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Thyroxine-derivatives of lipopeptides: bifunctional dimerization inhibitors of human immunodeficiency virus-1 protease

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Abstract

The structure of new lipopeptides targeting the enzymic dimer interface have been rationally improved resulting in dimerization inhibitors of the human immunodeficiency virus 1 protease (K_{id} = 5 nM for the best inhibitor). The contribution of each amino acid in inhibitory 3-mer lipopeptides was analyzed demonstrating that the C-terminal amino acid residue may preferably be replaced by thyroxine and thyronine. The negative charge of Glu is not essential. Lengthening of the peptidic chain may lead to a decrease of efficiency and a change in the mechanism (competitive inhibition instead of dimerization inhibition). The N-terminal blocking group can be replaced by 2-aminopalmitic acid. The mechanism of inhibition has been ascertained using Zhang's kinetic analysis combined with a physical method based on binding of 1-anilino-8-naphtalene sulfonate to enzyme. By targeting the hydrophobic pocket and the interface antiparallel β-sheet found relatively free of mutations in contrary to the active site, these efficient dimerization inhibitors may provide a way of overcoming the drug resistances observed with therapeutic antiproteases that bind to the active site.

Keywords: HIV-1 protease; Antiproteases; Lipopeptides; Dimerization inhibitors; Thyroxine; AIDS

1. Introduction

The use of active-site directed inhibitors of HIV-1 protease (PR) human immunodeficiency virus-1 protease in combination with reverse transcriptase inhibitors has confirmed PR as a target for antiviral therapy of proven benefit in individuals and communities with HIV infection and AIDS [1]. However, these drugs produce serious adverse reactions [2] and the emergence of PR inhibitor-resistant virus strains is a leading factor of treatment failure [3,4]. Cross-resistance to several antiproteases has been observed since they target the active-site pocket, an enzyme region highly likely to amino acid mutations [5]. PR is a homodimeric, aspartyl protease of which the monomers are inactive [6]. This interface region has been found to be relatively free of mutations [5]. Therefore, designing small molecules able to disrupt the dimer and/or bind to a protease monomer could constitute an alternative strategy to the conventional targeting of the active site and lead to potent inhibitors for PR mutants. The rationale behind the design of dimerization inhibitors is based on targeting of the antiparallel β-sheet formed by the interdigitation of the N-terminal (residues 1–4) and C-terminal (residues 96–99) β-strands of each monomer. This four stranded β-sheet is estimated to provide 75% of the energy of dimer stabilization [7]. N- and C-terminal mimetics of the monomer ends (also called 'interface peptides') [8–12] and interfacial peptides cross-linked with flexible alkyl tethers [13,14] or rigid spacers [15,16] have also led to dimerization inhibitors of PR.

It is interesting that lipopeptides displaying a large N-terminal aliphatic blocking group act as bifunctional dimerization inhibitors while the corresponding peptides without lipid group target the active site [10,11]. Thus, the

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Abbreviations: ANS, 1-anilino-8-naphtalene sulphonate; DABCYL, 4-(4-dimethylaminophenylazo)benzoic acid; EDANS, 5-(2-aminoethyl amino)-1-naphthalene sulfonic acid; HIV, human immunodeficiency virus-1.

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N°	Compounds	K_{id}[a](nM)	K_{ic}[a](nM)
1	Pam-Tyr-Glu-T(4)-OH	20	
2	Pam-(D)Tyr-Glu-T(0)-OH	80	
3	Pam-Tyr-Asn-T(0)-OH	50	
4	Apam(1)-Tyr-Glu-T(4)-OH	10	
5	Apam(2)-Tyr-Glu-T(4)-OH	5	
6	Pam-Thr-Tyr-Glu-T(0)-OH	90	300
		IC_{50}[b](nM)	K_{ic}[a](nM)
7	Pam-Thr-Val-Ser-Tyr-Glu-Leu-OH	500	
8	H-Thr-Val-Ser-Tyr-Glu-Leu-OH	1160	650
9	Pam-2(Nal)-Glu-Leu-OH	12500	
10	H-Tyr-Glu-Apam-OH	NI	

Fig. 1. Structure of peptide compounds and PR inhibition (inhibition constants at pH 4.7 and 30°). K_{id} and K_{ic} are the inhibition constants for dimerization and active-site inhibition, respectively. T(0), L-thyronine; T(4), thyroxine; Pam, palmitoyl; Apam, 2-aminopalmitoyl: Apam(1) and Apam(2) are enantiomers; (2-Nal), 2-naphtalenylalanine. [a]Standard errors on initial rates are less than 5%. [b]Standard errors are less than 15%.

