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Reduced-bond tight-binding inhibitors of HIV-1 protease

Fine tuning of the enzyme subsite specificity

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Truncation of a peptide substrate in the N-terminus and replacement of its scissile amide bond with a non-cleavable reduced bond results in a potent inhibitor of HIV-1 protease. A series of such inhibitors has been synthesized, and S2–S3′ subsites of the protease binding cleft mapped. The S2 pocket requires bulky Boc or PIV groups, large aromatic Phe residues are preferred in P1 and P1′ and Glu in P2′. The S3′ pocket prefers Phe over small Ala or Val. Introduction of a Glu residue into the P2′ position yields a tight-binding inhibitor of HIV-1 protease, Boc-Phe-[CH_2–NH]-Phe-Glu-Phe-OMe, with a subnanomolar inhibition constant. The relevant peptide derived from the same amino acid sequence binds to the protease with a K_i of 110 nM, thus still demonstrating a good fit of the amino acid residues into the protease binding pockets and also the importance of the flexibility of P1–P1′ linkage for proper binding. A new type of peptide bond mimetic, *N*-hydroxylamine -CH_2-N(OH)-, has been synthesized. Binding of hydroxylamino inhibitor of HIV-1 protease is further improved with respect to reduced-bond inhibitor.

Inhibitor; HIV-1 protease; Enzyme subsite specificity

1. INTRODUCTION

The virus-encoded protease (PR) catalyzing specific cleavage of the polyprotein precursors of human immunodeficiency virus (HIV-1) mature proteins has become an obvious target for anti-viral chemotherapy [2–4]. Understandably, most authors approached the design of an HIV-1 PR-specific inhibitor by replacing a scissile peptide bond of a proper peptide substrate by its non-cleavable isostere, e.g. reduced bond [5], statine and its derivatives [6], hydroxyethylene [7], hydroxyethylamine [8,9] or difluoroketone and phosphinates [10]. Alternative strategies, e.g. random screening of natural products [11] or the use of non-peptidic inhibitors of protease [12,13] have not yet been equally successful.

An obvious basis for designing substrate-based inhibitors has been the amino acid sequences spanning the cleavage sites in retroviral gag-pol polyprotein, mostly matrix–capsid or capsid–nucleocapsid protein boundaries. However, the peptide substrates derived from these processing sites exhibit relatively high K_m values, demonstrating thus rather poor binding to the protease binding cleft [3]. Inhibitors based on these substrates must comprise additional structural features capable of increasing the affinity of a substrate to the protease by a factor of 10^5 or 10^6 [10]. Intuitively, substrates exhibiting better binding to the enzyme should serve as a more advantageous basis for the rational design of inhibitors. Recently, we presented a series of chromogenic peptide substrates of both HIV-1 and HIV-2 proteases with K_m values in the micromolar range [14]. We used them for thorough mapping of subsite requirements of HIV-1 and -2 and myeloblastosis-associated virus proteases [15–17]. In the present study we show that information gained from such substrate specificity 'fine tuning' may open a way to the design of potent, specific inhibitors based on rather simple chemistry.

Abbreviations: DMSO, dimethylsulfoxide; Et, total enzyme concentration; K_i, inhibition constant, dissociation constant of enzyme-inhibitor complex; PR, protease; HIV, human immunodeficiency virus; Nle, norleucine; Nph, *p*-nitro-phenylalanine; Me, methyl; Ac, acetyl; Boc, tert-butoxycarbonyl; Z, benzyloxycarbonyl protecting group; PIV, pivaloyl, (*t*-butylcarbonyl); [CH_2–NH], reduced peptide bond. The nomenclature system of Schechter and Berger [1], i.e. P2–P1*P1′–P2′–P3′ is used to depict residues adjacent to the scissile (or reduced) bond situated between P1 and P1′ marked by an asterisk.

