Structure–activity studies with high-affinity inhibitors of pyroglutamyl-peptidase II

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Inhibitors of PPII (pyroglutamyl-peptidase II) (EC 3.4.19.6) have potential applications as investigative and therapeutic agents. The rational design of inhibitors is hindered, however, by the lack of an experimental structure for PPII. Previous studies have demonstrated that replacement of histidine in TRH (thyrotropin-releasing hormone) with asparagine produces a competitive PPII inhibitor (K_i 17.5 μ M). To gain further insight into which functional groups are significant for inhibitory activity, we investigated the effects on inhibition of structural modifications to Glp-Asn-ProNH₂ (pyroglutamyl-asparaginyl-prolineamide). Synthesis and kinetic analysis of a diverse series of carboxamide and C-terminally extended Glp-Asn-ProNH₂ analogues were undertaken. Extensive quantitative structure–activity relationships were generated, which indicated that key functionalities in the basic molecular structure of the inhibitors combine in a unique way to cause PPII inhibition. Data from kinetic and molecular modelling studies suggest that hydrogen bonding between the asparagine side chain and PPII may provide a basis for the inhibitory properties of the asparagine-containing

peptides. Prolineamide appeared to be important for interaction with the S₂' subsite, but some modifications were tolerated. Extension of Glp-Asn-ProNH₂ with hydrophobic amino acids at the C-terminus led to a novel set of PPII inhibitors active *in vitro* at nanomolar concentrations. Such inhibitors were shown to enhance recovery of TRH released from rat brain slices. Glp-Asn-Pro-Tyr-Trp-Trp-7-amido-4-methylcoumarin displayed a K_i of 1 nM, making it the most potent competitive PPII inhibitor described to date. PPII inhibitors with this level of potency should find application in exploring the biological functions of TRH and PPII, and potentially provide a basis for development of novel therapeutics.

Key words: pyroglutamyl-peptidase II (PPII), pyroglutamyl-peptidase II inhibitor, thyrotropin-releasing hormone (TRH), thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE), thyrotropin-releasing hormone-degrading ectoenzyme inhibitor.

INTRODUCTION

Enzymic degradation plays an important role in the regulation of biologically active peptides [1]. Extracellularly oriented, membrane-associated peptidases (ectopeptidases), in particular, are strategically located to terminate peptide-mediated cell signalling [2,3]. It is apparent that a relatively small number of ectopeptidases with broad specificities provide a key mechanism for controlling the levels of a large number of peptides [4,5].

PPII (pyroglutamyl-peptidase II) (EC 3.4.19.6), also known as TRH (thyrotropin-releasing hormone)-degrading ectoenzyme, is a highly unusual ectopeptidase in that it appears to display absolute functional specificity for its substrate, TRH [or Glp-His-ProNH₂ (pyroglutamyl-histidyl-prolineamide)] [6–9]. Thus PPII catalyses the removal of Glp (pyroglutamic acid) from TRH, but it does not appear to be responsible for the inactivation of other naturally occurring peptides that contain a Glp residue at the N-terminus [7,8,10]. The exclusivity of the relationship between PPII and TRH is emphasized further by the observation that extracellular TRH seems to be degraded only by PPII [7,8,10].

Selective inhibition of ectopeptidases as a means to modulate peptide levels has proved to be useful for investigating the biological functions of many peptidergic systems [11,12]. In addition, inhibitors of a number of such enzymes have found significant medical application. Notable among these are inhibitors of angiotensin-converting enzyme, which are now widely employed to treat hypertension [13]. The actions of TRH in the CNS (central nervous system) are recognized to be potentially beneficial in the treatment of CNS disorders, including CNS trauma, spinocerebellar degeneration, epilepsy, memory loss, and disorders of consciousness and mood [14–24]. It is acknowledged, however, that the rapid inactivation of TRH by PPII critically impedes both research into the functions of TRH within the CNS and its clinical use [17–19]. Thus PPII inhibitors, like those of other ectoenzymes, could have valuable application as both experimental tools and therapeutic agents.

To date, there is no experimental structure for PPII on which to base the rational design of active-site-directed inhibitors. Sequence alignment indicates that PPII is a member of the M1 family of zinc metallopeptidases [25], which includes LTA₄H

Abbreviations used: AMC, 7-amino-4-methylcoumarin; APA, aminopeptidase A; APN, aminopeptidase N; Bzl, benzyl; CNS, central nervous system; Dab, α , γ -diaminobutanoic acid; DCC, 1,3-dicyclohexylcarbodi-imide; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DPP-IV, dipeptidyl peptidase IV; eq., equivalent(s); Fmoc, fluorenylmethoxycarbonyl; Glp, pyroglutamic acid; Glp-Asn-ProNH₂, pyroglutamyl-asparaginyl-prolineamide; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; LHRH, luteinizing-hormone-releasing hormone; LTA₄H, leukotriene A₄ hydrolase; MBHA, 4-methylbenzylhydrazine; Me, methyl; MOE, Molecular Operating Environment; PDB, Protein Data Bank; PPII, pyroglutamyl-peptidase II; SAR, structure–activity relationship; TFA, trifluoroacetic acid; TRH, thyrotropin-releasing hormone; TRH-AMC, pyroglutamyl-histidyl-prolyl-7-amido-4-methylcoumarin; Trt, trityl; TFA, trifluoroacetic acid.

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(leukotriene A₄ hydrolase) (EC 3.3.2.6), APA (aminopeptidase A) (EC 3.4.11.7) and APN (aminopeptidase N) (EC 3.4.11.2). By analogy with other metalloproteases, a hypothetical model of the active site of PPII has been derived from mutational analysis [26], but this provides insufficient detail for directing inhibitor design. The M1 family displays most sequence conservation in the region of the zinc-binding motif, and a model of the zinc-binding domain of APA has been constructed based on the crystal structure of LTA₄H [27].

