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Characterization of two structurally novel HIV-1 protease inhibitors identified by rational selection

C.C. Humblet[a], E.A. Lunney[a], R.W. Buckheit Jr.[c], C. Doggett[c], R. Wong[b] and T.K. Antonucci[b]

[a]*Department of Chemistry and* [b]*Department of Biotechnology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, USA,* [c]*Retrovirus Research Section, Microbiology Research Department Southern Research Institute, 2000 North Ave. S., PO Box 55305, Birmingham, AL 35255, USA*

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Summary

The human immunodeficiency virus (HIV-1), associated with the AIDS (acquired immunodeficiency syndrome) epidemic, encodes an aspartyl protease that is essential for polyprotein processing in the virus (Navia et al., 1989). It has been demonstrated that inactivation of the protease either catalytically or by an inhibitor prevents infectious virion formation (Kohl et al., 1988; Darke et al., 1989). The acquired knowledge of key molecular interactions occurring between inhibitors and aspartyl proteases, as well as the structural similarities between HIV-1 protease and human renin was used to rationally select candidates for HIV-1 screening from the pool of analogs designed as renin inhibitors. A minimal number of chosen compounds were tested in an HIV-1 protease assay system. Two structurally novel peptides emerged as potent enzymatic protease inhibitors. This study highlights the selection process and characterizes the antiviral properties of the two novel analogs.

HIV-1 Protease; Inhibitor; Antiviral; Peptide; Renin; AIDS

Introduction

The AIDS epidemic, caused by the retroviruses HIV-1 and HIV-2, is

Correspondence to: T.K. Antonucci, Department of Biotechnology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, USA.

recognized as a major global health threat. Despite intense research carried out on the AIDS viruses only a relatively few, short-term therapies currently exist (Richman, 1991; Mitsuya et al., 1990). The merits of choosing the HIV-1 protease as a target for drug discovery have been reviewed elsewhere (Darke et al., 1989; Tomasselli et al., 1991). Since the viral protease is a member of the aspartyl protease family (Navia et al., 1989), it is not surprising that inhibitors of human aspartyl proteases such as renin are also capable of inhibiting HIV-1 protease (Darke et al., 1989; Tomasselli et al., 1991).

We took advantage of a collection of Parke-Davis compounds synthesized as potential human renin inhibitors through a procedure designed to be both effective and efficient. At the time of these studies, the X-ray crystal structure for HIV-1 protease had not been solved. However, the similarities of the 3-dimensional structures of human renin and HIV-1 protease laid the foundation for rational selection of possible inhibitors for HIV-1 protease. Knowledge of the critical interactions existing between aspartic proteases and known ligands, the established inhibition of HIV-1 protease by pepstatin, and the chemical structures of various HIV-1 protease substrate analogs enhanced our ability to identify HIV-1 protease inhibitors among the hundreds of compounds designed as human renin binders. Other factors such as the symmetry of the viral enzyme and its pH optimum of 5.5 were also considered. The first set of analogs screened by enzymatic assay for HIV-1 protease inhibition included only eleven compounds and resulted in the identification of a potent inhibitor. A batch of 22 compounds was subsequently chosen and an analog, structurally related to the inhibitor identified in the first set, also exhibited strong binding affinity. This article describes the characterization of these two peptides.

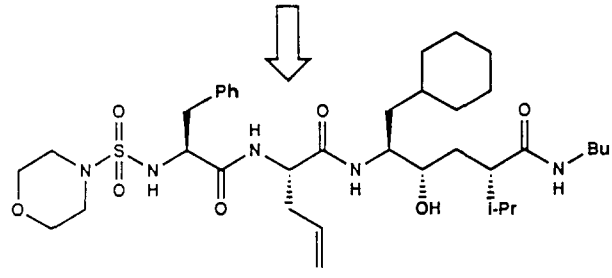
Materials and Methods

Compound

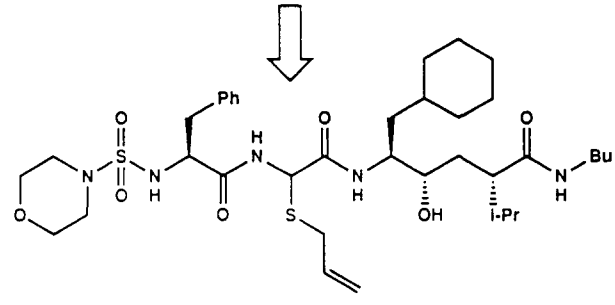
The compounds described in this study, PD 134922 and PD 135390, are small 'dipeptides' with molecular weights of 747 and 752, respectively. Their structures are shown in Fig. 1. PD 135390 is a diastereomeric mixture. Stock solutions in ethanol/5% dextrose (1:1) were used for this work; both peptides are soluble in EtOH/dextrose up to 5 mg/ml. The peptide solutions were diluted in assay buffer or in cell culture medium, as appropriate, to achieve final test concentrations.

