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Original article

HIV protease inhibitors: synthesis and activity of *N*-aryl-*N*′-hydroxyalkyl hydrazide pseudopeptides

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Abstract

We describe the synthesis and activities of a series of pseudopeptides containing an *N*-aryl-*N*′-hydroxyalkyl hydrazide core structure to inhibit human immunodeficiency virus protease and viral replication. Of the series, compound Hmb-Leu-N(Bzl)–N(CH_2–CH–OH)-rPro-Boc (**24**) displayed the greatest inhibitory potency (IC_{50} < 1 μM) and exhibited enzymatic resistance and stability in vitro.
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Keywords: HIV protease inhibitors; Hydrazide pseudopeptides; Synthesis; Antiviral activity

1. Introduction

The human immunodeficiency virus (HIV) has been recognized as the causative agent of acquired immunodeficiency syndrome (AIDS) and its related disorder [1–3]. Characterization of the HIV life cycle has highlighted many different targets for potential drug intervention. These include inhibition of virus adsorption, viral fusion, viral uncoating and inhibition of the viral replicative enzymes such as reverse transcriptase (RT), integrase (IN) and HIV protease (PR) [4]. Currently, there are several anti-retroviral drugs available, such as the introduction of protease inhibitors (PI) in combination with anti-retroviral therapy (HAART), which cause changes in the development of HIV infections [5,6]. Most of the problems associated with HIV therapy are the consequence of the long-term use of the drugs. The development of resistant variants of the virus limits the prolonged use of PI as therapeutic agents so it is necessary to produce new compounds that have efficacy in the control of HIV infection and are active against the resistant forms [7,8].

The HIV protease is an aspartic protease composed by two identical monomers. The monomers are 99 amino acids long and exist as a C2-symmetric homodimer in the active form of the protease. The active site, which consists of two catalytic aspartate residues, is located in the center of the binding site and is hidden under flaps. Substrates or inhibitors create tetrahedral transition-state intermediates through the development of hydrogen bonding networks between the flaps of the HIV binding site and water molecules of the substrates or inhibitors [9–11]. Several classes of PIs have been developed, showing excellent antiviral profiles. Nevertheless new potent inhibitors that show good pharmacokinetics and activity against mutant strains of HIV are required [12–14].

Initially, we developed a series of peptide-based inhibitors which showed significant activity and good metabolic stability following the Kempf strategy [15–23]. The last two series of pseudopeptides containing an *N*-hydroxyamino acid or *N*-benzyl hydroxyalkylamino acid core structure were able to inhibit isolated enzymes and viral replication in the nM range [24,25]. On the basis of these features, we designed a new series of pseudodipeptides which incorporate a hydrazide moiety in the structural core. Aryl and hydroxyalkyl functions are directly linked, respectively, to hydrazine *N* and *N*′ at the P1/$P1^I$ position (Fig. 1). 3-Hydroxy-2-methylbenzoyl (Hmb) in P2 and Boc-proline in $P1^I$/$P2^I$ were selected as they show close analogy with other substituents of potent HIV PI [26,27]. In the sequence of compounds **41**–**52** proline is condensed to the molecules in the opposite direction and is indi-

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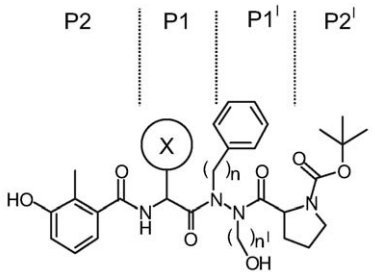


X = -CH_2-CH_2-S-CH_3, -CH_2-CH(CH_3)-CH_3, CH(OH)-CH_3; n = 0, 1; n' = 1, 2.

Fig. 1. General structure of *N*-aryl-*N*′-hydroxyalkyl hydrazide pseudopeptides **41**–**52**.

cated as ″rPro″. Different residues are situated at P1 in an aim to evaluate the most favorable side-chain for enzymatic interaction. Phenyl or benzyl and hydroxymethyl or hydroxyethyl groups are linked in all combinations to the central hydrazine to modulate the distance and flexibility of those functions responsible for the primary interactions with the catalytic pocket.

