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HIV proteinase inhibitors containing 2-aminobenzylstatine as a novel scissile bond replacement: biochemical and pharmacological characterization

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Abstract

Derivation of the 2-aminobenzylstatine containing HIV-1 proteinase (PR) inhibitor **I** led to a series of compounds with considerably improved antiviral activity, the most potent derivatives inhibiting HIV-1 with IC_{50} values below 25 nM. This was achieved by the combination of several structural modifications, most prominently by introduction of a benzimidazole heterocycle into the inhibitor. The mode of action of the 2-aminobenzylstatine PR inhibitors was demonstrated to be inhibition of *gag* precursor processing. The antiviral efficacy of the PR inhibitors was demonstrated in various cell lines, in primary T4 lymphocytes and in monocytes. The most potent compound (**XI**) inhibited replication of several HIV-1 clinical isolates in primary cells with IC_{50} values of 8 to 23 nM. The analysis of the pharmacokinetic behaviour of compounds **I** and **VII** revealed blood half-lives in rodents in the range of about 1.5 h. Compound **I** also showed appreciable oral uptake in mice (18%), but yielded no detectable blood levels in rats after oral administration. Benzimidazole containing compounds like **VII** were not orally bioavailable to a significant extent, neither in mice nor in rats. Thus, while introduction of a benzimidazole group

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into the PR inhibitors was a successful structural modification with regard to antiviral activity in cell culture, it completely abolished oral bioavailability.

Keywords: AIDS; HIV; Proteinase inhibitor

1. Introduction

The proteinase of the human immunodeficiency virus type 1 (HIV PR) is regarded as one of the most promising targets for anti-AIDS chemotherapy. Following the nucleoside analogue inhibitors of reverse transcriptase, proteinase inhibitors are envisioned to create a 'second front' against the AIDS epidemic (Blundell and Pearl, 1989). Recently, evidence for clinical efficacy of an HIV PR inhibitor in AIDS patients has been reported (Delfraissy et al., 1993; Kitchen et al., 1993). The proteinase is essential for production of infectious virus particles: It cleaves specifically *gag* and *gag-pol* precursor polyproteins to yield the enzymes required for viral replication, as well as the structural proteins of the mature viral core (Kohl et al., 1988; Seelmeier et al., 1988). The tertiary structure of HIV-1 proteinase and its inhibitor complexes has been elucidated in detail by X-ray crystallography (Appelt, 1993). This knowledge, together with the application of strategies used previously to design aspartic proteinase inhibitors, has led to the development of a variety of potent ($K_i < 1$ nM) and selective enzyme inhibitors, mostly based on incorporation of transition-state mimetics as scissile bond replacements into substrate analogues (for reviews see Huff, 1991; Meek, 1992; Debouck, 1992; Robins and Plattner, 1993). Several of these compounds also proved to be inhibitory to HIV replication in cellular assays in nanomolar concentrations. For a compound, however, intended as a drug for long term use in chronic HIV-disease, additional properties are required. Besides being a potent and selective inhibitor of replication of a variety of HIV-1 strains and isolates in various cell types, including primary cells, a promising candidate should exhibit metabolic stability, favorable pharmacokinetic behavior, good bioavailability, preferentially by the oral route, and finally good tolerability in long term use. While rational techniques (namely systematic derivation and molecular modeling) have been used successfully to design potent inhibitors of the enzyme, the structure-activity relationships leading to favorable biological properties of compounds can, in most cases, only be investigated by an empirical survey of a given set of inhibitors. Only recently, detailed reports describing the evaluation of relevant pharmacological properties of HIV PR inhibitors have been published (Kort et al., 1993; Alteri et al., 1993; Otto et al., 1993, Lam et al., 1994; Galpin et al., 1994; Kempf et al., 1991).

In a separate publication (Scholz et al., 1994) we describe the synthesis of HIV PR inhibitors containing 2-hetero-substituted statine as a novel scissile bond replacement. For a large series of derivatives the structural parameters determining inhibitory potency against the enzyme and against HIV-1, strain IIIB, replication in a T cell line were identified. Here, a detailed biochemical and pharmacological characterization of inhibitors containing 2-aminobenzylstatine is reported. Antiviral activity is demonstrated against further HIV-1 strains in various cell types, including primary T4 lymphocytes and monocytes. Furthermore, pharmacokinetic behavior of compounds after oral admin-

istration is investigated. Mode of action studies indicate, that a late step in virus replication is the point of interference of the proteinase inhibitors described here.

2. Materials and methods

2.1. Compounds

The synthesis of compounds **I-VIII** and **X-XIV** is described elsewhere (Scholz et al., 1994); compound **IX** was prepared by an analogous procedure. All derivatives were dissolved in dimethyl sulfoxide to 10 mM. For cellular assays, they were diluted from this stock solution into cell culture medium containing 5% fetal bovine serum and for enzyme assays into appropriate buffer (see below); the concentration of dimethyl sulfoxide did not exceed 0.3% in cellular assays and 5% in enzymatic assays.

2.2. Viruses

The HIV-1, strains IIIB and BaL, have been described (Gartner et al., 1986; Popovic et al., 1984). The origin of the clinical HIV-1 isolates was as follows: HIV-1 (K31), isolated in 1988 from a patient from Zaire; HEM, isolated in 1992 from a patient in Vienna (Austria); SF-162 isolated in 1988 from a patient in San Francisco, USA (Cheng-Mayer and Levy, 1988), and D747, isolated in 1992 from a patient in Bombay, India. All clinical isolates were propagated up to three times in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC). Virus dose (multiplicity of infection) was adjusted individually for each cell type such that exponential virus growth was observed for two to four sampling points.