Cell lines

The human T-cell lines used in this study included CEM (Nara and Fischinger, 1988), H9 (Popovic, 1984; Mann, 1989) and MT-2 (Harada, 1985). These cells were grown in standard RPMI-1640 medium with 10% fetal calf serum (20% for H9 cells), penicillin (100 units/ml), streptomycin (100 μm/ml) and 25 mM HEPES buffer. Cultures were maintained in disposable tissue culture labware at 37°C in a humidified atmosphere of 5% CO_2 in air.



PD 134922



PD 135390

Fig. 1. Structures of PD 134922 and PD 135390. These hybrid peptides were originally designed to inhibit human renin.

Chronically HIV-infected cell lines were obtained from the acute infection of H9 cells as described (Buckheit and Swanstrom, 1991).

Virus strains

The HIV-1 virus utilized in both acute and chronic infection systems was HTLV-III$_B$ which was propagated in both the H9 and the CEM cell lines. The virus inoculum consisting of supernatant fluids from acutely infected CEM cells was prepared as described previously (Buckheit and Swanstrom, 1991).

In vitro protease assay

The Bachem HIV Protease Assay Kit was used to screen 150 peptides synthesized at Parke-Davis for potential protease inhibition. The assay was performed as described in the Kit's instructions: briefly, a synthetic substrate

His-Lys-Ala-Arg-Val-Leu-(pNO_2-Phe)-Glu-Ala-Nle-Ser-NH_2 was dissolved in 50 mM NaOAc (pH 5.5), at 2 mg/ml. The substrate plus synthetic HIV-1 protease were incubated together at 37°C for 2 hours. Approximately 50% of the reaction volume (75 μl) was injected into a reverse phase high-pressure liquid chromatography column (C_{18}) using a linear gradient of 5–50% in 12 minutes. The areas under the substrate and product peaks were quantitated in order to calculate the percent of substrate converted to product. Acetyl-pepstatin (10 μl of a solution at 0.1 mg/ml in dimethyl sulfoxide) was used with each assay set as the negative control.

Each peptide screened as a possible inhibitor of HIV protease activity was tested initially at a concentration of 10 micromolar in the assay system described above. Dilutions from stock solutions were made so that the stock buffer was present as a maximum of 1% total assay volume. Significant inhibition was scored if the compound resulted in at least a 50% reduction of HIV protease activity. Compounds which significantly inhibited the protease were retested to confirm the result. After confirmation, the inhibitors were tested at the following micromolar concentrations: 10, 1, 0.1, 0.01 and 0.001. The concentration at which HIV protease was inhibited by 50% (IC_{50}) was determined by the linear regression analysis available in the CricketGraph Software Analysis Program.

Cytotoxicity

The quantitative assessment of cytotoxicity resulting from the compounds was made using the XTT colorimetric method (Weislow et al., 1989). The XTT-PMS solution used in this assay consisted of the tetrazolium reagent, XTT, and phenazine methosulfate (PMS) mixed at final concentrations of 200 μm/ml and 0.02 mM, respectively. Quantitation of cell viability was performed spectrophotometrically with a Molecular Devices V_{Max} Plate Reader at 450 and at 650 nm. The values for % cell viability were calculated by linear regression analysis.

Virus CPE inhibition assay

For acutely infected cells (CEM and MT2) the inhibition of virus-induced cytopathic effects by the peptides was measured by the tetrazolium dye assay described previously (Weislow et al., 1989). Briefly, 5×10^3 CEM cells were plated in each well of a 96-well round bottom microtiter plate. Drug was added at six different dilutions with a high test concentration of 13 μm and 5-serial half-log dilutions. The cells were infected at an MOI of 0.12 TCID50/cell on CEM cells and 0.03 TCID50/cell on MT2 cells. Each drug concentration was tested in triplicate. The plates were incubated for 6 days after which time viability was quantitated by the XTT-PMS method described above.