2. Chemistry

Hydrazide derivatives **41**–**52** were synthesized (Scheme 1) starting from phenylhydrazine or benzylhydrazine (**1**, **2**), that was monoacylated in N′ with $(Boc)_2O$ (1.1 equiv) to give intermediates **3** or **4** [28]. Coupling with the corresponding Fmoc-protected amino acids activated by HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) gives the selective protected hydrazinoamino acids **5**–**10**. After cleavage of the Fmoc group (fluorenylmethoxycarbonyl), condensation of the 3-hydroxy-2-methyl benzoic acid via active ester resulted in compounds **11**–**16**. After TFA treatment, hydrazide intermediate was acylated by ethyl chloroformate or alkylated by ethyl bromoacetate to yield esters **17**–**22** or **23**–**28**, respectively. Reduction by $LiAlH_4$ gave the corresponding *N*′-hydroxymethyl (**29**–**34**) or *N*′-hydroxyethyl (**35**–**40**) intermediates. Finally, condensation of the Nα-protected proline using HBTU, yielded the target products (**41**–**52**). Crude products were purified by preparative RP-HPLC, lyophilized, and their structure verification was achieved by mass spectrometry and NMR spectroscopy. HPLC capacity factors (K^I) and other physicochemical properties of pseudopeptides are summarized in Table 1.

3. Biological activity

IC_{50} and ED_{50} values, respectively, against HIV-1 protease and for inhibition of viral replication in cell culture for hydrazyde derivatives **41**–**52** are shown in Table 2. Inibitory potencies (IC_{50}) against purified recombinant HIV-Pr were determined using synthetic peptide H-His-Lys-Ala-Arg-Val-Leu-Phe(pNO_2)-Glu-Ala-Nle-Ser-OH (Bachem Bioscience) as a substrate. The C-terminal pentapeptide cleaved product was measured at 220 nm by reversed-phase HPLC analysis at different times during incubation. The data were plotted as percent control (the ratio of percent conversion in the presence and absence of inhibitor) vs. inhibitor concentration and fitted with the Eq. $Y = 100/1 + (X/IC_{50})^A$, where IC_{50} is the inhibitor concentration at 50% inhibition and *A* is the slope of the inhibition curve.