2.3. HIV proteinase inhibition assay

HIV-1 proteinase was expressed in *Escherichia coli*, strain JM 105, using the expressor plasmid pTZprt$^+$ (Seelmeier et al., 1988) and was purified to homogeneity as described before (Billich et al., 1990). Enzymatic activity was measured by monitoring cleavage of the substrate H-Lys-Ala-Arg-Val-Leu-pNph-Glu-Ala-Nle-NH_2, originally described by Richards et al. (1990). Briefly, HIV-proteinase was incubated at 37°C in 0.1 M 2-morpholinoethanesulfonic acid, 0.37 M NaCl, 4 mM EDTA, pH 6.25, with 280 μM of substrate in the presence or absence of inhibitors. From the decrease of absorbance at 298 nm initial reaction rates were calculated.

IC_{50}-values for test compounds were obtained by fitting the initial velocity data (V) from the inhibition of substrate hydrolysis to the equation $V = V_0 \cdot IC_{50}/(I + IC_{50})$ where I denotes the inhibitor concentration and V_0 the velocity of the uninhibited reaction. Kinetic constants K_i were calculated (Cha et al., 1975) from IC_{50} values by using the equation $IC_{50} = E_t/2 + K_i \cdot (1 + S/K_m)$, where E_t is the total enzyme concentration, S is the substrate concentration, and K_m is the Michaelis constant for the substrate. K_i-values reported here are the mean of two determinations, which usually yielded the same result within limits of $\pm 20\%$.

Recombinant HIV-2 proteinase, kindly supplied by P. Strop, Prague, was assayed as described for the HIV-1 enzyme, but in 0.1 M NaAc, pH 4.7, 0.37 M NaCl, 4 mM EDTA.

2.4. Inhibition of HIV-1 induced cytopathic effect in MT4 cells

The assay procedure (Pauwels et al., 1988) was used with minor modifications. Briefly, the HTLV I transformed cell line MT4 was used as the target cell. Inhibition of HIV-1, strain IIIB, induced cytopathic effect was determined by measuring the viability of both HIV- and mock-infected cells. Viability was assessed spectrophotometrically via in situ reaction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Virus-infected and uninfected cultures without compound were included as controls as were uninfected cells treated with compound. The cell concentration was chosen so that the number of cells per ml increased by a factor of 10 during the 5 days of incubation in mock-infected cultures. Virus inoculum was adjusted such to cause cell death in 90% of the target cells after 5 days of incubation. The virus was adsorbed to a cell suspension containing 1×10^6 cells/ml at 37°C for 1 h. Then, the infected cells were added to microtiter plates containing the test compounds to give 1×10^5 cells/ml. Thus, compounds were added post adsorption. The IC_{50} values reported here are the mean of at least five determinations.

2.5. Inhibition of HIV replication in cell lines

MT4 cells were infected with HIV-1, strains IIIB or RF, or HIV-2, strains ROD or EHO, by suspending the cells at a concentration of 1×10^6 cells/ml in virus suspension. Adsorption was allowed for 2 h at 37°C. Virus inoculum was adjusted such to give a linear increase of p24 antigen concentration in the supernatants of infected cells up to day 4 post infection. After adsorption the cells were spun down, the inoculum was removed by washing, and the infected cells were added to 6-well plates containing the test compounds at the appropriate concentrations to give 1×10^5 cells/ml in a volume of 5 ml. At days 3 and 4 post infection aliquots were removed, the cells were spun down and the supernatants were analyzed for p24 antigen concentration by means of a commercial ELISA kit (Coulter). Infection of HUT78 and U937 cells with HIV-1, strain IIIB, and incubation in the absence or presence of compound was performed in analogous manner. Every 3 to 4 days aliquots of the supernatants were taken for determination of p24 antigen; cells were counted and adjusted to 2×10^5 cells/ml by adding fresh medium containing the test compound at the particular concentration. P24 antigen concentrations were plotted vs. compound concentrations for those days post infection, when p24 was increasing exponentially, e.g., in the experiment depicted in Fig. 1 for days 5, 8 and 12. IC_{50} and IC_{90} values (50% and 90% inhibitory concentrations) were calculated by comparing p24 antigen concentrations in supernatants of treated, infected cells to those of untreated, infected cells.

2.6. Inhibition of HIV-$1_{(IIIB)}$ induced infectious particle formation in MT4 cells

The supernatants from infected, compound-treated cells as well as those from infected control cells, harvested on day 4 post infection, were incubated in serial

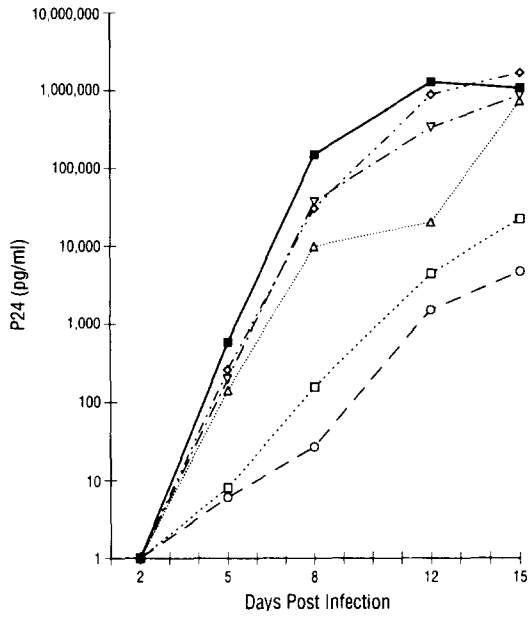


Fig. 1. HIV-1, IIIB, infection of HUT78 cells: Effect of **XI**. P24 antigen concentrations were determined in the supernatants of infected cells after various times post infection in the absence or presence of **XI**. Aliquots were taken at the days indicated before adjustment of the cell cultures to 2×10^5/ml. ■ ——— ■ Untreated control; ○---○ treated with 0.1 μM **XI**; □...□ treated with 0.03 μM **XI**; △...△ treated with 0.01 μM **XI**; ▽-·-·-▽ treated with 0.003 μM **XI**; ◇-·-·-◇ treated with 0.001 μM **XI**.

dilutions with fresh MT4 cells in the absence of compound. Five days post inoculation virus-induced cytopathic effects were quantitatively determined by viability staining with MTT.