Reverse transcriptase assay

In chronically infected H9 cells, virus quantitation was achieved by the measurement of virus reverse transcriptase activity which has been described elsewhere (Buckheit and Swanstrom, 1991).

Immunoprecipitations

Acutely infected CEM or H9 cells were treated with several concentrations of the peptide PD 134922 for 5 or 7 days, respectively, after which time the supernatants were removed. These supernatants were pelleted at 13 000 rpm for 2 hours and resuspended in phosphate-buffered saline solution for subsequent immunoprecipitation. To each virus suspension, 10 μg of anti-p24 and anti-p17 monoclonal antibody (DuPont) was added and incubated overnight at 4°C. Protein A-Sepharose was added to each suspension in a volume of 50 μl and was incubated for an additional 4 hours with gentle shaking. The mixture was washed three times with wash buffer (0.4 M NaCl, 0.05 M Tris-HCl, 0.5% sodium deoxycholate, 0.5% NP-40). Following washing, 100 μl of sample buffer was added to each pellet and the solution was boiled for 4 minutes. The Sepharose was pelleted and the supernatant was carefully removed. The supernatant was frozen until performance of gel electrophoresis. The frozen samples were thawed and boiled for 90 seconds before electrophoresis. A 12% SDS-polyacrylamide gel was utilized for the electrophoresis. The SDS-PAGE, the transfer to nitrocellulose and the Western immunostaining were performed as described (Buckheit and Swanstrom, 1992).

Results

In vitro protease assays

Among the thirty-three proprietary renin inhibitor peptides assayed for inhibition of HIV-1 protease activity, two analogs, PD 134922 and PD 135390, were identified as potent inhibitors. The dose-response data for HIV-1 protease inhibition determined from enzymatic assays by these two active peptides are shown in Table 1. The IC_{50}s for PD 134922 and PD 135390 were 15 nM and 2 nM, respectively, thus ranking this structural class of peptide inhibitors among

TABLE 1

HIV-1 protease activity

Compound	Dose (μm)	% Reaction
PD 134922	10	N.D.[a]
	1	N.D.[a]
	0.1	34
	0.01	47
	0.001	51
	0	100
PD 135390	10	15
	1	25
	0.1	46
	0.01	54
	0.001	59
	0	100

[a]Not tested.

TABLE 2

Antiviral activity in cells acutely infected with HIV-1 (HTLV III_B)

Drug	Conc. (μm)	CEM cells		MT-2 cells	
		% Cell viability	% CPE reduction	% Cell viability	% CPE reduction
PD 134922	0.043	98	5	96	0
	0.13	96	5	100	0
	0.43	96	6	98	1
	1.3	98	41	100	27
	4.3	96	97	100	77
	13	100	100	93	88
PD 135390	0.043	99	3	96	0
	0.13	98	2	100	0
	0.43	96	1	100	0
	1.3	98	1	100	0
	4.3	100	6	100	1
	13	58	14	25	0

the most potent of those reported (Blundell et al., 1990, Connolly and Hammer, 1992).

Acutely infected cells: CEM

Both peptides were tested in two independent test sets which consisted of six different concentrations of the test compounds in triplicate, as shown in Table 2. PD 134922 did not cause significant cytotoxicity at the concentrations tested. This peptide displayed antiviral activity (IC_{50} 1.9 μm) as measured by % CPE reduction. However, PD 135390 was cytotoxic at its highest concentration (13 μm) and showed no significant antiviral effect.

Acutely infected cells: MT-2

For the MT-2 cell line, only PD 135390 appeared to cause loss of viability at the highest concentration tested (see Table 2). PD 134922, however, showed a specific antiviral effect with the estimated IC_{50} (2.7 μm) in good agreement when compared to its IC_{50} in CEM cells.

Chronically infected H9 cells

PD 134922 showed antiviral activity in chronically infected cells at the highest concentration (13 μm) tested (see Table 3). Table 3 also shows that PD 134922 was not significantly toxic to cells at any concentration tested, whereas PD 135390 resulted in an 18% loss of cell viability at 13 μm. Since 13 μm was the only concentration at which PD 135390 correlated to a reduction in virus reverse transcriptase (RT) activity, it is most likely that this racemic mixture does not have a specific antiviral effect, but is merely toxic to the infected cells.