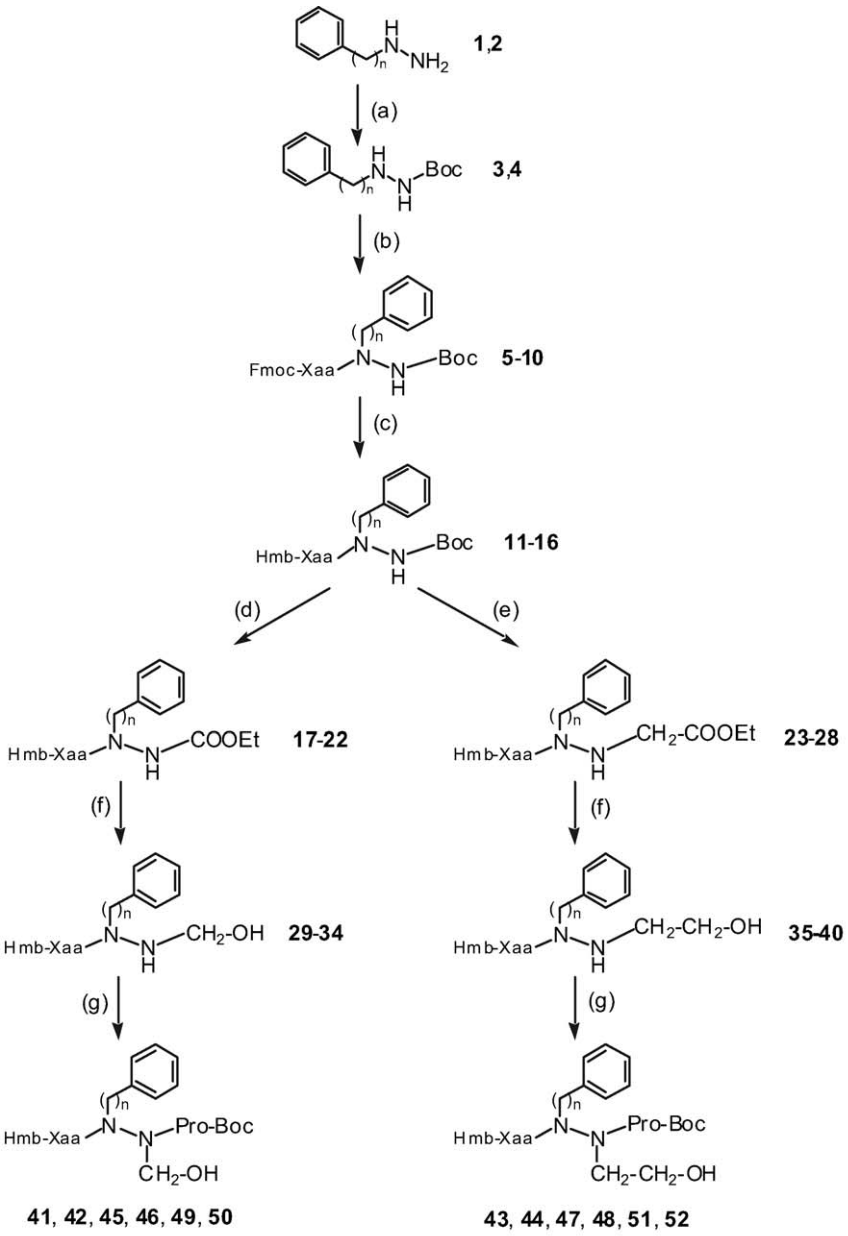
Better isolated enzyme hydrazide inhibitors were also tested for their capacity to inhibit viral replication in the HIV-1 strain IIIB in CEM cells. HIV-1 IIIB was obtained from HIV-1 IIIB chronically infected Molt-4 cells as a supernatant fluid. The ED_{50} (50% dose) values were calculated as the dose of inhibitor that resulted in a 50% reduction in p24 levels as compared to those in control wells.

In vitro metabolic stability of compounds **41**–**52** was evaluated in cell culture medium (RPMI) and in human plasma. Each inhibitor was incubated in RPMI containing 20% fetal calf serum or in human plasma. In both cases, incubations were performed at 37 °C for different periods up to a maximum of 360 min. The incubation was terminated by addition of ethanol, following centrifugation aliquots of the clear supernatant were added to the RP-HPLC column.

Table 1
Analytical data and physicochemical properties of pseudopeptides **41**–**52**

Numbers	Compound	HPLC [a]		M.p.	$[\alpha]_D^{20}$	MS Maldi-Tof
		K^I (a)	K^I (b)	(°C)	(*c* = 1, MeOH)	(M + H^+)
41	Hmb-Met-N(Ph)–N(CH_2–OH)-rPro-Boc	7.35	5.87	183–185	–3.4	601.7
42	Hmb-Met-N(Bzl)–N(CH_2–OH)-rPro-Boc	7.56	6.11	167–169	–3.7	615.6
43	Hmb-Met-N(Ph)–N(CH_2–CH_2–OH)-rPro-Boc	8.03	6.74	172–176	–4.9	615.6
44	Hmb-Met-N(Bzl)–N(CH_2–CH_2–OH)-rPro-Boc	8.45	6.89	154–157	–4.3	629.6
45	Hmb-Leu-N(Ph)–N(CH_2–OH)-rPro-Boc	9.13	8.02	143–145	–8.5	583.6
46	Hmb-Leu-N(Bzl)–N(CH_2–OH)-rPro-Boc	9.33	8.14	136–139	–9.9	597.5
47	Hmb-Leu-N(Ph)–N(CH_2–CH_2–OH)-rPro-Boc	9.80	8.38	128–131	–10.1	597.4
48	Hmb-Leu-N(Bzl)–N(CH_2–CH_2–OH)-rPro-Boc	10.11	8.96	122–125	–9.4	611.5
49	Hmb-Thr-N(Ph)–N(CH_2–OH)-rPro-Boc	6.74	4.97	187–189	–11.7	571.3
50	Hmb-Thr-N(Bzl)–N(CH_2–OH)-rPro-Boc	6.90	5.32	179–183	–12.4	585.4
51	Hmb-Thr-N(Ph)–N(CH_2–CH_2–OH)-rPro-Boc	7.19	5.70	170–172	–7.6	585.3
52	Hmb-Thr-N(Bzl)–N(CH_2–CH_2–OH)-rPro-Boc	7.28	5.79	159–162	–8.2	599.5

[a] HPLC conditions (a) and (b) are defined in Section 6.



