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Development of a microtiter plate fluorescent assay for inhibition studies on the HTLV-1 and HIV-1 proteinases

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Abstract

The proteinase of human T-cell leukemia virus type-1 (HTLV-1), similar to the proteinase of human immunodeficiency virus type-1 (HIV-1), is a potential target for chemotherapy, since the virus is associated with a number of human diseases. A microtiter plate fluorescent assay was developed for the HTLV-1 and HIV-1 proteinases for direct comparison of the inhibition profiles of the enzymes. It was established that, except for Indinavir, none of the inhibitors designed against the HIV-1 proteinase were able to inhibit the HTLV-1 proteinase in the studied concentration range, while two reduced peptide bond-containing peptides having the sequence of HTLV-1 cleavage sites were inhibitors of the HTLV-1 proteinase. One of these was potent enough to be used for active site titration of the HTLV-1 proteinase.

Keywords: HTLV-1 proteinase; HIV-1 proteinase; Oligopeptide substrates; Fluorescent assay; Substrate specificity; Inhibitors

1. Introduction

Human T-cell leukemia virus type-1 (HTLV-1) is a retrovirus that has been etiologically associated with a number of diseases including adult T-cell leukemia and HTLV-1 associated myelopathy (for recent reviews, see Johnson et al., 2001; Gallo, 2002). HTLV-1 infection is a global epidemic: 10–20 million individuals are estimated to be carriers of the virus and the risk of developing disease is estimated to be 5% in infected patients (Macchi et al., 2003). Studies indicate that viral replication is critical for the development of HTLV-1 associated myelopathy, and initial studies suggested that blocking replication with AZT had a therapeutic effect (Sheremata et al., 1993).

The retroviral proteinase (PR) is responsible for the processing of viral Gag and Gag-(Pro)-Pol polyproteins during maturation, hence catalyzing essential steps of virion replication (for a review, see Oroszlan and Luftig, 1990). Therefore, the HIV-1 PR has proved to be an important target for antiretroviral therapy of AIDS, and various PR inhibitors are now in clinical use (for a review, see Swanstrom and Eron, 2000). HTLV-1 PR, like HIV-1 PR, is a homodimeric aspartyl proteinase. Recently we have cloned and partially characterized the HTLV-1 PR (Louis et al., 1999; Tözsér et al., 2000). While HTLV-1 PR shares 28% sequence identity with HIV-1 PR, the substrate binding region is more conserved showing 45% sequence identity, nevertheless, the substrate specificities of the two enzymes are different (Louis et al., 1999; Tözsér et al., 2000). Furthermore, initial tests suggested that the inhibition profile of the two enzymes was substantially different (Louis et al., 1999; Hruskova-Heidingsfeldova et al., 1997; Ding et al., 1998).

A fluorogenic method for measuring protease activity utilizing a synthetic peptide with an acceptor and a quenching molecule attached to either end of the substrate was first described for HIV-1 PR (Matayoshi et al., 1990; Wang et al.,

Abbreviations: HTLV-1, human T-cell leukemia virus type-1; HIV-1, human immunodeficiency virus type-1; PR, proteinase; MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; TFA, trifluoroacetic acid; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; Dabcyl, 4-(4-dimethylaminophenylazo) benzoic acid; Edans, 5-((2-aminoethyl)amino)naphtalene-1-sulfonicacid

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1990). The sequence of the substrate they used was based on the HIV-1 MA↓CA cleavage site, and it contained two covalently modified residues, a Glu modified with the fluorophore 5-((2-aminoethyl)amino)naphtalene-1-sulfonic acid (Edans) and a Lys modified with the acceptor chromophore 4-(4-dimethylaminophenylazo)benzoic acid (Dabcyl). The acceptor molecule was chosen for maximal overlap of its absorbance with the emission spectrum of the fluorophore, resulting in quenching of the fluorophore, through resonance energy transfer. As a result of proteolytic cleavage, the full fluorescence quantum yield of Edans is restored. Later assays based on the same principle were also developed for other proteases including cytomegalovirus protease (Holskin et al., 1995) and hepatitis C virus NS3 protease (Liu et al., 1999). Here we describe a fluorescent plate reader assay for the direct comparison of the inhibition profiles for the HTLV-1 and HIV-1 proteinases.