Immunoprecipitations

Fig. 2 shows that treatment with PD 134922 caused a dose-dependent

TABLE 3

Antiviral activity in chronically infected H9 cells

Drug	Conc. (μm)	% Cell viability	RT cpm/15 μl cell-free supernatant
PD 134922	0.043	92	51,429
	0.13	94	44,349
	0.43	96	59,254
	1.3	96	48,832
	4.3	91	50,937
	13	93	18,624
PD 135390	0.043	92	47,193
	0.13	95	44,945
	0.43	94	45,305
	1.3	96	47,245
	4.3	93	48,832
	13	82	30,637
No Drug		95	52,428

reduction of p24 processing in both acutely infected CEM cells and acutely infected H9 cells. This result confirms that the antiviral activity observed with PD 134922 is indeed directed at the virus protease. PD 135390 relative to that in PD 134922 and the stereochemistry at P_2 is 'S' in PD 134922, while the center

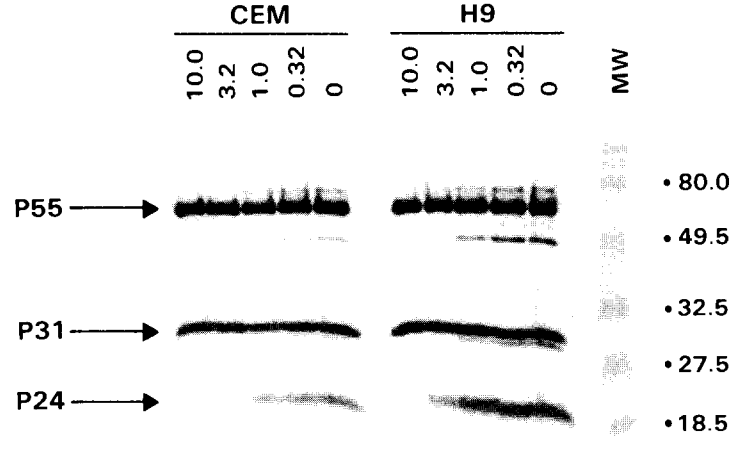


Fig. 2. Polyacrylamide gel electrophoresis of supernatants from acutely infected cells. Both CEM and H9 cells which had been acutely infected with HIV-1 strain III_B were grown in the presence of the peptide shown to inhibit HIV-1 protease in vitro (PD 134922) at the following concentrations (μm): 13, 4.3, 1.3 and 0.43. Molecular weight markers are shown on the right. Three virus gag proteins (p55 gag precursor, p31 processing intermediate and p24 mature core protein) are shown on the left.

is racemic in PD 135390. The 'S' diastereomer of the latter is predicted to be the more potent inhibitor (Repine et al., 1992). Subsequent structure-activity relationship (SAR) studies for HIV-1 protease inhibitors (Gustchina and Weber, 1990), have indicated that the large hydrophobic groups are favored in the P_1 and $P_{1'}$ sites, while the P_4 to P_2 and $P_{4'}$ to $P_{2'}$ residues remain less selective. This SAR is shown with PD 134922 and PD 135390. For both analogs, hydroxyethylene isostere is the transition state mimic at the active site for the two analogs and the hydrophobic groups, cyclohexylmethylene and isopropyl, occupy the S_1 and $S_{1'}$ pockets, respectively. In completing the characterization of these inhibitors, with the exception of the morpholinylsulfonyl group at P_4, the remaining sites in the two compounds are hydrophobic.

Although both PD 134922 and PD 135390 were potent inhibitors of enzyme activity, in the antiviral assays with the acutely infected cell lines, CEM and MT-2, and the chronically infected H9 cells, the analogs differed significantly in their relative activities. In all three assay systems, PD 134922 was not cytotoxic at the concentrations tested and displayed selective antiviral activity. The opposite was found for PD 135390. This analog was cytotoxic in the three assay systems at 13 μm and thus exhibited no specific antiviral inhibition. Interestingly, these major disparities in the cellular activity data result from one or both structural changes at the P_2 site in the inhibitors: the addition of sulfur and the presence of both the 'R' and 'S' stereocenter in PD 135390. Although the allyl group of PD 134922 is less lipophilic and confers a different structural geometry than the thioallyl side chain in PD 135390, the 'R' stereochemical component is postulated to trigger the cytotoxic behaviour observed with PD 135390. It is therefore speculated that the isolation of the 'S' enantiomer of PD 135390 would result in an inhibitor as potent if not more potent than PD 134922 in the antiviral assays. Furthermore, specific structural modifications of PD 134922 and the 'S' enantiomer of PD 135390 could lead to compounds with even greater inhibitory and antiviral activities. The results reported here clearly highlight the P_2 group as a key site for structural exploration in efforts to enhance cellular activity of HIV-1 protease inhibitors.