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2. MATERIALS AND METHODS

2.1. *HIV-1 protease expression and purification*

High-level expression of HIV-1 PR was achieved in an adapted T7 RNA polymerase/promoter system [18] and will be described in detail elsewhere. Briefly, bacterially expressed enzyme was purified from intracellular inclusion bodies isolated from isopropylthiogalactoside-induced *E. coli* BL21 (DE3) cells [18] transformed with recombinant

plasmid pT7Q10H1EG (M. Andreansky, Ph.D. Thesis, 1990; manuscript in preparation). A 390 bp *Nde*I–*Eco*RI DNA fragment in the plasmid pT7Q10H1EG encodes mature HIV-1 protease (99 amino acid sequence corresponding to bru isolate); these are preceded by initiator methionine and an additional 20 upstream amino acids. The processing cleavage site contained in this artificial precursor is functional and allows autocatalytic processing, leading to liberation of a mature 99 amino acids-long protease molecule. Its carboxy-terminus is created by a translation termination codon in the construct.

2.2. *Synthesis of inhibitors*

Inhibitors containing a reduced peptide bond were synthesized as follows: corresponding tripeptides were synthesized in solution using *t*-butyloxycarbonyl protection for alpha-amino groups. Reduced bonds were introduced by reductive amination of appropriate N-protected or acylated alpha-aminoaldehydes in the presence of cyanoborohydride. N-protected aminoaldehydes were obtained by oxidation of corresponding N-protected aminoalcohols with periodinane. This method proved to yield optically pure protected amino aldehydes (details will be published elsewhere).

The *N*-methyl group in compound 14 (Table II) was introduced using paraformaldehyde and cyanoborohydride. The *N*-hydroxylamine moiety in compound 15 was introduced via oxidation with *m*-chloro-perbenzoic acid in dichloromethane. The synthesis of hydroxyethylene isoster 12 and other synthetic details will be published elsewhere. All products were purified on reversed phase HPLC (C-18 VYDAC column with 0.05% aqueous TFA-methanol as the mobile phase, UV detection at 220 nm) and characterized by FAB mass spectrometry, quantitative amino acid analysis and elemental analysis. All the inhibitors were readily soluble in DMSO. Typically, 2 mM stock solutions were prepared and used for inhibition measurements.

2.3. *Inhibition studies*

Inhibition constants of the inhibitors were determined using a spectrophotometer assay with chromogenic peptide substrate Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-NleNH_2 [14]. Typically, 5 pmole of HIV-1 PR was added to 1 ml of a 10 μM solution of the substrate and various concentration of an inhibitor in 0.1 M sodium acetate buffer, pH 4.7, with 0.3 M NaCl and 4 mM EDTA. The initial velocities (the depletion of the substrate less than 10%) were measured on a UV-VIS spectrophotometer Aminco DW 2000 as a decrease of absorbance at 305 nm [14,17] at 37°C and fitted to the Dixon analysis [19], i.e. $1/v$ vs. the concentration of I. The K_i values lower than 5 nM were assessed using the equation [20]:

$$[E]= ([E]_o+[I]_o+K_ia)/2 + \mathrm{sqrt}(\mathrm{sqrt}([E_o]+[I]_o+K_ia) - 4[E]_o*[I]_o/2$$

where the apparent equilibrium dissociation constant K_ia should be corrected into the true K_i as $K_i= K_ia/(1+[S]/K_m)$.

The HIV-1 PR concentration was evaluated by titrating with subnanomolar concentrations of inhibitor, quinoline-2-carbonyl-Asn-Phe-psi ($CHOH-CH_2N$)-decahydro-isoquinoline-carbonyl-NH-*t*-butyl [8], and independently with compound 19 or 20 (Table III). The titration of the HIV-1 PR with inhibitor 19 (shown in Fig. 1) was performed under the following conditions: 1–10 μl of 0.6 μM inhibitor Boc-Phe [CH_2-NH] Phe-Glu-Phe-OMe (compound 19) in DMSO were added to a cuvette with 3.3 nM solution of HIV-1 PR in 100 μl of 0.1 M acetate buffer, pH 4.7, with 0.3 M NaCl and 4 mM EDTA. The reaction was started by addition of 3 μl 5 mM solution on chromogenic substrate Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-Nle-NH_2 and followed on a spectrophotometer Aminco DW 2000 at 305 nm. Remaining enzyme activity was plotted against concentration of the inhibitor.