Scheme 1. Synthesis of the hydrazide derivatives **41**–**52**.

4. Results and discussion

In general, the HIV Pr inhibition results showed moderate activity for all compounds, in the order of μM concentrations. Derivatives **44**–**48** were the best inhibitors of the first series. In P1, the branched aliphatic side chain (Leu) is favorable to hydrophilic (Thr) or sulfur containing linear (Met) side chains. The greater length of the hydroxyalkyl chain in the structural core probably promotes easier hydrogen bond formation with the catalytic aspartic acid of HIV. The larger flexibility of the benzyl group over the phenyl in the *N*′ hydrazine moiety favors interactions with the catalytic groove of HIV. Hydrazide pseudodipeptide **48** was the best PI of the second series, and can be used as a model to summarize—the favorable molecular features for enzymatic interaction. Molecular modeling studies have defined the interactions between *N*-benzyl-*N*′-hydroxyethyl hydrazide derivative and the HIV-1 protease active site, and the results are schematically shown in Fig. 2. In comparison with the previous *N*-hydroxy and *N*-benzyl derivatives, the *N*-aryl-*N*′-

Table 2
Inhibitory potencies and metabolic degradation of compounds **41**–**52**

No	IC_{50} ± S.D. (μM) [a]	ED_{50} ± S.D. (μM) [a]	Half-life (min)	
			Culture medium	Human plasma
41	430 ± 3.53	ND [b]	>360	287
42	135 ± 6.36	ND [b]	>360	255
43	275 ± 4.94	ND [b]	>360	341
44	44 ± 2.83	32.4 ± 2.26	>360	>360
45	77 ± 4.24	28.0 ± 2.50	>360	310
46	23 ± 4.82	18.4 ± 2.69	>360	>360
47	85 ± 6.82	26.7 ± 1.98	>360	>360
48	5 ± 0.82	0.3 ± 0.02	>360	>360
49	>1000	ND [b]	>360	277
50	214 ± 5.65	ND [b]	>360	316
51	205 ± 7.15	ND [b]	>360	>360
52	131 ± 4.90	103.5 ± 4.15	>360	>360

[a] Values are the average of at least two determinations ($n = 2$) unless otherwise noted.
[b] Not determined.

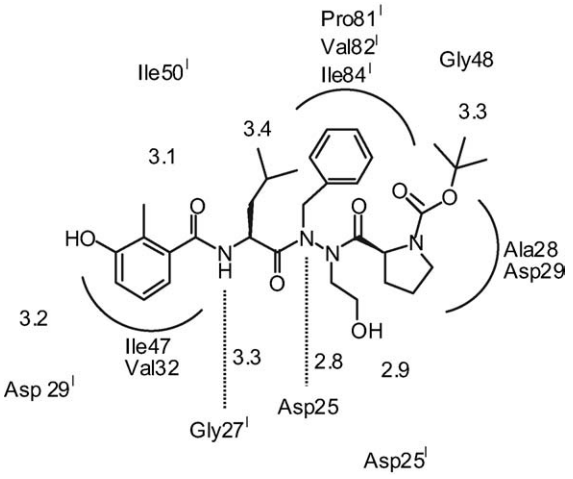


Fig. 2. Schematic drawing showing the expected interactions between *N*-aryl-*N*′-hydroxyalkyl hydrazide derivative **48** and the HIV-1 protease active site. Hydrogen bonds are shown as dashed lines with distances in Å. Data were obtained by means of binding model previously suggested by our *N*-benzyl inhibitors dynamic simulations [24].

hydroxyalkyl hydrazide pseudodipeptides are less active, indicating that a reduction of molecular size decreases the number of interactions between enzyme and inhibitor, especially at the P2 site.