2.7. Antiviral activity assays in primary T4 lymphocytes and primary monocytes

Primary T4 lymphocytes were purified from human spleens obtained from healthy donors by using a commercial kit ('Lympho-Kwik'), which combines reaction of cells with specific monoclonal antibodies and density gradient centrifugation to separate the cells. Preparations obtained by this procedure contained 60–80% CD4 positive cells as analyzed by FACS. Cells were stimulated with 2 μg/ml of phytohemagglutinin (PHA) for 24 h. Then, they were infected with HIV-1 by suspending the cells in virus solution. Adsorption was allowed for 2 h at 37°C. The inoculum was removed and the cells were resuspended in fresh culture medium containing IL-2 (40 units/ml; Genzyme). Test compound was added after stimulation and virus adsorption. Every 3 to 4 days half of the supernatant of the infected cultures was removed and replaced by fresh medium containing IL-2 and the test compound at the particular concentration.

Preparation and infection of monocytes were performed essentially as described by Perno et al. (1989). Mononuclear cells were isolated from healthy, HIV negative donors using Ficoll density separation. Cells were incubated for 5 days in 48-well plates in

monocyte medium, consisting of RPMI1640, supplemented with 20% FCS and 10% human serum. On day 5, non-adherent cells were washed out with PBS containing 2% human serum. Preparations obtained by this procedure were > 95% positive for nonspecific esterase. Cell viability (as determined by trypan blue exclusion) was always > 95%. Adherent monocytes were exposed to 100 μl/well of HIV-1 for 30 min; subsequently, monocyte medium was added to 1 ml/well. Adsorption was allowed for 48 h at 37°C. Then, excess virus was removed, and the cells were cultivated in the presence of different drug concentrations. Every 3 to 4 days, the supernatant of the infected cultures was removed and replaced by fresh medium containing the test compound at the particular concentration.

The concentration of viral p24 antigen was determined in supernatants by ELISA (Coulter). IC_{50} and IC_{90} values were calculated by comparing p24 antigen concentrations in supernatants of treated, infected cells to those of untreated, infected cells at days post infection, when p24 production was increasing exponentially.

2.8. Cytotoxicity assay

To investigate cytotoxic and cytostatic effects of the HIV PR inhibitors, exponentially growing cells, namely two T4 cell lines (MT4 and HUT78) and one monocytic cell line (U937), were used. Cell suspensions were adjusted to 10^5 cells/ml and incubated in the presence of various concentrations of test compounds. After 2, 3 and 4 days aliquots of the cells were stained with MTT and the optical densities obtained were compared to that of the untreated control cells. The inhibitory effects were measured at a time point when untreated cells showed exponential growth, and the concentrations of test compound reducing cell proliferation by 50% (TC_{50}) were calculated.

2.9. Assay of virus production by chronically-infected cells

The chronically-infected monocytic cell line U1 was diluted to 10^5 cells/ml at day 0 in fresh culture medium containing either phorbol 12-myristate 13-acetate (PMA) at 10 ng/ml or IL-6 at 1 ng/ml plus various concentrations of test compound. P24 antigen concentrations were measured in the supernatants at day 4 post seeding by ELISA (Coulter). Chronically HIV-1, IIIB,-infected Jurkat cells (Jurkat/IIIB), isolated in our laboratories from a de novo HIV-1, IIIB, infection of Jurkat cells, were washed twice with culture medium to remove cell-free virus. They were resuspended at 10^5 cells/ml in fresh medium containing the test compound at the appropriate concentration. After 48 h of incubation at 37°C, the cells were removed by centrifugation and the supernatants were analyzed for both p24 antigen and infectious virus. Viral p24 antigen was measured by ELISA (Coulter), infectious virus titrations were done in MT4 cells. Therefor, 10^6 MT4 cells were infected by suspending them in 1 ml of 1:3 diluted supernatant from the Jurkat/IIIB cells. After virus adsorption the inoculum was removed and the cells (2×10^5/ml) were resuspended in fresh medium without compounds. At day 4 post infection aliquots of the MT4 cell suspensions were harvested, and p24 antigen concentration was measured in the supernatants by ELISA.

2.10. Analysis of gag processing in HeLa-HXB3 cells

Confluent monolayers of HeLa/HXB3 cells, which had been generated by transfection with DNA of HIV-1, HXB3, and contain a stably integrated copy of the viral genome, were trypsinized and seeded 1:5 diluted in cell culture flasks. At day 1 post seeding compound **VII** was added from a 1000-fold concentrated stock solution to give the appropriate final concentration. At day 8 post seeding debris was removed from the supernatant by centrifugation ($1200 \times g$, 10 min) and the virus particles were pelleted from this clarified supernatant by ultracentrifugation ($160\,000 \times g$, 1 h at 4°C). The pellet from 8 ml of supernatant was dissolved in 100 μl of sample buffer (130 mM Tris-HCl, pH 6.8, 4.2% SDS, 10.5% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue). Proteins were denatured (95°C, 5 min), separated by SDS gel electrophoresis, and then blotted onto nitrocellulose. Blots were incubated with rabbit anti-p24 antiserum and protein bands were detected with a second anti-rabbit antibody conjugated with horse-radish peroxidase, using the enhanced chemiluminescence Western blotting kit (Amersham).

For analysis of reversibility of cleavage inhibition by **VII** the virus pellet was redissolved in PBS with or without inhibitor and incubated for various times at 37°C. Then, the particles were pelleted again by ultracentrifugation, were dissolved in sample buffer, and the protein patterns were analyzed as described above.

2.11. Pharmacokinetic studies

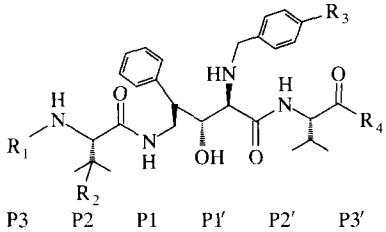
Female Balb/c mice or Wistar rats were used. For oral administration (by gavage) the animals were fasted for 24 h prior to the start of and throughout the experiment; water was given ad libitum. Intraveneous injection was given into the tail vein (mice) or femoral vein (rats).