palmitoyl group acts in a dual way, it improves the inhibitory potency and acts as an interface directing group. Some lipopeptidic compounds are able to abolish viral replication at concentrations with negligible toxicity [17]. In order to improve metabolic stability and bioavailability of short lipopeptides as antiproteases capable of circumventing drug resistance, their structural features have been further defined using D-amino acids and non-natural amino acids such as L-thyronine, L-thyroxine, naphthalenylalanine and 2-aminopalmitic acid (Fig. 1). The aim was to analyze the influence of modifications on the power and specificity of binding by variation of the short related peptide sequence and the attachment of the palmitoyl or 2-aminopalmitoyl group at the N- or C-terminal end. It was important to clearly establish the mechanism of inhibition since only dimerization inhibitors are highly likely to circumvent resistance observed with the active-site targeting pseudopeptides used in therapy. To do this, kinetic analyses according to Zhang *et al.* [9] of newly designed lipopeptides were combined with a direct structural investigation (ANS binding).

2. Experimental procedures

2.1. Materials

HIV-1 protease was produced by bacterial expression in *E. coli* [10,18]. Peptides were purchased from Biosyntan GmbH (Berlin, Germany). Their purities were 95% (**1**, **3**), 93% (**2**, **5**), 87% (**4**), >95% (**6**) and >70% for other compounds. Lipopeptides **4** and **5** are diastereoisomers. 1-Anilino-8-naphtalene sulphonate (ANS) was purchased from Sigma-Aldrich Chimie, and DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS and acetylpepstatin from Bachem-Biochimie. The fluorescence-based assays were performed using a LS 50B Perkin–Elmer luminescence spectrofluorometer equipped with a thermostated cell holder. Fluorescence of ANS were measured with a Jasco FP-777 spectrofluorometer. The spectral evolution were followed using an Uvikon 941 spectrophotometer.

2.2. Enzyme inhibition

Enzymatic assays and inhibition studies were performed as in Bouras [15] at pH 4.7 and 30° using 100 mM sodium acetate, 1 mM EDTA, 0.1 M NaCl. Inhibitors and the fluorogenic substrate DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS were dissolved in DMSO before being used (final concentration: 3% DMSO v/v). For the determination of the IC_{50} values, 0.52 μL of a 3 mM substrate solution (final concentration 5.2 μM) was added to 8.5 μL of 7–11 different concentrations of inhibitor (final volume 300 μL). The enzymatic reaction was initiated by adding of enzyme (pre-diluted in buffer containing 1 mg/mL BSA). The final enzyme concentration was 7.5 nM. The increase of fluorescence was followed for 3 min at 30°. A correction was made for the intrinsic fluorescence of compound **9**. IC_{50} values were calculated by fitting the experimental data to the equation: % inhibition = 100 $[I]_0/(IC_{50} + [I]_0)$. The Zhang's kinetic analyses were carried out at a constant initial substrate

concentration and using at least four concentrations of enzyme (4.7–23.6 nM) for two different concentrations of inhibitor. The experimental data were fitted to Eq. (1) corresponding to the minimal kinetic scheme of Eq. (2).

$$\frac{[E]_T}{\sqrt{v_i}} = \frac{K_m}{k_{cat}[S]_0}\sqrt{v_i} + \sqrt{\frac{K_m K_d}{4[S]_0 k_{cat}}}\left(1 + \frac{[I]_0}{K_{id}}\right) \quad (1)$$

$$M + MI \overset{K_{id}}{\rightleftarrows} I + 2M \overset{K_d}{\rightleftarrows} D + S \overset{K_m}{\rightleftarrows} DS \overset{k_{cat}}{\rightarrow} D + P \quad (2)$$

In each case, blanks without inhibitor were performed.

2.3. ANS binding

Emission spectra of ANS were measured using excitation and emission wavelengths of 370 and 470 nm, respectively, and excitation and emission bandwidths of 10 and 5 nm, respectively. PR was diluted into assay buffer (100 mM sodium acetate, 1 mM EDTA, 0.1 M NaCl, pH 4.7) and incubated at 25° with or without the inhibitor, acetylpepstatin (100 nM) or Pam-Tyr-Glu-Leu (400 nM), before regularly adding 2 μL ANS (final concentration, 10–40 μM). In all cases, the final DMSO concentration was 0.2% (v/v). Each fluorescence measurement was made twice and averaged. The fluorescence intensity was corrected from the fluorescence due to the same concentration of ANS without enzyme (experiment without inhibitor), and without enzyme and with inhibitor (experiment with inhibitor).