All selected compounds displayed a capacity to inhibit viral replication in cultured cells close to their IC_{50} values for HIV PR inhibition. In particular *N*-benzyl-*N*′-hydroxyethyl hydrazide pseudopeptide **48** shows acceptable potency ($ED_{50} = 0.3$ μM) confirming its favorable pharmacokinetic properties, especially in the important cell membrane penetration employed here.

In the enzymatic hydrolysis tests, all compounds are stable in cell culture medium, while in human plasma only derivatives **41**–**43**, **45**, **49** and **50** exhibit a half-life less than 360 min (Table 2). The data of the metabolic stability demonstrate that the presence of the hydrazine moiety in the structural core stabilizes these new pseudodipeptides against enzymatic hydrolysis.

5. Conclusion

We designed, synthesized and tested new pseudodipeptides containing a *N*-aryl-*N*′-hydroxyalkyl hydrazide moiety in the core structure on the basis of our previously studies concerning the development of HIV PI. Our results show that the hydrazide derivatives possess elevated metabolic stability and maintain appreciable antiviral activity. In order to improve inhibitory potency, further structural modification around the hydrazide template in P2/$P2^I$ positions are required in an aim to develop novel agents for the treatment of HIV.

6. Experimental protocols

6.1. General

Amino acids, amino acid derivatives, resins and chemicals were purchased from Bachem, Novabiochem and Fluka (Switzerland).

Crude pseudopeptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 A 15 μm spherical particle size column). The column was perfused at a flow rate of 50 ml/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0% to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA), 25 min was adopted for the elution of compounds. HPLC analysis was performed by a Beckman System Gold with a Hypersil BDS C18 column (5 μm; 4.6 × 250 mm). Analytical determination and capacity factor (*K*′) of the hydrazide derivatives were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at flow rates of 1 ml/min using the following linear gradients: a) from 0% to 100% B for 25 min and b) from 10% to 70% B for 25 min. All pseudopeptides showed less than 1% impurities when monitored at 220 and 254 nm.

Molecular weight of compounds were determined by a MALDI-TOF analysis using a Hewlett Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as a matrix. The values are expressed as MH^+. TLC was performed in precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: c) AcOEt/*n*-hexane (1:1, v/v), d) CH_2Cl_2/methanol (9.5:0.5, v/v), e) CH_2CL_2/methanol (9:1, v/v), f) CH_2CL_2/methanol/toluene (17:2:1, v/v/v). Ninhydrin (1%) or chlorine iodine spray reagents were employed to detect the peptides. Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined by a Perkin–Elmer 141 polarimeter with a 10-cm water-jacketed cell. ^{1}H-NMR spectroscopy was obtained on a Bruker AC 200 spectrometer.

6.2. Chemistry

*6.2.1. General procedure for compounds **17–28***

6.2.1.1. Introduction of Boc (a). Phenylhydrazine or benzylhydrazine (2 mmol) and $(Boc)_2$ O (2.2 mmol) were dissolved in 20 ml of a mixture H_2O/*tert*-butanol. NMM (2.2 mmol) were added, and the resultant mixture was stirred for 2 h at 0 °C and further 8 h at room temperature (RT). Following ilution with a saturated solution of NaCl, the mixture was extracted with AcOEt (2 × 50 ml). The combined extracts were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resultant oil was used without any further purification for the next step.

6.2.1.2. Coupling with HBTU (b, g). The hydrazide component (1 mmol), NMM (1 mmol) and HBTU (1 mmol) were added to a solution of Fmoc-Xaa-OH or Boc-Pro-OH (1 mmol) in DMF (3 ml) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 18 h at RT; then the solution was diluted with AcOEt (70 ml) and washed consecutively with HCl 0.1 N, brine, $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was treated with Et_2O and resulting solid separated by centrifugation.

6.2.1.3. Fmoc removal (c.1). The Fmoc group was cleaved from **5**–**10** with a solution of 20% piperidine in DMF for 25 min. After evaporation the residue was triturated with Et_2O, centrifugated and the resulting solid was collected and dried.