For analysis, rat or mouse blood was collected in heparinized tubes. Samples (typically 0.4–1.0 ml) were frozen in liquid nitrogen and stored at −70°C. After thawing, 200 μl of a suitable internal standard (0.01 mg/ml in methanol) and 4 vol. of methanol were added. For calibration, blood samples were spiked with 10 to 10 000 ng of test compound, then internal standard and methanol were added as above. Samples were mixed for 10 min, and then were centrifuged at 4000 rpm, 4°C, for 10 min. The supernatants were removed and dried in vacuo.

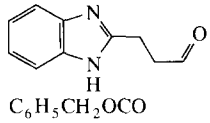
For solid-phase extraction of the compounds, 1-ml disposable sulfobenzyl extraction columns (Baker) were used. The dried supernatant of the methanol extraction, resuspended in 10% methanol in 10 mM NH_4Ac, pH 4.0, was applied, followed by successive washes (1 ml each) with 10 mM NH_4Ac, pH 4.0 (twice), methanol, hexane, and 10% trifluoroacetic acid. Compounds were eluted with 5% ammonia in methanol. Eluates were dried in vacuo.

For HPLC analysis, residues were taken up in 100 μl 0.1% trifluoroacetic acid, 10% methanol in water. Aliquots (20–100 μl) were injected into the HPLC system (HP1500; column: Vydac RP-C18, 5 μ, 4×250 mm; isocratic elution with 50 or 60% acetonitrile in 10 mM NH_4Ac, pH 4.0; flow: 1 ml/min; 25°C; UV-detection at 200 nm). Drug concentration in the samples was calculated by least-squares linear regression analysis of

Table 1
Activity of proteinase inhibitors against HIV-1 proteinase (K_i) and against HIV-1, strain IIIB, replication in MT4 cells (IC_{50}), measured by different endpoints



P3 P2 P1 P1′ P2′ P3′

No.	R_1	R_2	R_3	R_4	K_i [nM]	IC_{50} [nM]	
						CPE [a]	p24 [b]
I	$C_6H_5CH_2OCO$	H	H	$NHCH_2C_6H_6$	6.1	580	315
II	$C_6H_5CH_2OCO$	CH_3	H	$NHCH_2C_6H_6$	9.2	320	95
III	$C_6H_5CH_2OCO$	H	OCH_3	$NHCH_2C_6H_6$	7.4	320	132
IV	$C_6H_5CH_2OCO$	H	Cl	$NHCH_2C_6H_6$	17.0	400	109
V	$C_6H_5CH_2OCO$	H	Br	$NHCH_2C_6H_6$	23.0	350	147
VI	$C_6H_5CH_2OCO$	H	H	2(NH-CH_2)benzimidazolyl	13.0	140	13.3
VII	$C_6H_5CH_2OCO$	H	OCH_3	2(NH-CH_2)benzimidazolyl	3.4	25	7.3
VIII	$C_6H_5CH_2OCO$	H	Cl	2(NH-CH_2)benzimidazolyl	6.1	120	35.0
IX	$C_6H_5CH_2OCO$	H	Br	2(NH-CH_2)benzimidazolyl	8.0	110	20.3
X		H	H	$NHCH_2C_6H_6$	9.9	180	54.5
XI	$C_6H_5CH_2OCO$	CH_3	OCH_3	2(NH-CH_2)benzimidazolyl	4.6	12	5.4
XII	$C_6H_5CH_2OCO$	CH_3	Cl	2(NH-CH_2)benzimidazolyl	7.5	34	11.3
XIII	(S)-phenyllactoyl	CH_3	OCH_3	2(NH-CH_2)benzimidazolyl	6.0	38	7.9
XIV	p-(OH)-C_6H_4-C_2H_4CO	CH_3	OCH_3	2(NH-CH_2)benzimidazolyl	13.0	39	21.0

[a] The concentrations of compounds reducing virus-induced cytopathic effect by 50% are shown. Given are mean values of at least 5 determinations.
[b] The concentrations of compounds reducing p24 antigen concentrations in the supernatants of infected cultures by 50% are shown. Given are mean values of 2–4 determinations at times post infection, when p24 antigen concentration in the untreated control culture increased exponentially.

the peak area ratio (inhibitor/internal standard) of the spiked blood standards vs. concentration.

3. Results

3.1. Structure-activity relationships

The prototype compound **I** (Table 1) is a potent inhibitor of HIV-1 proteinase ($K_i = 6.1$ nM), but blocks HIV-1 induced cytopathic effect in MT4 cells only with an IC_{50} value of 580 nM. From a series of derivatives of **I**, discussed in detail elsewhere (Scholz et al., 1994), we learned that the following structural modifications lead to

increased antiviral activity while inhibitory potency against the enzyme is usually not enhanced but in some cases even lowered: (i) Replacement of valine in the P2 position by tert. leucine (compound **II**); (ii) para-substitution of the benzylamino moiety in P1′ with a methoxy- (**III**), chloro- (**IV**), or bromo group (**V**); (iii) change of the benzylamino group in P3′ to the 2-methylaminobenzimidazole moiety (**VI** to **IX**). The enhancement of antiviral activity following introduction of the benzimidazole heterocycle into the inhibitor is exemplified by comparing compounds **I** and **VI**, **III** and **VII**, **IV** and **VIII**, **V** and **IX** (Table 1). Compound **VII**, which is also the most potent enzyme inhibitor in this series, brings about 20-fold more effective inhibition of virus replication than **I**. Compound **X**, too, in which the benzimidazole moiety is placed in the P3 position, is considerably more potent (about 10 times) than the prototype inhibitor **I**. Compounds **XI** to **XIV** are selected examples of potent proteinase inhibitors which contain combinations of structural changes enhancing antiviral activity.