2. Materials and methods

2.1. Oligopeptides

Unmodified oligopeptide substrates used in this paper were described previously (Louis et al., 1999). The donor/acceptor (Edans/Dabcyl) containing peptides and the fluorescent control peptide RE(Edans) were synthesized by Dr. Ivo Blaha (Ferring Leciva, Prague). Stock solutions of the fluorescent peptides were made in water.

2.2. HPLC assays

Purified HIV-1 and HTLV-1 proteinases, containing stabilizing mutations were prepared as described previously (Louis et al., 1991, 1999; Mahalingam et al., 2001). The protein concentrations for the PR preparations were determined by amino acid analysis. The PR assays were initiated by the mixing of 5 μl (8–140 nM) purified PR with 10 μl 2× incubation buffer (0.5 M potassium phosphate buffer, pH 5.6, containing 10% glycerol, 2 mM EDTA, 10 mM dithiothreitol, 4 M NaCl) and 5 μl (0.01–1.3 mM) substrate. The active amount of the enzymes were determined by active site titration, for these assays the volume of the substrate was 4.8 μl and 0.2 μl inhibitor solution of various concentrations were also added. Compound 3 (Grobelny et al., 1990) and IB-268 were used as inhibitors for HIV-1 and HTLV-1 proteinases, respectively. The substrate concentration range for the kinetic determinations was selected depending on the approximate K_m values. The reaction mixture was incubated at 37 °C for 1 h and the reaction was stopped by the addition of 180 μl 1% trifluoroacetic acid (TFA), and an aliquot was injected onto a Nova-Pak C_{18} reversed-phase chromatography column (3.9 mm × 150 mm, Waters Associates Inc.) using an automatic injector. Substrates and the cleavage products were separated using an increasing water–acetonitrile gradient (0–100%) in the presence of 0.05% TFA. Cleavage products of PR-catalyzed hydrolysis for the peptides were identified by amino acid analysis. Kinetic parameters were determined by fitting the data obtained at less than 20% substrate hydrolysis to the Michaelis–Menten equation (or by linear fitting for k_{cat}/K_m values determined under pseudo first order conditions) by using the Fig. P program (Fig. P Software Corp.). The standard errors of the kinetic parameters were below 20%. The catalytic constants were calculated using the active enzyme amounts determined by active site titration.

2.3. Fluorescent microtiter plate assay

The PR assays were performed in microtiter plate wells, by mixing 10 μl (60–4800 nM) purified HTLV-1 PR with 100 μl PNF buffer (250 mM phosphate buffer, pH 5.6 containing 5% glycerol, 1 mM EDTA, 5 mM DTT, 500 mM NaCl). The mixture was preincubated at 37 °C for 5 min and the reaction was initiated by the addition of 90 μl fluorescent peptide substrate (2–40 μM) in PNF buffer. The increase of fluorescence was detected at 460 nm, using 355 nm excitation wavelength in a Wallac 1420 Victor^2 fluorimeter-luminometer (Wallac Oy, Turku, Finnland) at 37 °C. Inhibition assays were performed in the same way at 200 μl final concentration, but the reaction mixture contained 2 μl DMSO or inhibitor solution diluted in DMSO. Inner filter effect was determined by measuring the fluorescence as a function of the concentration for RE(Edans), at the substrate concentration range used for the kinetic measurements. Data analysis was performed with the program KiDet (an in house Fortran program), which fitted lines to the fluorescent intensity versus time data, corrected them with the inner filter effect then calculated the k_{cat} and K_m values with nonlinear regression analysis of Michaelis–Menten equation. Inhibition constants as well as the active enzyme concentrations were calculated with the same program using the equation of Williams and Morrison (Williams and Morrison, 1979). To validate the fluorescent method, 100 μl aliquot was removed at the end of measurement, the reaction was stopped by the addition of 100 μl stop solution (7.8 M guanidine-HCl, 2% TFA), and the samples were subjected to reversed-phase HPLC separation.