The cleavage of the peptide 21 was performed under similar conditions as described above. After 2 h incubation the reaction mixture was quenched by the addition of 20% TFA to a final concentration of 5% and applied on reversed phase HPLC. The peaks corresponding to the hydrolysis products were collected and the actual cleavage site determined by N-terminal sequencing and amino acid analysis.

3. RESULTS AND DISCUSSION

In this paper we have compared the series of inhibitors which map requirements in S2, S1 and S1′ enzyme binding subsites. We designed a series of N-protected tetrapeptides, based on an amino acid sequence of inhibitor 1 (Table I), which have a reduced peptide bond proximal to the N-terminus. Structure-activity data obtained on this set of inhibitors based on simple chemistry could be used for the design of potentially even more active compounds, e.g. hydroxyethylene- or hydroxyethyl- amino-isosteres.

The reduced bond is situated between two phenylalanine residues that we found to be favorable in P1 and P1′ positions [16,22]. This led to an inhibitor with a remarkably lower K_i value than found for any inhibitor based on the reduced bond analogue. Table I summarizes the influence of the nature of the protecting group on binding of an inhibitor to the S2 protease subsite. The bulky tert-butyl-oxy-carbonyl group fits to the S2 subsite better than the large hydrophobic benzyl-oxy-carbonyl or small acetyl group. Charged, unprotected N-terminus (compound 5), prolongation or extension of the amino acid chain at the N-terminus (compounds 6 and 7) sharply decreases binding and shifts the K_i value to the higher range. A representative of a conventional inhibitor derived from the sequence spanning the matrix–nucleocapsid (MA–CA) boundary of HIV-1 polyprotein is compound 8 (Table I). Micromolar IC_{50} or K_i values were reported also for other reduced-bond inhibitors derived from different processing sites in HIV-1 polyprotein [6,24]. The weaker binding indicates that the extended peptide structure of the inhibitor on the non-prime side orients the inhibitor differently and to some extent disturbs optimal binding of the rest of the inhibitor.

Inhibitors shown in Table II map the requirements for S1–S1′ subsites neighboring the reduced peptide bond. All the replacements (compounds 9, 10, 11, 13, 14) lead to lower inhibition activity of resulting inhibitors which correlates with similar findings of increasing of K_m values of HIV-1 PR peptide substrates when sim-

Table I

Reduced-bond inhibitors of HIV-1 protease with variations in the N-terminus

Number	Sequence			K_i (nM)
	P1		P1′ P2′ P3′	
1	Boc-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	23
2	Piv-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	36
3	Z-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	200
4	Ac-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	800
5	Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	11,000
6	Val-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	2,700
7	Boc-Val-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	750
8	Ser-Gln-Asn-Tyr	[CH_2–NH]	Pro-Ile-Val-Gln	9,000

All details are given in Materials and Methods.

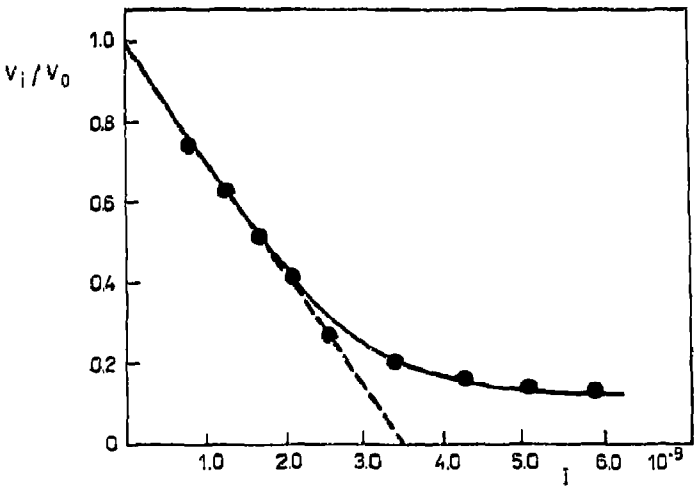


Fig. 1. Active-site titration of HIV-1 PR by inhibitor 19, Boc-Phe-[CH_2–NH]-Phe-Glu-Phe-OMe. For conditions see Materials and Methods.