3. Results and discussion

The inhibitor potency of the molecules was checked at pH 4.7 and 30° using the fluorogenic substrate DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS. Except for compound **10**, all the tested molecules inhibited PR (Fig. 1). The mechanism of inhibition was identified for the efficient compounds **1–6** and **8** using Zhang's kinetic analysis [9]. Plots of $[E]_0/\sqrt{v_i}$ vs. $\sqrt{v_i}$ when the enzyme is incubated in the presence of various concentrations of molecules **1–5** gave straight lines with the same slope as without inhibitor (Fig. 2A and B). This is consistent with molecules acting as pure 'dissociative' inhibitors. Different patterns have been obtained with compounds **6** and **8**: non-parallel lines (**6**, data non shown) and lines with altered slopes and unaltered *y*-axis intercept (**8**, Fig. 2C) which were consistent with mixed-type inhibition and competitive inhibition, respectively. In the mixed inhibition process, the inhibitor may bind to the active site as well as the interfacial region whereas a competitive inhibitor interacts only with the active site.

The favorable effect of the large N-terminal palmitoyl blocking group on the inhibition process is confirmed (compound **7** vs. **8**). It was previously reported that the K_{id} inhibition constant was decreased about 200-fold by

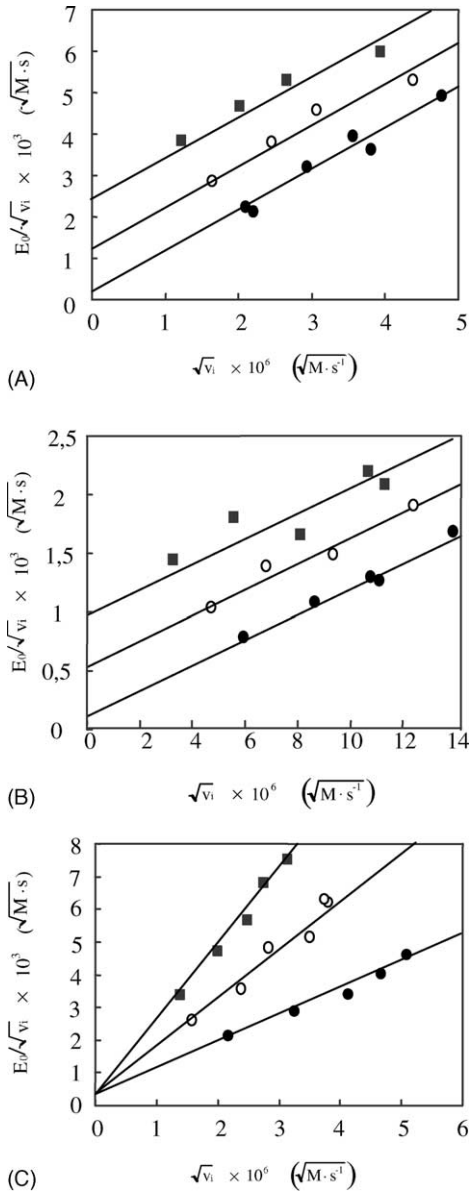
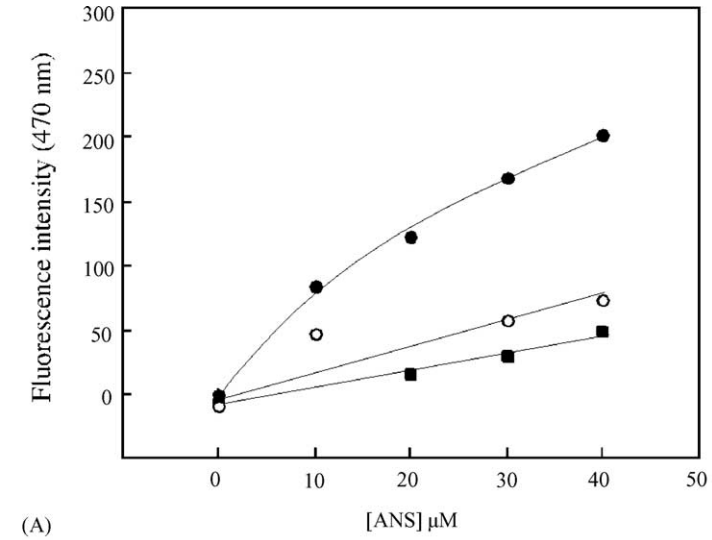