To assess the specificity of the compounds for inhibition of HIV-1 proteinase we also tested compounds **I**, **II**, **VII** and **X** to **XIV** against a set of other proteinases; no inhibition was observed at concentrations of up to 10 μM in the case of serine proteinases (trypsin, chymotrypsin, kalikrein, plasmin, thrombin, elastase, cathepsin G), a cysteine proteinase (cathepsin B), a metalloproteinase (aminopeptidase M) and the aspartic proteinases renin and pepsin. Another acid proteinase, cathepsin D, however, was inhibited by the prototype compound **I** with $IC_{50} = 35$ nM. As described in detail elsewhere (Scholz et al., 1994), subsequent modifications led to compounds which only marginally inhibited the enzyme (e.g., IC_{50} above 1 μM for **IX** to **XIV**).

3.2. Evaluation of antiviral potential

Initially, all derivatives synthesized were compared for inhibition of HIV-1 (strain IIIB)-induced cytopathic effect in one specific cell line (MT4), as described in detail elsewhere (Scholz et al., 1994). To evaluate the antiviral potential of this series of proteinase inhibitors, a selection of compounds containing 2-aminobenzylstatine as a scissile bond replacement and exhibiting promising antiviral activity was further characterized pharmacologically. First, inhibition of virus replication was measured by using different parameters: p24 release from MT4 cells infected with HIV-1 (strain IIIB) in the presence of the 2-aminobenzylstatine derivatives was compared with the inhibition of virus-induced cyctopathic effect (CPE) in these cells (Table 1); IC_{50} values determined by p24 ELISA generally were 2- to 10-fold lower than those calculated from the inhibition of CPE. For one of the compounds (**VII**), also inhibition of infectious particle formation was determined by infectivity titration of the cell supernatants in fresh MT4 cells (see Section 2); here, an IC_{50} value of 1.8 nM was obtained, which is 5-fold lower than the value calculated from p24 antigen measurement in the same supernatants.

The antiviral effects of a selection of compounds were further demonstrated in another T4-cell line, HUT78, and in the monocytic cell line U937 using p24 antigen shedding into the supernatant as parameter for virus replication. Fig. 1 illustrates the time course of p24 production by HIV-1, IIIB, infected HUT78 cells in the absence and presence of a series of concentrations of **XI**. In this particular experiment IC_{50} values were calculated from p24 concentrations measured at days 5, 8 and 12 post infection. In

Table 2
Activity of proteinase inhibitors against HIV-1, strain IIIB, replication in three cell lines

No.	MT4	IC_{50} [nM] [a]	
		HUT78	U937
I	315	173	300
II	95	145	76.6
VI	13.3	64.7	48.0
VII	7.3	20.3	13.7
IX	20.3	25.0	49.7
X	54.5	92.0	- [b]
XI	5.4	5.9	10.7
XII	11.3	20.3	16.0
XIII	7.9	26.8	- [b]
XIV	21.0	34.7	- [b]

[a] The concentrations of compounds reducing p24 antigen concentrations in the supernatants of infected cultures by 50% are shown. Given are mean values of 2–4 determinations at those times post infection, when p24 antigen concentration in the untreated control culture increased exponentially.
[b] Not determined.

the two cell lines used, IC_{50} values were in most cases comparable or slightly higher than those observed in MT4 cells (Table 2). The inhibitory effect of proteinase inhibitors on proliferation of uninfected cells was assessed in the same three cell lines (Table 3); TC_{50} values in the range of 5–30 μM were determined. For the most potent derivative, **XI**, a selectivity index of 1100–2200 was calculated.

The antiviral efficacy of proteinase inhibitors also extends to the laboratory strain RF (Table 4); here, however, 50% inhibition of p24 production was achieved only at 3–10-fold higher concentrations. Replication of HIV-2, strains EHO and ROD, was also inhibited to some extent by the test compounds, but IC_{50} values were in the range of 0.2–1 μM. We have reported elsewhere (Scholz et al., 1994) that this group of

Table 3
Inhibitory effect of proteinase inhibitors on proliferation of three cell lines

No.	MT4	TC_{50} [μM] [a]	
		HUT78	U937
I	$\geq$ 30	14.0	29.8
II	6.2	8.0	9.1
VI	6.0	5.6	6.1
VII	13.3	12.1	11.3
VIII	9.9	10.8	9.1
IX	8.3	7.4	5.8
X	14.8	15.8	14.0
XI	12.1	11.9	12.2
XII	5.6	4.9	4.5
XIII	9.3	10.9	9.5
XIV	17.1	18.8	14.6

[a] The concentrations of compounds reducing cell density by 50% are shown.

Table 4
Activity of proteinase inhibitors against HIV-1 and HIV-2 replication in MT4 cells

No.	IC_{50} [nM] [a]			
	HIV-1 strain		HIV-2 strain	
	IIIB	RF	EHO	ROD
I	315	1005	> 3000	> 3000
II	95	534	714	1170
VI	13.3	140	805	1100
VII	7.3	45	306	300
VIII	35.0	110	550	415
IX	20.3	140	680	1200
X	54.5	185	620	665
XI	5.4	23	385	755
XII	11.3	38	263	300
XIII	7.9	59	470	770
XIV	21.0	140	380	700

[a] The concentrations of compounds reducing p24 antigen concentrations in the supernatants of infected cultures by 50% are shown. Given are mean values of 2–4 determinations at those times post infection, when p24 antigen concentration in the untreated control culture increased exponentially.

derivatives represents less potent inhibitors of the HIV-2 enzyme than of the HIV-1 coded proteinase; furthermore, different structure-activity relationships govern the interaction with this enzyme. The most effective inhibitor of HIV-2 replication, derivative **XII,** was also the most potent HIV-2 PR inhibitor (K_i = 17 nM).