3. Results and discussion

3.1. Assay of modified cleavage site peptides by HTLV-1 and HIV-1 proteinases using an HPLC assay

It was shown previously (Louis et al., 1999) that the peptide representing the HTLV-1 CA↓NC cleavage site (KTKVL↓VVQPK) was an efficient substrate of both proteinases (SP-997 in Table 1). This peptide was later modified by introduction of amino acid substitutions to characterize the subsite specificity of the two proteinases (Tözsér et al., 2000). We have selected this substrate for further modifications. The fluorescent donor group Edans,

Table 1
Kinetic parameters for the hydrolysis of fluorescent peptide substrates representing HTLV-1 and HIV-1 cleavage sites by HTLV-1 and HIV-1 proteinases using HPLC detection assay (at high salt)

Code	Sequence	HTLV-1 PR			HIV-1 PR		
		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
SP-997	KTKVL↓VVQPK	0.051[a]	7.68[a]	150.6[a]	0.182[a]	2.60[a]	14.3[a]
FSP-354	RE(Edans)TKVL↓VVQPK(Dabcyl)R	0.032	0.02	0.7	0.050	0.23	4.6
FSP-404	RE(Edans)TKVF↓VVQPK(Dabcyl)R	0.053	0.05	0.9	0.014	0.23	16.4
FSP-405	RE(Edans)KTKVF↓VVQK(Dabcyl)R	0.020	0.001	0.06	N.D.[b]	N.D.	0.2[c]
SP-211	VSQNY↓PIVQ	Not hydrolyzed			0.150[d]	6.80[d]	45.3[d]
FSP-407	RE(Edans)SQNY↓PIVRK(Dabcyl)R	Not hydrolyzed			0.038	7.79	205.0

[a] These data were taken from Louis et al., 1999.
[b] N.D., not determined.
[c] This value was determined under pseudo first order reaction condition.
[d] These data were taken from Tözsér et al., 1991.

and a quenching acceptor group Dabcyl were built into the N- and C-terminal part of the peptide, respectively, to obtain the peptide designated FSP-354. This peptide was assayed as a substrate of the HTLV-1 PR by the conventional HPLC method, to compare its kinetic parameters to that of the unmodified substrate. The HPLC assay also allowed the direct identification of the site of cleavage, by determining the amino acid composition of the product peaks, as exemplified in Fig. 1. The K_m value of the substrate was low, similar to the original peptide (Table 1, Louis et al., 1999), but the catalytic constant was much lower, together yielding an approximately 200-fold lower specificity constant (k_{cat}/K_m). Nevertheless, the catalytic constants obtained for this modified substrate were within the range obtained for naturally occurring cleavage site substrates with the respective retroviral proteinases (Tözsér et al., 1991, 1993, 1996). Substitution of P1 Leu to Phe in this sequence (yielding FSP-404) did not alter substantially the kinetic parameters for HTLV-1 PR, although it more than doubled the specificity constant when introduced into the original substrate sequence (Tözsér et al., 2000). Our previous study indicated that the HTLV-1 PR may require more residues at the N-terminus than HIV-1 PR (Tözsér et al., 2000), therefore another version of the P1 Phe substituted peptide was synthesized, in which the N-terminal sequence was extended by one more residue, at the expense of shortening the C-terminal part. However, this peptide (FSP-405) was a much worse substrate for HTLV-1 PR, than FSP-404, indicating that the proper length at the C-terminus is also important for efficient catalysis. Interestingly, FSP-354 and FSP-404 were much better substrates for HIV-1 PR than for HTLV-1 PR, even though their nonfluorescent analogs were substantially better substrates for the HTLV-1 PR (Tözsér et al., 2000).