Fig. 2. Zhang plots for the inhibition of PR by compounds **1** (A), **5** (B) and **8** (C) at pH 4.7 and 30°. Protease activity was measured at different concentrations of inhibitor: 133 nM (○), 400 nM (■) for **1**; 11 nM (○), 33 nM (■) for **5**; 500 nM (○), 1500 nM (■) for **8**. In all cases, activity measurements were also made in the absence of inhibitor (●). The straight lines are curves calculated by regression fit to the data.

adding the palmitoyl group to the amino terminus of the peptide H-Tyr-Glu-Leu-OH [10]. The 2-aminopalmitoyl group may also be used instead of the palmitoyl group (compounds **4** and **5** vs. compound **1**) favoring by factors of 2–4 the inhibitory potency. Because of the insolubility of sample **10** at pH 4.7, no conclusion can be drawn about the attachment of the large aliphatic blocking group (2-aminopalmitoyl, 2-Apam) at the C-terminal position 99—as H-Tyr-Glu-(2-Apam)-OH—and the possible sterical fit of this long side-chain. The peptide length seems to be essential for dissociative inhibitors. Tripeptide derivatives (**1–5**) behave as strict dimerization inhibitors whereas the tetramer **6** is a mixed inhibitor interacting with both sites,



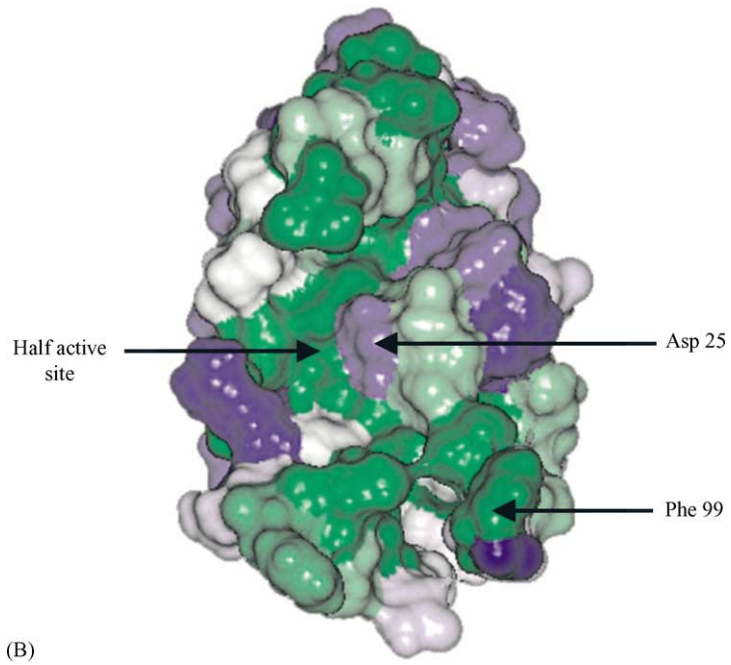


Fig. 3. (A) ANS emission fluorescence measured at pH 4.7 and 25°. The excitation wavelength was 370 nm. PR (350 nM) was pre-incubated in the absence of inhibitor (○) or in the presence of acetylpepstatin (■, 100 nM) or Pam-Tyr-Glu-Leu-OH (●, 400 nM), prior to the addition of ANS. In all cases, the final DMSO concentration was 0.2% (v/v). The fluorescence intensity was corrected from the fluorescence due to the same concentration of ANS without enzyme (○), and without enzyme plus inhibitor (■ and ●). (B) Schematic representation of the interface region of the monomer showing the half active site, the catalytic residue Asp25 and the C-terminal residue Phe99. Conolly surfaces were computed using the INSIGHT II software (Biosym/Molecular Simulations Inc., San Diego, version 98.0). The PR structure was the X-ray structure solved by Jaskolski *et al.* [26]; the complexed inhibitor was removed and the hydrogen atoms added. The hydrophilic surfaces are colored in purple and the hydrophobic ones in green.

the active site and the dimer interface. Nevertheless, some tetramers have previously been reported to be as effective dimerization inhibitors as some trimers [10]. The longer lipopeptidic molecule **8** (a hexamer peptide) is a competitive inhibitor. With compounds presenting a lipid-blocked core trimer, the influence of the nature of the C-terminal amino acid was further explored by introducing biphenyl derivatives, thyronine T(0) and thyroxine (T4). Interestingly, the bulkier thyroxine group is quite as favorable as thyronine (compound **1** vs. Pam-Tyr-Glu-T(0)-OH, [11]) demonstrating that the pocket accommodating the C-terminal residue is large and hydrophobic. The best inhibitors **4** and **5** display thyroxine at the C-terminus and the D,L-2-aminoplamitoyl residue at the N-terminus. Substitutions of Tyr in Pam-Tyr-Glu-T(0)-OH by D-Tyr (**2**) is not unfavorable, neither is the suppression of the negative charge of Glu (mutation to Asn in **3**). Both changes preserve the mechanism of inhibition with only moderate increase of the inhibition constant K_{id}. The importance of the anchoring Tyr, interacting within the binding pocket (normally