T4 lymphocytes and monocytes are the predominant target cells for HIV-1 infection in vivo. Compound **XI**, which was the most active derivative in the assays described above, inhibits HIV-1, IIIB, replication also in primary T4 lymphocytes with an IC_{50} value of 3 nM and an IC_{90} value of 4.5 nM (Table 5). A monocytotropic strain of HIV-1, BaL, is inhibited by **XI** in primary monocytes with an IC_{50} value of 7.6 nM and an IC_{90} value of 21 nM. Low passage clinical isolates of T-lymphotropic and monocytotropic strains of HIV-1 from different geographic locations were also inhibited in T4 cells and monocytes with IC_{50} values in the range of 8 to 23 nM and IC_{90} values in the

Table 5
Activity of HIV proteinase inhibitor **XI** against HIV-1, strains IIIB and BaL, and against clinical HIV-1 isolates in primary cells

Cell type	Virus strain (HIV-1)	IC_{50} [nM] [a]	IC_{90} [nM] [a]
T4-lymphocytes	IIIB	3.0	4.5
	K31	8.1	15
	HEM	10	17
Monocytes	BaL	7.6	21
	SF162	18	46
	D747	23	84

[a] The concentrations of compounds reducing p24 antigen concentrations in the supernatants of infected cultures by 50 or 90%, respectively, are shown. Given are mean values of 2–4 determinations at those times post infection, when p24 antigen concentration in the untreated control culture increased exponentially.

range of 15 to 84 nM. The proteinase inhibitor Ro 31-8959 (compound **XVII** in Roberts et al., 1990) was tested as a reference compound in these assays. It showed IC_{50} and IC_{90} values of 3–10 nM and 5–14 nM, respectively in T4 lymphocytes, and of 1.4–8.9 nM and 4.6–39 nM in monocytes.

3.3. Studies on the mode of action

Since the 2-aminobenzylstatine containing compounds studied here are inhibitors of HIV-1 proteinase in enzymatic assays, their mode of antiviral action is expected to be due to blockage of the maturation of *gag* and *gag/pol* precursor proteins. Thus, they should exert their effect at a late stage of viral replication. In line with this expectation, compound **VII** not only inhibited HIV replication in *de novo* infected MT4 cells, as measured by p24 release (IC_{50} = 7.3 nM), but also p24 antigen production by various chronically infected cells.

The chronically-infected monocytic cell line U1 shows minimum constitutive expression of HIV-1 genes, but upon stimulation with various cytokines or with phorbol 12-myristate 13-acetate (PMA), HIV-1 gene expression is enhanced (Folks et al., 1987). In U1 cells stimulated with IL-6 or PMA, **VII** dose-dependently reduced p24 shedding into the culture medium (IC_{50} = 240 nM and 150 nM, respectively). Also, in chronically infected primary human monocytes/macrophages, exposed to compound **VII**, dose-de-

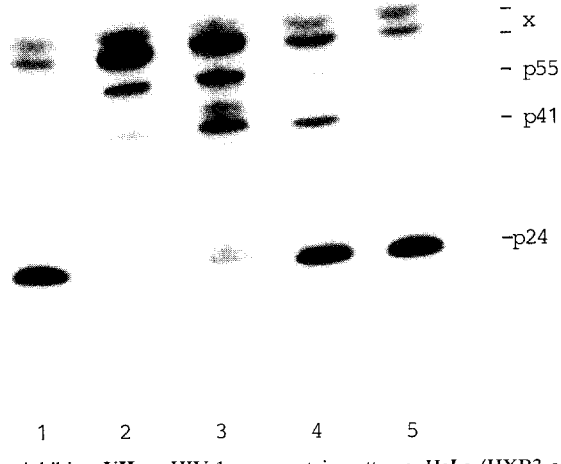


Fig. 2. Effect of protease inhibitor **VII** on HIV-1 *gag* protein patterns. HeLa/HXB3 cells were cultivated in the presence of **VII**; supernatants were harvested and protein contents of virus particles were analysed by immunoblotting with anti-p24 antibody (see Section 2). Positions of *gag* proteins p24, p41, and p55 are indicated. X indicates two cross-reacting bands which occur also in HeLa cells not expressing the HIV genome. Lane 1, no inhibitor; Lane 2, 10 μM **VII**; Lane 3, 3 μM; Lane 4, 1 μM; Lane 5, 0.3 μM.

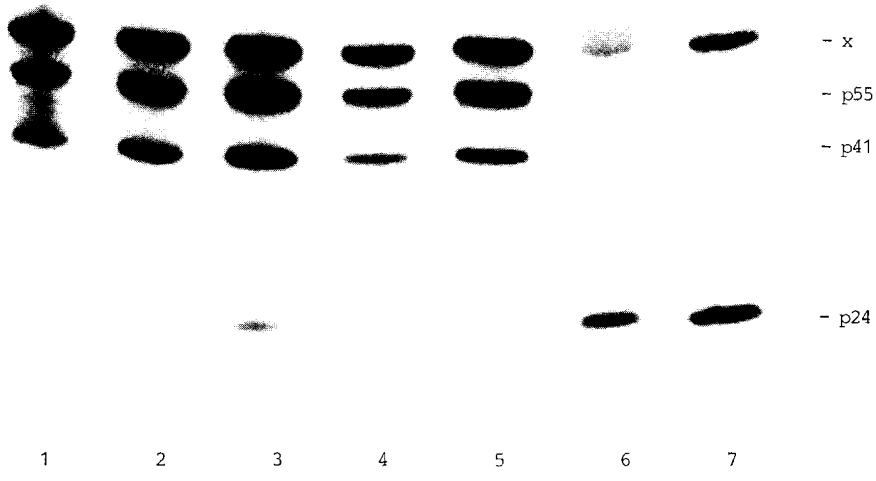


Fig. 3. Persistence of proteinase inhibition by compound **VII** in immature viral particles. Lane 1, virions pelleted from culture supernatants of HeLa/HXB3 grown in presence of 10 μM **VII**; Lane 2, same as 1, but virions resuspended in PBS and incubated for 6 h, then concentrated again; Lane 3, same as 2, but incubation for 24 h; Lane 4, same as 1, but virions resuspended in PBS containing 10 μM **VII** and incubated for 6 h, then concentrated again; Lane 5, same as 4, but incubation for 24 h; Lane 6, virions pelleted from culture supernatants of HeLa/HXB3 (untreated control); Lane 7, same as 6, but virions resuspended in PBS and incubated for 24 h, then concentrated again.

pendent inhibition of virus production was observed (IC_{50} about 1000 nM) (C. Perno, personal communication).