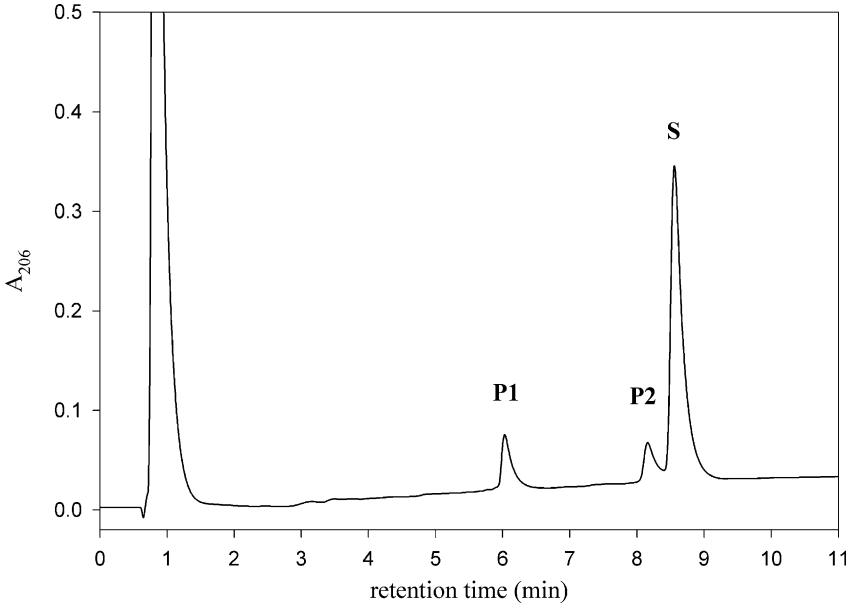


Fig. 1. HPLC assay of the fluorescent substrate FSP-354. The substrate was incubated with HIV-1 PR for 1 h under high salt conditions (see Section 2), then the reaction was terminated by the addition of TFA. An aliquot was injected to the HPLC reversed-phase column, and peaks were identified and quantitated by amino acid analysis. P1, RE(Edans)TKVL; P2, VVQPK(Dabcyl)R; S, RE(Edans)TKVLVVQPK(Dabcyl)R.

We have also tested a donor/acceptor substrate version of the HIV-1 MA↓CA (VSQNY↓PIVQ) cleavage site. Similar to the unmodified substrate (SP-211), the fluorescent peptide was not hydrolyzed by the HTLV-1 PR, while it was an even better substrate of the HIV-1 proteinase than the unmodified one (Table 1).

3.2. Calibration of the inner filter effect for the fluorescent plate reader assays

The inner filter effect associated with intramolecular fluorescence energy transfer (FRET)-based proteinase substrates has prevented some researchers for measuring kinetic parameters from the fluorescent signal. However, empirical procedures for correcting inner filter effects have been used in fluorescence spectroscopy including a method for microtiter plate reader applications (Liu et al., 1999). Here we present an alternative method for inner filter calibration in which a small modified peptide, RE(Edans) was used to mimic the cleavage products. At each substrate concentration of FSP-354, the fluorescence of RE(Edans) peptide was measured as a function of its concentration (Fig. 2A) and the decrease in slope (Fig. 2B) was converted to a correction factor (Fig. 2C), by which the individually measured fluo-