occupied by the isobutyl side chain of Leu 97), was explored by introducing the large and hydrophobic amino acid 2-naphthalenylalanine (**9**); however the peptide **9** is 83-fold less potent than Pam-Tyr-Glu-Leu-OH (IC_{50} = 150 nM [10]). The tyrosine residue with its ability to allow hydrogen bonding is obviously preferred.

Several new results confirm the basic approach of dimerization inhibition. The genetic selection for dissociative inhibitors of protein–protein interactions has produced peptides which are able to dissociate PR dimers [19]; these inhibitors produce monomeric PR complexes identified by analytical gel chromatography confirming Zhang's kinetics. Monomers were also found by analytical ultracentrifugation in the presence of H-Thr-Leu-Asn-Phe-OH [9]. The intravirion display of the C-terminal segment of PR as an extension to the viral protein Vpr (Vpr-(spacer)-Thr-Leu-Asn-Phe-OH) attenuates HIV-1 replication confirming the activity of the interface peptides as PR inhibitors within the cell [20]. An alternative but related method of inhibition is to produce dominant negative forms of PR [21]. Targeting of the dimerization interface for irreversible inhibition (using affinity labeling by a peptide of the interface residue Cys 95) is a confirmatory result defining the expected structural target [14]. It can further be shown by calculations that interface targeting is possible with a nM (or even pM) K_d of the protein complex since the small-molecule concentration is relatively high and the concentration of the protein very low [22]. These findings demonstrate the solidity of the approach and the growing acceptance of the Zhang–Poorman test for dissociative inhibition [23].

To further probe the mechanism of inhibition by lipopeptides, the fluorescence emission for the binding of the fluorescent probe ANS to PR was measured in the presence of acetylpepstatin, an active-site inhibitor [24] and Pam-Tyr-Glu-Leu-OH identified as dimerization inhibitor [11]. Fluorescence intensity at 470 nm is enhanced in the presence of a fixed Pam-Tyr-Glu-Leu-OH concentration and various ANS concentrations when compared with that observed in absence of inhibitor (Fig. 3A). Conversely, a decrease of fluorescence intensity is observed in the presence of the active site inhibitor acetylpepstatin. It is known that ANS exhibits an overall enhancement of fluorescence intensity upon binding to hydrophobic surfaces [25]. Upon binding to the active site of PR, inhibitors induce a noticeable conformational change [6] with closing of the hydrophobic active site by flap movements. Conversely, in the presence of the inhibitor Pam-Tyr-Glu-Leu-OH identified as 'dimerization inhibitor' using Zhang's plot, more hydrophobic surfaces are found exposed. This is in agreement with a sequestration of the monomer by the inhibitor or disruption of the dimer interface preventing the correct assembly of the inactive monomers to active enzyme. In both cases, the interface region which displays large hydrophobic pockets (Fig. 3B) may be more accessible to ANS.

4. Conclusions

Lipopeptides are the best of known dimerization inhibitors when compared to cross-linked interface peptides (K_{id} = 220 nM [12,13], 780 nM [19]) and conformationally constrained hairpins (K_{id} = 560 nM [15], 5400 nM [16]). This study shows that: (i) the further amino acid elongation of the Pam-tripeptides is not necessary to enhance dimerization inhibitory efficiency; (ii) the binding pocket for the third C-terminal amino acid in 3-mer lipopeptides is hydrophobic and large enough to accommodate a variety of groups, confirming computer design [10,27]. Since the mutation-prone PR parts (active site, flap regions), are not involved in binding, these antidimers may lead to valuable anti-HIV drugs active also against therapy mutants. Their small size is already near to the (''rule of 5'') desired M_r value of about 500 [28].

The principle of creating potent inhibitors by adding non-specific lipidic re-enforcement groups to specificity conveying structural parts may also be applied to other composite proteins, e.g. to multienzyme complexes. The necessary steps are: identification of weakly binding peptides, transformation into 'modified peptides' or peptidomimetics with improved stability and bioavailability and further unspecific attachment of lipid (or other) residues as auxiliary groups—additional 'glue'—for binding. The application of this principle can be found in many bioactive natural compounds.

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