The results of this study are consistent with earlier work (Hui et al., 1991 and McGowan et al., 1990) in demonstrating the powerful potential that exists in identifying inhibitors of a targeted enzyme by selectively screening compounds designed as ligands for another member of the protein family. The selection of compounds is made by applying what is known about the structure and binding modes of homologous enzymes to the targeted enzyme. Once an inhibitor is identified, increased potencies could be obtained through structural modifications of the initial lead compound. With this use of information regarding the enzyme and its ligands, and the screening of available analogs, the identification of therapeutic leads from a large collection of compounds can be potentially realized in a minimal amount of time.

Discussion

Since the crystal structure of HIV-1 protease had not been solved, the pertinent information available at the time was applied to the selection of the first series of renin inhibitors to be tested against HIV-1 protease. This information included the structural similarities between the two enzymes, the symmetry and optimum pH for the HIV-1 protein, and the structures of pepstatin and known HIV-1 protease substrates (Darke et al., 1989). Through the resolution of crystal structures (Blundell et al., 1986), aspartyl proteases had been shown to bind their inhibitors in a conserved extended structure within the active site cleft. The peptide backbone is engaged in a series of hydrogen bonds with a flap segment and the main region of the enzyme, and the side chains occupy subsites alternating above and below the main chain. Key interactions occur between the catalytic aspartic acids of the protease and the transition state mimetics found in most inhibitors, including pepstatin. This knowledge was applied to predict the subsite occupation and the electrostatic interactions of known binders of HIV-1 protease (i.e., identified substrates and pepstatin). The octapeptide substrates bind either with the cleavage site flanked by four residues on each side or by five amino acids at the N-terminus and three residues on the C-terminus. For this series, the Tyr-Pro amide bond is the scissile bond at the P_1-$P_{1'}$ site and thus an aromatic group is positioned in the S_1 pocket. The minimal peptide fragment to be efficiently cleaved was reported to be seven residues (Darke et al, 1989). The in-house compounds, designed as renin inhibitors, incorporated transition state mimetics that are known to enhance binding relative to the parent substrates (Boger et al., 1983). Therefore, it was speculated that potent inhibitors of the HIV-1 protease could be found in the collection of renin analogs, even though they did not span the minimal length observed for the substrates. Furthermore, to probe the selectivity of the enzyme at the catalytic site, analogs presenting different transition state mimetics were included in the series selected for screening. These P_1-$P_{1'}$ groups included diol and hydroxyethylene isosteres, and statine derivatives. The pH optimum of 5.5 for the viral protease and the premise that symmetrical enzymes may prefer symmetrical ligands were also considered in selecting the potential HIV-1 protease inhibitors.

Two peptide inhibitors of HIV-1 protease were identified by this process, PD 134922 and PD 135390. Both compounds are potent enzymatic inhibitors (IC_{50} = 15 nM and 2 nM, respectively) and structurally novel at the P_2 site with respect to other HIV-1 protease inhibitors (McQuade et al., 1990; Meek et al., 1990; and Roberts et al., 1990). The two compounds differ slightly in their structures in that sulphur is incorporated into the P_2 side chain in PD 135390 relative to that in PD 134922 and the stereochemistry at P_2 is 'S' in PD 134922, while the center is racemic in PD 135390. The 'S' diastereomer of the latter is predicted to be the more potent inhibitor (Repine et al., 1992). Subsequent structure-activity relationship (SAR) studies for HIV-1 protease inhibitors (Gustchina and Weber, 1990), have indicated that the large hydrophobic

groups are favored in the P_1 and $P_{1'}$ sites, while the P_4 to P_2 and $P_{4'}$ to $P_{2'}$ residues remain less selective. This SAR is shown with PD 134922 and PD 135390. For both analogs, hydroxyethylene isostere is the transition state mimic at the active site for the two analogs and the hydrophobic groups, cyclohexylmethylene and isopropyl, occupy the S_1 and $S_{1'}$ pockets, respectively. In completing the characterization of these inhibitors, with the exception of the morpholinylsulfonyl group at P_4, the remaining sites in the two compounds are hydrophobic.

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