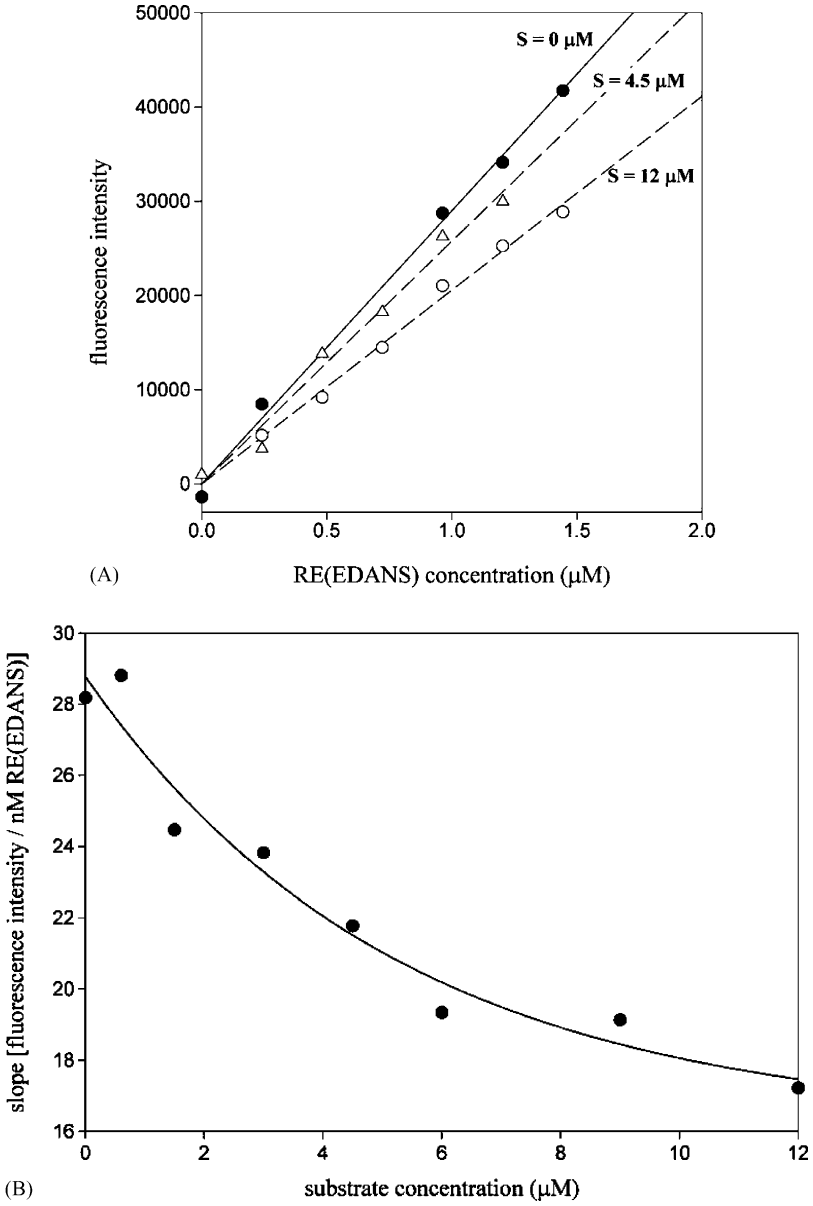
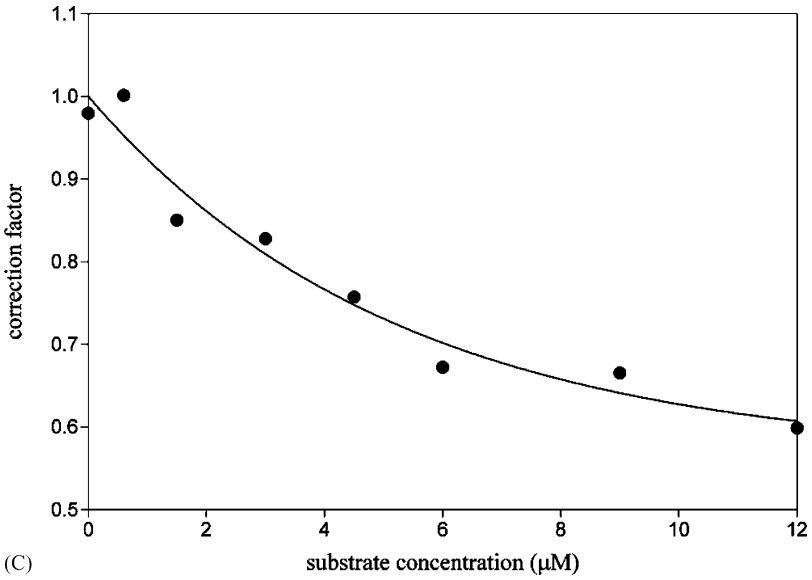


Fig. 2. Determination of the inner filter effect. The fluorescence of the RE(Edans) dipeptide was measured as a function of its concentration at the substrate concentrations used in kinetic measurements (A). Only three representative lines are shown, and lines were moved to zero fluorescence intensity at zero RE(Edans) concentration. The slopes obtained from these lines were plotted as a function of the substrate concentration (B). Exponential decay curve with three parameters was fitted to the measured points and the slope values were converted to correction factor (cf) values using the assumption that the fitted curve should start from cf = 1 point (C).



(C)

Fig. 2. (*Continued*).

rescent intensity values were divided. The inner filter effect was calibrated for each substrate used in this study by the same procedure.

