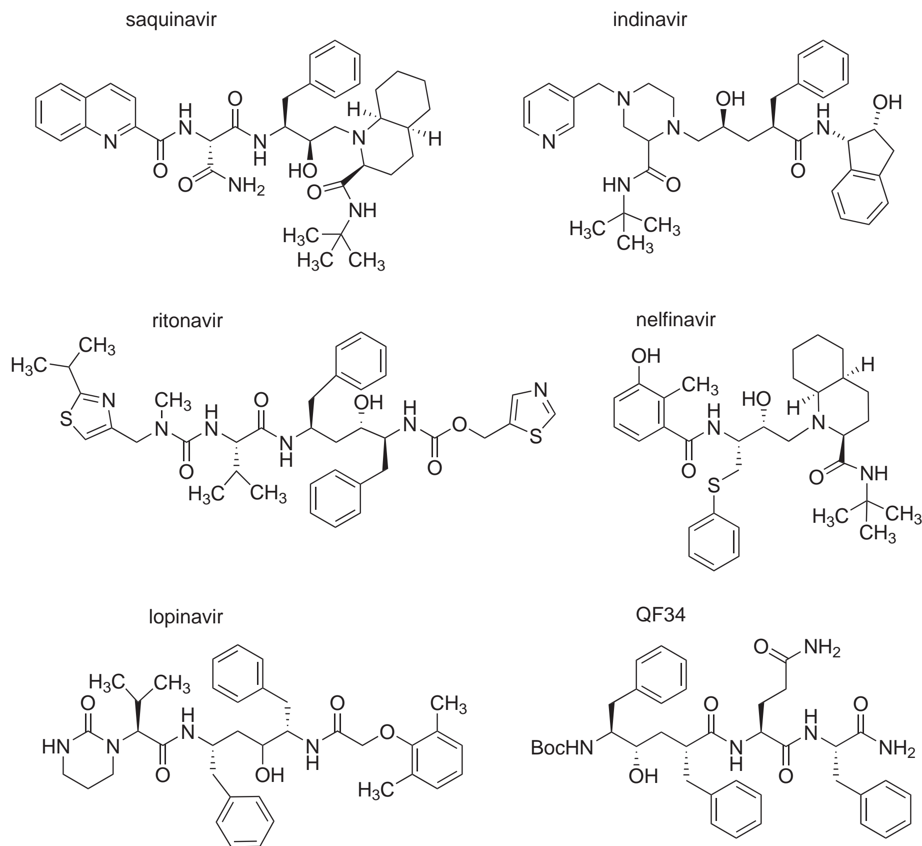

FIG. 3
Chemical structures of the inhibitors used in the study

was determined for saquinavir, which had been used in the treatment of this patient for 25 months. A slightly lower decrease in sensitivity was observed for indinavir (23 months of treatment). The patient was not treated with ritonavir before the sample was taken: the resistance to this drug is probably due to the cross resistance caused by the mutations selected by indinavir. Very low vitality values are observed for lopinavir and QF34. The data summarised in Fig. 4 suggest that lopinavir would be a useful inhibitor for patient CZ101.

TABLE III
Inhibition constants for QF34 and 5 clinically used inhibitors of HIV PR with wt HIV-1 PR and patient-derived PRs CZ101 and CZ15 (for details, see Experimental)

Inhibitor	K_i, nmol l^{-1} CZ101	K_i, nmol l^{-1} CZ15	K_i, nmol l^{-1} wt HIV-1 PR
Saquinavir (SQV)	23.3 ± 2.6	11.7 ± 1.1	0.04 ± 0.01
Ritonavir (RTV)	1.5 ± 0.2	3.8 ± 0.4	0.015 ± 0.003
Indinavir (IDV)	42.3 ± 7.2	24.0 ± 1.4	0.12 ± 0.02
Nelfinavir (NFV)	16.9 ± 1.7	13.4 ± 1.9	0.07 ± 0.01
Lopinavir (LPV)	0.71 ± 0.12	0.57 ± 0.12	0.110 ± 0.007
QF34	0.85 ± 0.16	0.28 ± 0.05	0.021 ± 0.005

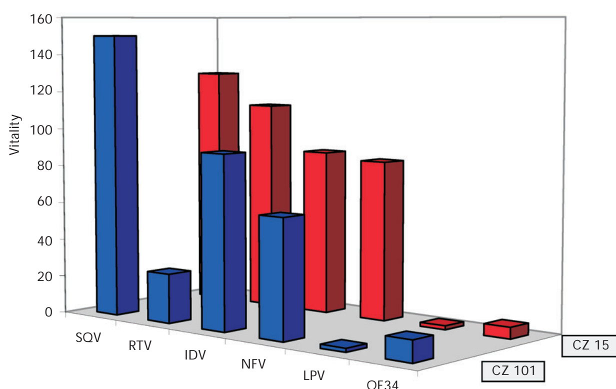

FIG. 4
Vitality of HIV PR mutants CZ101 and CZ15 and inhibitors used in the study (for details, see Experimental)

Patient CZ15 was treated with saquinavir, ritonavir, indinavir and nelfinavir. The PR mutant prepared from the patient shows high vitality values for all these inhibitors (Fig. 4). The patient was also treated with lopinavir: the 2-months period clearly did not suffice to select mutations rendering resistance to the drug. Again, the enzymatic analysis suggests lopinavir as a favourable treatment for this patient.

Inhibitor QF34 was shown to retain subnanomolar K_i values for multi-drug resistant HIV PR species isolated from patients treated with various protease inhibitors for a prolonged period of time. It remains to be elucidated if this relaxed substrate specificity would be sufficient to block the protease variants with mutations in position 50, known to be resistant to the new PIs atazanavir and amprenavir.

Interpretation of these data could be provided only when 3-D structure of the inhibitor with the studied PR mutants is available. Our previous structural studies[18] suggest that the flexibility and unusual binding mode of the inhibitor in the PR binding cleft are responsible for its potency and wide specificity.

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