ilar replacements in substrate analogues (Phe-Nle, Phe-Gly) were introduced [14,16]. The 50-fold drop in K_i from compound 11 to 12 documents particularly the important role of the nature of the P1-P1′ peptide bond mimetic and superiority of hydroxyisostere over the reduced bond in binding to protease active cylinder. Hydroxyisostere 12 clearly compensates for the disadvantage of the absence of the side chain in P1′ and restores essentially the inhibition potency of a prototype compound 1. Insertion of *N*-methylated peptide bond (compound 14), on the other hand, results in a 10-fold increase in K_i. The introduction of hydroxy-ethylidene isostere into compound 13 leads to fixed non-flexible conformation and decrease of inhibitory activity.

N-Hydroxylamine isostere (Scheme I), inhibitor 15 (Table II), is synthesized via oxidation of the reduced bond inhibitor 1. This peptide bond mimetic improves binding by a factor of 50, similar to the improvement observed upon insertion of hydroxyethylene isostere instead of a reduced peptide bond (compounds 11 and 12). This suggests that the hydroxyl group of hydroxylamine 15 binds between the two catalytical aspartates and excludes the water molecule from the protease active site [23].

Inhibitors shown in Table III were designed in order to investigate the nature of the residue occupying the P2′ and P3′ positions. The P2′ position was found to be particularly critical. Leu or Val (compound 16 and 17) had a minor effect on K_i, in good agreement with data published on synthetic peptide substrates [22]. Ala in the same position (compound 18) increases K_i of the resulting inhibitor by an order of magnitude.

Previously, we have reported the design of a sensitive chromogenic substrate of HIV-1 PR [14] exhibiting a 20-times lower K_m value than other substrates described

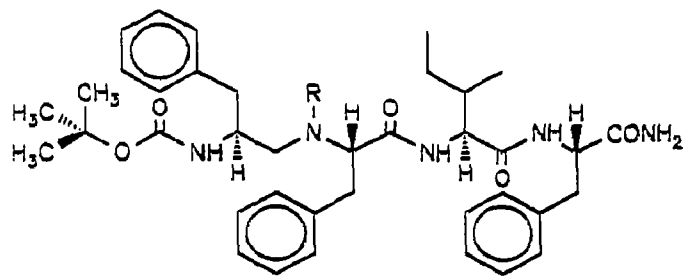
Table II

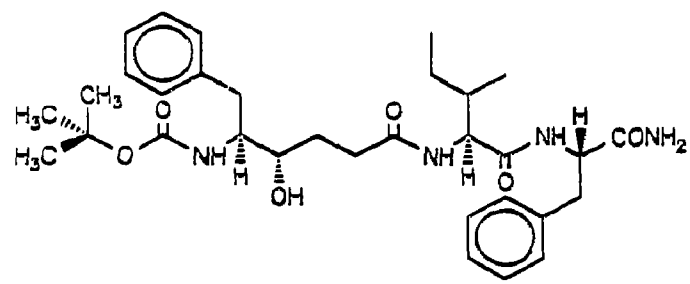
Reduced-bond inhibitors of HIV-1 protease with variation in P1′–P1′ positions

Number	Sequence			K_i (nM)
	P1		P1′ P2′ P3′	
1	Boc-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	23
9	Boc-Nle	[CH_2–NH]	Phe-Ile-Phe-OMe	110
10	Boc-Nle	[CH_2–NH]	Nle-Ile-Phe-OMe	450
11	Boc-Phe	[CH_2–NH]	Gly-Ile-Phe-OMe	1,650
12	Boc-Phe	[CH(OH)–CH2]	Gly-Ile-Phe-OMe	35
13	Boc-Phe	[CH(OH)–CH=]	Gly-Ile-Phe-OMe	5,000
14	Boc-Phe	[CH_2–N(Me)]	Phe-Ile-Phe-OMe	390
15	Boc-Phe	[CH_2N(OH)]	Phe-Ile-Phe-OMe	0.5

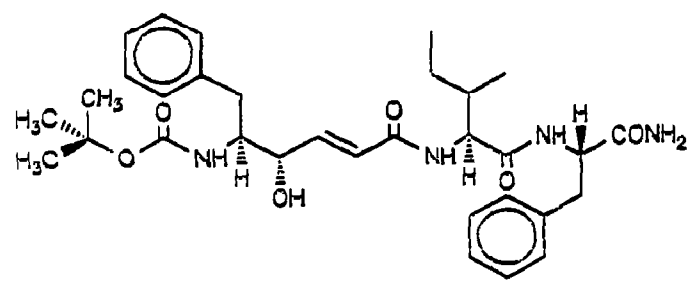
All the details are given in Materials and Methods.