For the analysis of the effect of the proteinase inhibitors on polyprotein processing, a HeLa cell line containing a stably integrated copy of HIV-1, HXB3, genome was used. Since these cells lack the CD4 receptor, re-infection is not possible, thus the study of exclusively late events is feasible. As is depicted in Fig. 2, only particles containing the mature capsid protein p24, but no $p55^{gag}$ are shed into the culture medium by these cells. In the presence of **VII**, *gag* precursor proteins p55 and p41 dose-dependently increase while the amount of p24 decreases. This indicates that the proteinase inhibitor indeed blocks *gag* protein maturation in chronically infected HeLa/HXB3 cells. From the intensities of protein bands (Fig. 2) it is estimated that the concentration of **VII** inhibiting 50% of p55 cleavage is approximately 3 μM.

We wondered whether the block of *gag* cleavage in viral particles produced by HeLa/HXB3 cells in presence ofPR inhibitor would persist for a prolonged period of time. To test this, we harvested the immature particles obtained in the presence of **VII** and incubated them in phosphate-buffered saline to eventually wash out the inhibitor. As seen in Fig. 3, p24 protein was not detectable after 6 h of incubation and only a minute amount of this protein appeared after 24 h. This indicates a continued inhibition of proteinase function. When particles were incubated in presence of **VII** no cleavage at all was detectable during 24 h.

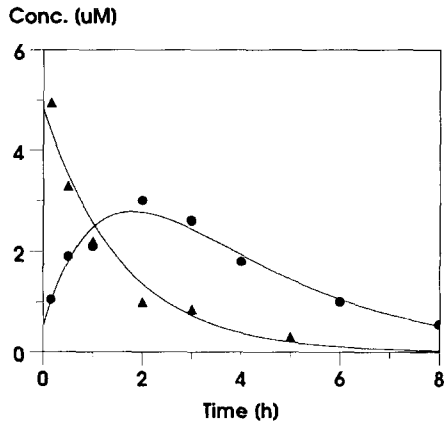


Fig. 4. Pharmacokinetics of HIV proteinase inhibitor I in mice. Blood levels following intraveneous (▲) or peroral administration (●) of I at doses of 10 and 125 mg/kg, respectively, are depicted.

Surprisingly, the IC_{50} values determined for inhibition of p24 release from chronically infected cells as well as that estimated for p55 cleavage in particles from HeLa/HXB3 cells were in all cases 20- to 100-fold higher than those obtained in the de novo infection experiments. In these experiments the endpoint is the result of several rounds of virus replication, i.e. the virus particles produced in the first replication cycle have to infect de novo fresh cells, while in chronically infected cultures the early steps of the viral replication cycle are missing. A possible explanation for the observed divergent IC_{50} values might be that the virus particles, though containing normal amounts of p24, are less infectious. Therefore, we tested the infectivity of viral particles produced in the presence of proteinase inhibitors. For this purpose, a high producer HIV-1, IIIB, chronically-infected Jurkat cell line was used. The supernatants of these cells, which had been incubated in the presence of various concentrations of **XI** were analyzed for p24 content and for infectious particles by titration in MT4 cells. At a concentration of 1 μM **XI** inhibited p24 production by 89%, but these particles were considerably less infectious than those derived from untreated cells: MT4 cells infected with this supernatant yielded only 0.09% of virus particles of that of MT4 cells infected with the control supernatant. Thus, infectivity of the p24 containing virus particles grown in the presence of **XI** was 100-fold lower than that of control virus particles.

3.4. Pharmacokinetic studies

The parent compound (**I**) of the heterostatine-containing proteinase inhibitors and a benzimidazole-substituted analogue (**VII**) were compared with respect to their pharmacokinetic properties. First, they were administered to rats by intraveneous injection; terminal elimination half-lives were determined to be 100 min for both compounds (Table 6). However, when given *per os* to rats, both compounds were not detectable in the blood after administration of a dose of 125 mg/kg, the detection limit being 0.1 μM. Thus, both proteinase inhibitors were not orally bioavailable in rats.

Table 6
Pharmacokinetic parameters of 2-aminobenzylstatine-containing HIV proteinase inhibitors

Species	Compound	$T^{\beta}_{1/2}$(i.v.) [a] [min]	F [b][%]
Rat	**I**	100	0
	VII	100	0
Mouse	**I**	100	18.3
	VII	88	0.4
	XIV	95	0

[a] Compounds were given i.v. as a solution in Cremophor EL/ethanol 7:3 at a dose of 10 mg/kg; from these experiments terminal elimination half-lives ($T^{\beta}_{1/2}$) were determined.
[b] Compounds were given p.o. at a dose of 125 mg/kg (formulation on file at Sandoz). Bioavailability (F) was calculated from the ratio of area-under-the curve (AUC) following p.o. and i.v. administration with correction for different doses.

Unexpectedly, a different situation was encountered in mice: Here, compound **I** exhibited 18% oral bioavailability (Fig. 4, Table 6), while with **VII** still only marginal oral uptake was observed. Other derivatives containing the benzimidazole moiety (e.g., **XIV**), too, showed no or little oral bioavailability, while their elimination half-lifes following i.v. dosage were comparable to that of **I** (Table 6).