3.3. Fluorescent plate reader assays of the peptides by HTLV-1 and HIV-1 proteinases

The fluorescent substrates were also assayed in a microtiter plate assay, in which the appearance of the fluorescence of the Edans group was detected after cleavage. The conditions of the fluorescent assay were somewhat different from the conditions of the HPLC method, since the high salt concentration (2 M), which was previously found to be optimal for the retroviral proteinases interfered with the fluorescent detection (data not shown). To validate the fluorescence microtiter plate method, aliquots were injected at the end time point of the measurements, and the specificity constants were calculated based on peak quantitation, as done in the conventional HPLC assays. The values obtained with the HPLC quantitation agreed with the values obtained with the fluorescent readings corrected with the inner filter effect (Table 2). Interestingly, the values obtained in low salt (0.5 M) conditions (Table 2) were similar to those obtained with the HPLC assay at high salt (2 M) concentration (Table 1) indicating that hydrolysis of these substrates was not improved by high ionic strength. Recently fluorogenic substrates were used to test the salt effect on HTLV-1 protease mediated cleavage (Ha et al., 2002). Those substrates were based on the MA↓CA cleavage site of HTLV-1, and the specificity constants were in the same range we obtained for the CA↓NC cleavage site substrate. They did not find a salt enhancing effect on the substrate hydrolysis. However, our study indicates that this is also the case with the HIV-1 PR for the studied substrates, therefore it may be a characteristic feature of the substrate sequences rather than feature of the enzymes.

3.4. Comparison of the inhibition profile of HTLV-1 and HIV-1 proteinases

We have selected FSP-354 for assay of various inhibitors designed against HIV-1 PR or designed on the basis of HTLV-1 cleavage sites. Only Indinavir was able to inhibit HTLV-1 PR in the studied concentration range among the tested HIV-1 PR inhibitors used in clinical practice (Table 3). These findings are in good agreement with the findings that clinically used HIV-1 PR inhibitors failed to inhibit Gag processing in HTLV-1 infected cells (Pettit et al., 1998). We have tested two statine-containing peptides based on HTLV-1 cleavage sites (compounds 7 and 8 in Table 3) as inhibitors:

Table 2
Comparison of the kinetic parameters determined for HIV-1 proteinase in lower salt using fluorescent and HPLC detection

Code	Sequence	HTLV-1 PR			HIV-1 PR		
		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m[a] ($mM^{-1}\ s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m[a] ($mM^{-1}\ s^{-1}$)
FSP-354	RE(Edans)TKVL↓VVQPK(Dabcyl)R	0.015	0.02	1.21 (1.37)	0.015	0.09	6.0 (5.4)
FSP-404	RE(Edans)TKVF↓VVQPK(Dabcyl)R	0.012	0.006	0.49 (0.51)	0.038	0.43	11.3 (12.4)
FSP-407	RE(Edans)SQNY↓PIVRK(Dabcyl)R	Not hydrolyzed			0.031	7.61	245 (258)

[a] Values in parentheses are those determined by HPLC, taking aliquots from the fluorescently assayed solutions at the end of the measurements.

Table 3
Inhibition of the HTLV-1 and HIV-1 proteinases by inhibitors specific to HIV-1 proteinase and by HTLV-1 cleavage site-based substrate analogs

Compound number	Inhibitor	HTLV-1 PR, K_i (nM)	HIV-1 PR, K_i (nM)
1	Saquinavir[a]	>20000	0.001
2	Ritonavir	>20000	0.366
3	Nelfinavir	>20000	0.088
4	Amprenavir	>20000	0.001
5	Indinavir	3475	0.440
6	DMP-323[a]	>20000	0.914
7	Ala-Pro-Gln-Val-Sta-Val-Met-His-Pro[a,b]	2300	530
8	Lys-Thr-Lys-Val-Sta-Val-Gln-Pro-Lys[a,b]	>20000	>20000
9	IB-269: Ala-Pro-Gln-Val-Leu-r-Pro-Val-Met-His-Pro[c]	465	>20000
10	IB-268: Lys-Thr-Lys-Val-Leu-r-Val-Val-Gln-Pro-Lys[c]	298	11 215

[a] These inhibitors were also tested previously in the HPLC-based assay system (Louis et al., 1999).
[b] Sta denotes a statin residue.
[c] r denotes a reduced peptide bond.

as we found previously with the conventional HPLC method (Louis et al., 1999), one of them was a moderate inhibitor of the HTLV-1 PR in the fluorescent assay. Two new compounds, containing reduced peptide bonds in place of the cleavable bond (compounds 9 and 10 in Table 3) were also assayed. These were so far the best inhibitors of the HTLV-1 PR we have tested. In the conventional HPLC assay at high salt concentration (which is not suitable for the fluorescent detection) IB-268 had sufficiently low K_i (48 nM) to allow for its use for active site titration of the HTLV-1 proteinase.

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