6.2.1.4. Coupling with WSC/HOBt (c.2). To a solution of the 3-hydroxy-2-methyl benzoic acid (1.5 mmol) in DMF (5 ml) were added the amine component (1.5 mmol), HOBt (1.5 mmol) and WSC (1.5 mmol) in this order at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 12 h at RT; then the solution was diluted with AcOEt (100 ml) and worked up as described in Section 6.2.1.2.

6.2.1.5. TFA deprotection (d.1; e.1). Boc was removed by treating intermediates **7**, **8** with aqueous 90% TFA (1:10, w/v) for 30–40 min. After evaporation, the residue was worked up as described in Section 6.2.1.3.

6.2.1.6. N-*Acylation (d.2).* TEA (2 mmol) and ethyl chloroformate (1 mmol) were added to a solution of hydrazide intermediate (1 mmol) in DMF (10 ml). The resultant mixture was stirred for 2 h at RT and refluxed for 1 h; after evaporation the residue was diluted with AcOEt (50 ml) and washed with H_2O (2 × 10 ml). The organic phase was dried ($MgSO_4$), filtered, evaporated to dryness and the residue was used for the following reduction.

6.2.1.7. N-*Alkylation (e.2).* The reaction was conducted as described in Section 6.2.1.6. using ethyl bromoacetate as alkylating agent.

6.2.1.8. Reduction of ethyl ester (f). The hydrazide ester intermediate (0.4 mmol) in THF (3 ml) was added dropwise to a cold solution of $LiAlH_4$ (0.5 mmol) in THF (3 ml). The mixture was stirred for 2 h at RT and then evaporated. The residue was suspended in H_2O (5 ml) and extracted with AcOEt (2 × 20 ml); the organic phase was dried, filtered and evaporated. The residue was triturated with Et_2O, centrifugated and the resulting solid was collected and used for the final condensation.

6.2.1.9. ^1H NMR data of compounds. **Hmb-Met-N(Ph)-N(CH_2-OH)-rPro-Boc (41).** ^{1}H NMR ($CDCl_3$): δ 1.29 (s, 9H), 1.58–1.77 (m, 4H), 1.94 (s, 3H), 2.23–2.51 (m, 7H), 3.28 (bs, 1H), 3.74 (t, 2H), 4.12 (m, 1H), 4.49 (m, 1H), 5.27 (s, 2H), 5.54 (bs, 1H), 6.67–7.19 (m, 8H), 7.63 (s, 1H).

Hmb-Met-N(Bzl)-N(CH_2-OH)-rPro-Boc (42). ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 9H), 1.54–1.86 (m, 4H), 2.03 (s, 3H), 2.17–2.29 (m, 7H), 2.74 (bs, 1H), 3.27 (t, 2H), 4.08–4.37 (m, 4H), 5.13 (bs, 1H), 5.42 (s, 2H), 7.03–7.32 (m, 8H), 7.81 (s, 1H).

Hmb-Met-N(Ph)-N(CH_2-CH_2-OH)-rPro-Boc (43). ^{1}H NMR ($CDCl_3$): δ 1.19 (s, 9H), 1.47–1.79 (m, 4H), 1.98 (s, 3H), 2.13–2.25 (m, 7H), 2.81 (bs, 1H), 3.35 (t, 2H), 3.71 (t, 2H), 4.15–4.36 (m, 2H), 4.98 (bs, 1H), 5.35 (t, 2H), 6.95–7.18 (m, 8H), 8.01 (s, 1H).

Hmb-Met-N(Bzl)-N(CH_2-CH_2-OH)-rPro-Boc (44). ^{1}H NMR ($CDCl_3$): δ 1.11 (s, 9H), 1.57–1.83 (m, 4H), 1.95 (s, 3H), 2.33–2.47 (m, 7H), 3.01 (bs, 1H), 3.44 (t, 2H), 3.59 (t, 2H), 4.05–4.35 (m, 4H), 5.05 (bs, 1H), 5.46 (t, 2H), 7.12–7.40 (m, 8H), 8.04 (s, 1H).