As a possible explanation for the species dependent variability of the oral bioavailability, we wondered whether compounds **I** or **VII** would be unstable under the conditions of the intestinal tract. The compounds were incubated with simulated gastric or intestinal juice (US Pharmacopoeia XXI, 1985), with 0.1 N HCl, and with various digestive enzymes (pepsin, trypsin, chymotrypsin, elastase). We did not find any degradation of the proteinase inhibitors under these conditions as judged from HPLC analysis (data not shown). Thus, at present we cannot provide an explanation for the low or absent oral bioavailability of the inhibitors.

4. Discussion

Derivation of the 2-aminobenzylstatine-containing compound **I**, which is a potent inhibitor of HIV-1 proteinase ($K_i = 6.1$ nM), but blocks HIV-1 replication only with IC_{50} values of 173 to 580 nM, led to a series of compounds with considerably improved antiviral activity, the most potent derivative (**XI**) exhibiting IC_{50} values of 3.0 to 23 nM. This increase in activity was achieved by the combination of several structural modifications, most prominently by introduction of a benzimidazole heterocycle into the inhibitor. The improved potency cannot be explained by higher activity against the enzyme since this usually is not enhanced (only a factor of 2 for **VII** as compared to **I**) but in some cases even lowered. It may be explained by improved cell penetration and/or favorable intracellular distribution and stability.

The antiviral effect of the inhibitors was measured by different end-points: virus-induced cytopathic effect, p24 production, and infectious virus particle formation. The most relevant parameter for the in vivo situation clearly is mature, infectious virus

progeny production, since this will determine the rate of spread of the virus. Here, the lowest IC_{50} value (1.8 nM for **VII**) was determined. Kaplan et al. (1993) reported that even partial inhibition of *gag* processing leads to aberrant and non-infectious virus particles. The p24 antigen ELISA used to assess the antiviral effect of proteinase inhibitors does also detect unprocessed or partially cleaved *gag* protein. Furthermore, cell death in the de novo infected MT4 cells may also be caused to some extent by the first round of virus replication. Thus, it can be explained, why for proteinase inhibitor the lowest IC_{50} values were obtained for inhibition of infectious virus formation, followed by p24 determination and then by assessment of the cytopathic effect.

The mode of antiviral action of the 2-aminobenzylstatine PR inhibitors was demonstrated to be inhibition of *gag* precursor processing. The antiviral effect was shown to persist for at least 24 h when isolated viral particles, generated in the presence of PR inhibitors, were washed and incubated in the absence of inhibitor. The hydrophobic nature or tight binding of the inhibitor to the enzyme (or both) can be envisaged to lead to capture of the compound in the virus core where it successfully detains the proteinase from its enzymatic action. Since the 24-h period of inhibition is expected to be longer than the biological life-time of the virus, e.g., in blood, it can be concluded that immature viral particles shed by an inhibitor-treated cell are unlikely to gain infectivity later.

The inhibitory potency of PR inhibitors in chronic virus infection determined by p24 release or cleavage of the p55 precursor protein in all cases was considerably lower than that in de novo infection. Concentrations of inhibitors being effective to $> 90\%$ in de novo infection allowed normal amounts of viral particles to be released to the supernatant of chronically infected cells, and these particles contained normal amounts of cleaved *gag* protein. This discrepancy could be resolved when infectivity of progeny particles from chronically infected cells produced in the presence of PR inhibitors was measured: These particles were about 100-fold less infectious than those from untreated cultures relative to p24 concentration. Obviously, though these particles are shed into the supernatant and contain normal amounts of cleaved *gag* protein, they are defective in their capacity to infect cells de novo.

The antiviral efficacy of PR inhibitors, and in particular of the most potent derivative **XI**, was further demonstrated in various cell lines, in primary T4 lymphocytes and in primary monocytes. Compound **XI** inhibited replication of several HIV-1 clinical isolates from different geographic locations in primary cells with IC_{50} values of 8 to 23 nM, while cytostatic effects in proliferating cell lines were detected only at 12 μM. The primary cell cultures under our experimental conditions did not proliferate, but cells were viable to $> 80\%$, as shown by staining, for the 20 days of the experiment. This group of PR inhibitors shows preferential inhibition of HIV-1 over HIV-2 strains which is explained by the observed weaker effect on HIV-2 proteinase. Thus, the antiviral effect of this group of PR inhibitors is highly selective for HIV-1. Importantly, all HIV-1 isolates tested were inhibited with similar efficacy by the PR inhibitors; thus, no natural occurring resistance was encountered.

The analysis of the pharmacokinetic behavior of compounds **I** and **VII** revealed reasonable blood half-lives in rodents of about 1.5 h. Thus, these compounds are not so rapidly excreted as reported for other peptide-like drugs, including some types of HIV

proteinase inhibitors (Kempf et al., 1991). However, unexpected results were obtained, when oral bioavailability was studied: (i) Compound **I** showed appreciable oral uptake in mice (18%), but yielded no detectable blood levels in rats. At present, no obvious explanation for these findings is at hand. Since the blood half-life after intraveneous administration of compound **I** is identical in mice and rats, there may be a problem with intestinal resorption in rats as compared to mice. (ii) Surprisingly, benzimidazole containing compounds like **VII** are not orally bioavailable to a significant extent, neither in mice nor in rats. An extensive first-pass excretion of **VII** after successful intestinal resorption can be excluded as an explanation for this finding, since **I** and **VII** have the same plasma half-lives after intraveneous injection. The benzimidazole group, obviously, is deleterious to the capability of compounds to pass the mucosal membranes, even in mice. Thus, while introduction of a benzimidazole group into the PR inhibitors was a very successful structural modification with regard to antiviral activity – presumably by enhancing cell permeability – it completely abolished oral bioavailability.

To conclude, we have been successful in creating a class of inhibitors with potent antiviral activity against HIV-1, low cytotoxicity, adequate selectivity for the viral enzyme, and promising blood half-lives. In the series of derivatives described here, however, we were unsuccessful to identify a compound exhibiting both, potent antiviral activity and good oral bioavailability. Further derivation is ongoing to combine these two properties to obtain a promising candidate for clinical use in HIV disease.

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