Scheme 1.





INHIBITOR 12



INHIBITOR 13

so far. The particular feature of that substrate was a glutamic acid residue in the P2′ position. Insertion of the same residue into the P2′ position of a reduced-bond inhibitor (compound 19, Table III) dramatically decreased the K_i value, thus showing the ideal fit of the peptide chain into the active site binding pockets. Compound 19 is a tight-binding competitive inhibitor of HIV-1 protease and is suitable for its active site titration (Fig. 1). Removal of the negative charge by Glu-Gln replacement (compound 23) results in a 20-fold decrease in binding which is then further lowered by a change to the homological Asn residue (compound 24). A remarkable drop in the inhibitory activity can be seen in the case of Glu-Asp replacement when the negative charge of the P2′ side chain is preserved (compound 22). The increase of K_i by four orders of magnitude achieved by the removal of a single methylene in the side chain of an amino acid in the P2′ position shows high specificity of the interaction of Glu in P2′. Compounds 25 and 26 are analogues of the parent compound 1 having the Phe in P3′ replaced by Ala and Val, respectively. Both replacements lead to higher K_i values.

To confirm the ideal fit of the amino acid sequence of the titrating inhibitor 20 into the protease binding cleft we synthesized tetrapeptide 21 (Boc-Phe-Phe-Glu-Phe-NH_2, Table III), derived from inhibitor 20. The binding constant of this peptide, as expressed by the K_i value (110 nM), is two orders of magnitude lower than the K_m value of other peptide substrates designed so far [3]. On the other hand, the K_i of this compound is three orders of magnitude higher than that of inhibitor 20, which differs from it by only a single oxygen. The flexibility of C–N linkage, (achieved here by linearization of an sp3 tetraheder via reduction), is probably crucial for inhibitor action.

Prolonged digestion of peptide 21 resulted in a partial cleavage between both Phe residues. The correct cleavage site is an indirect proof of our assumption that the reduced bond or hydroxylamine isosteres of the 'truncated' inhibitors are located between the active site aspartate.

Table III

Reduced-bond inhibitors with variations in P2′ and P3′

No.	Sequence			K_i (nM)
	P1		P1′ P2′ P3′	
1	Boc-Phe	[CH_2–NH]	Phe-Ile-Phe-OMe	23
16	Boc-Phe	[CH_2–NH]	Phe-Leu-Phe-OMe	37
17	Boc-Phe	[CH_2–NH]	Phe-Val-Phe-OMe	35
18	Boc-Phe	[CH_2–NH]	Phe-Ala-Phe-OMe	320
19	Boc-Phe	[CH_2–NH]	Phe-Glu-Phe-OMe	0.2
20	Boc-Phe	[CH_2–NH]	Phe-Glu-Phe-NH_2	0.2
21	Boc-Phe	–	Phe-Glu-Phe-NH_2	110
22	Boc-Phe	[CH_2–NH]	Phe-Asp-Phe-NH_2	1,100
23	Boc-Phe	[CH_2–NH]	Phe-Gln-Phe-NH_2	4
24	Boc-Phe	[CH_2–NH]	Phe-Asn-Phe-NH_2	960
25	Boc-Phe	[CH_2–NH]	Phe-Ile-Ala-OMe	300
26	Boc-Phe	[CH_2–NH]	Phe-Ile-Val-OMe	340

All the details are given in Materials and Methods. Compound 21 is a peptide without the reduced bond. Amide 20 was included in the series to prove that more convenient and, potentially, more stable amides can be used instead of methyl esters.

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