Hmb-Leu-N(Ph)-N(CH_2-OH)-rPro-Boc (45). ^{1}H NMR ($CDCl_3$): δ 0.99 (d, 6H), 1.08 (s, 9H), 1.58–1.95 (m, 7H), 2.02 (bs, 1H), 2.33 (s, 3H), 3.29 (t, 2H), 4.25 (m, 1H), 4.39 (m, 1H), 4.96 (bs, 1H), 5.34 (s, 2H), 7.03–7.41 (m, 8H), 8.05 (s, 1H).

Hmb-Leu-N(Bzl)-N(CH_2-OH)-rPro-Boc (46). ^{1}H NMR ($CDCl_3$): δ 1.05 (d, 6H), 1.15 (s, 9H), 1.55–1.65 (m, 4H), 1.67–1.78 (m, 3H), 1.99 (bs, 1H), 2.30 (s, 3H), 3.33 (t, 2H), 4.40–4.52 (m, 4H), 5.03 (bs, 1H), 5.51 (s, 2H), 7.10–7.57 (m, 8H), 8.02 (s, 1H).

Hmb-Leu-N(Ph)-N(CH_2-CH_2-OH)-rPro-Boc (47). ^{1}H NMR ($CDCl_3$): δ 0.98 (d, 6H), 1.13 (s, 9H), 1.50–1.81 (m, 7H), 1.99 (bs, 1H), 2.42 (s, 3H), 2.76 (d, 2H), 3.44 (d, 2H), 4.29–4.40 (m, 2H), 4.97 (bs, 1H), 5.45 (t, 2H), 7.07–7.49 (m, 8H), 7.99 (s, 1H).

Hmb-Leu-N(Bzl)-N(CH_2-CH_2-OH)-rPro-Boc (48). ^{1}H NMR ($CDCl_3$): δ 0.99 (d, 6H), 1.08 (s, 9H), 1.78–1.84 (m, 3H), 2.03–2.11 (m, 7H), 2.28–2.35 (m, 3H), 3.24 (d, 2H), 3.52 (d, 2H), 4.38 (m, 1H), 4.47 (s, 2H), 4.55 (m, 1H), 5.04 (bs, 1H), 6.93–7.27 (m, 8H), 7.97 (s, 1H).

Hmb-Thr-N(Ph)-N(CH_2-OH)-rPro-Boc (49). ^{1}H NMR ($CDCl_3$): δ 1.03 (m, 3H), 1.24 (s, 9H), 1.38 (m, 2H), 1.82 (m, 2H), 2.14 (bs, 2H), 2.42 (s, 3H), 3.20 (t, 2H), 4.14 (m, 1H), 4.33–4.46 (m, 2H), 4.88 (s, 2H), 5.11 (bs, 1H), 6.99–7.28 (m, 8H), 8.12 (s, 1H).

Hmb-Thr-N(Bzl)-N(CH_2-OH)-rPro-Boc (50). ^{1}H NMR ($CDCl_3$): δ 1.08 (m, 3H), 1.27 (s, 9H), 1.78–1.84 (m, 4H),

2.11 (bs, 2H), 2.36 (s, 3H), 3.28 (t, 2H), 4.28–4.57 (m, 5H), 4.61 (s, 2H), 5.04 (bs, 1H), 6.87–7.21 (m, 8H), 8.07 (s, 1H).

Hmb-Thr-N(Ph)-N(CH_2-CH_2-OH)-rPro-Boc (51). ^{1}H NMR ($CDCl_3$): δ 1.04 (m, 3H), 1.30 (s, 9H), 1.73–1.81 (m, 4H), 2.07 (bs, 2H), 2.29 (s, 3H), 3.33 (t, 2H), 3.41 (t, 2H), 3.82 (t, 2H), 4.31–4.47 (m, 3H), 4.98 (bs, 1H), 7.03–7.25 (m, 8H), 8.03 (s, 1H).

Hmb-Thr-N(Bzl)-N(CH_2-CH_2-OH)-rPro-Boc (52). ^{1}H NMR ($CDCl_3$): δ 1.01 (m, 3H), 1.28 (s, 9H), 1.53 (m, 2H), 1.82 (m, 2H), 2.11 (bs, 2H), 2.35 (s, 3H), 3.27 (t, 2H), 3.39 (t, 2H), 3.76 (t, 2H), 4.39–4.52 (m, 3H), 4.70 (s, 2H), 5.13 (bs, 1H), 6.89–7.26 (m, 8H), 7.95 (s, 1H).

6.3. Test for the inhibition of HIV-1 protease

For determination of IC_{50} values, affinity-purified HIV-1 protease (Bachem Bioscience) 1.1 nM final concentration, was added to a solution (100 μl final volume) containing inhibitor, 4 mM peptide substrate (His-Lys-Ala-Arg-Val-Leu-p-nitro-Phe-Glu-Ala-Nle-Ser, Bachem Bioscience), and 1.0% dimethyl sulfoxide in assay buffer: 1.0 mM dithiothreitol, 0.1% glycerol, 80 mM sodium acetate, 160 mM sodium chloride, 1.0 mM EDTA, all at pH 4.7. The solution was mixed and incubated for 25 min at 37 °C and reaction quenched by the addition of trifluoroacetic acid, 2% final concentration. The Leu-Phe(p-NO_2) bond of the substrate was cleaved by the enzyme. The cleavage products and substrate were separated by RP-HPLC. Absorbance was measured at 220 nm, peak areas were determined, and percent conversion to product was calculated using relative peak areas. The data were plotted as percent control (the ratio of percent conversion in the presence and absence of inhibitor) vs. inhibitor concentration and fitted with the Eq. $Y = 100/1 + (X/IC_{50})^A$, where IC_{50} is the inhibitor concentration at 50% inhibition and A is the slope of the inhibition curve.

6.4. Cell culture activity against HIV-1 IIIB

HIV-1 IIIB was obtained from HIV-1 IIIB chronically infected Molt-4 cells as a supernatant fluid. The 50% tissue culture infection dose (TC ID_{50}) was determined by an end point titration procedure. CEM cells (5000 per ml) were exposed to HIV-1 IIIB fluid at a multiplicity of infection (m.o.i.) 0.001 TC ID_{50} (ml). Aliquots (0.2 ml) of cells were placed in 96 well microtitre plates with 2 ml of the appropriate concentrations of inhibitors dissolved in DMSO. After incubation for 6 days in RPMI-1640 medium containing 10% fetal calf serum, the p24 antigen of HIV in the supernatant was determined by an ELISA assay kit (RETRO-TEK, Cellular Products Inc., Buffalo, USA). The ED_{50} (50% dose) values were calculated as the dose of the inhibitor that resulted in a 50% reduction in p24 levels as compared to those in control wells.

6.5. Metabolic stability assay

The kinetics of new inhibitors degradation were studied in culture medium (RPMI) and human plasma. 0.1 ml of a solution of each compound (10 mg/ml in acetonitrile/H_2O 1:1) was added to 1 ml of RPMI containing 20% fetal calf serum. Alternatively, test compounds were incubated with plasma (0.6 ml) in a total volume of 1.5 ml of 10 mM Tris–HCl buffer, pH 7.5. Incubation was performed at 37 °C for different periods up to a maximum of 360 min in the case of human plasma and up to 2 days in the case of RPMI containing 20% FCS. The incubation was terminated by addition of ethanol (0.2 ml), the mixture poured at 21 °C and after centrifugation (5000 rpm for 10 min), aliquots (20 μl) of the clear supernatant were injected into RP-HPLC column. HPLC was performed as described above (see Section 6, general).

6.6. Modeling

Molecular modeling was carried out using the Macromodel 5.5 software, using the AMBER force field. Structure of compound **48** was created by modifying the X-ray structure of A-98881 [29] and superimposing the new structures to the azacyclic urea-inhibitor. Energy minimization and molecular dynamics were run. Only the atoms of the inhibitors and the atoms of the enzyme at a maximum distance of 6 Å from the inhibitor were allowed to freely move during these operations, while the rest of the enzyme was held fixed (Fig. 2).

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