Few PPII inhibitors have been identified [8,28]. Until recently, the most potent of these was CPHNA (*N*-[1-carboxy-2-phenylethyl]-*N*-imidazole benzyl histidyl- β -naphthylamide) with a K_i of 8 μ M [28,29]. Through a kinetic study of PPII specificity, using a directed peptide library based on the structure of the endogenous ligand, TRH, we previously discovered two novel competitive PPII inhibitors: Glp-Asn-ProNH₂ (K_i = 17.5 μ M) and Glp-Asn-Pro-AMC (where AMC is 7-amido-4-methylcoumarin) (K_i = 0.97 μ M) [9]. Glp-Asn-Pro-AMC was the most potent PPII inhibitor then identified. Subsequent pilot studies indicated that well-defined central effects of TRH in rat could be enhanced by both intracerebroventricular and intraperitoneal administration of these inhibitors [30]. A recent paper has described the isolation of a PPII inhibitory activity from a marine invertebrate [31]. However, the type of inhibition has not been defined, its molecular structure is not yet known, and it is not certain that inhibition is due to a single chemical entity [31].

Our earlier study [9] showed that Glp-Asn-ProNH₂ was the only member of the tripeptide library with the structure Glp-Xaa-ProNH₂ to be resistant to hydrolysis and to bind to PPII with greater affinity than TRH. Furthermore, our results indicated that it might be possible to improve inhibitor potency by optimizing binding to what appeared to be a hydrophobic pocket in the area of PPII to which the C-terminus of PPII inhibitors bind, as seen by the lower K_i value for Glp-Asn-Pro-AMC as compared with that of Glp-Asn-ProNH₂. Although the hydrolytic activity of PPII appears to be restricted to tripeptides, tripeptide amides and tetrapeptides [9,32–35], the existence of an extended ligand-binding region close to the molecular recognition site for small peptides may also be suggested by the fact that LHRH (luteinizing-hormone-releasing hormone) binds to the enzyme with slightly higher affinity than does TRH [7,35]. TRH and LHRH share the same N-terminal dipeptide sequence, but LHRH is a decapeptide with the primary structure Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH₂ and is not hydrolysed by PPII [7,32,35].

To probe further the binding capacity offered by the region in the vicinity of the S₂' binding site of PPII, we determined quantitative SARs (structure–activity relationships) for a series of structurally diverse carboxamide and C-terminally extended analogues of Glp-Asn-ProNH₂ by means of kinetic assays using PPII purified from porcine brain. Significantly, we have found that substantial improvement in potency can be achieved through C-terminal extension of Glp-Asn-ProNH₂. This has led to the development of a uniquely effective set of novel, competitive PPII inhibitors that are active at nanomolar levels, the best of which has a K_i value of 1 nM, thus making it the most potent PPII inhibitor to date. Furthermore, we explored the relative importance of particular functional groups within the structure of Glp-Asn-ProNH₂ for binding to PPII and inhibiting its activity. A putative binding mode for the inhibitors was also generated using a homology model for PPII based on LTA₄H. Data from these studies provide new insights into the SARs for molecular recognition and inhibition of PPII, as well as critical information for subsequent design of site-directed PPII inhibitors and possible drug development. Moreover, results of the present study show

that such inhibitors of PPII enhance the recovery of TRH released from rat brain slices.

EXPERIMENTAL

Materials

All reagents were of analytical grade and were purchased from Sigma–Aldrich Chemical Company (Dublin, Ireland) unless stated otherwise.

Peptide synthesis and analysis

Peptides were synthesized using solution- and/or solid-phase methods and were purified, analysed and judged to be homogeneous by HPLC. HPLC analyses were conducted using a Thermo Separation Products Spectra System HPLC. In each case, products obtained by precipitation with diethyl ether, as described below, were purified using a semi-preparative C₁₈ reverse-phase HPLC column (μ Bondapak; Waters, Milford, MA, U.S.A.) and a linear gradient of 0–70% B at a flow rate of 2.5 ml · min^{−1} [solvent A = 0.08% TFA (trifluoroacetic acid) in water; solvent B = 40% acetonitrile in 0.08% TFA]. Peptide purity was confirmed by analytical HPLC analysis as described previously [9] and by MS.

Standard solid-phase Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry was employed using the bubbler system under nitrogen gas [36]. Rink amide MBHA (4-methylbenzylhydramine) resin was used for the synthesis of peptide amide sequences, such as Glp-Asn-AlaNH₂ and Glp-Asn-HomoProNH₂. H-Pro-2-Cl Trt (where Trt is trityl) resin was used for the synthesis of peptides with a C-terminal carboxylic acid. These resins, as well as Fmoc amino acid derivatives and Glp, were purchased from Novabiochem (Merck Biosciences, Poole, Dorset, U.K.). All amino acids were in the L configuration. Tri-functional amino acids were purchased with side-chain-protecting groups as follows: Fmoc-Asp(OtBu)OH, Fmoc-Asn(Trt)OH, Fmoc-Dab(Boc)OH (where OtBu is t-butylester, Dab is α,γ -diaminobutanoic acid and Boc is *N*- α -t-butyloxycarbonyl).

Synthesis of peptide amides was carried out using Rink amide MBHA resin (loading capacity: 0.73 mmol · g^{−1}). This was swollen using DMF (*N,N*-dimethylformamide), deprotected with 20% piperidine in DMF for 30 min and then washed with DMF. Each amino acid [3 eq. (equivalents), i.e. 3-fold excess over the resin-loading capacity] was coupled to the resin with HBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]/HOBt (*N*-hydroxybenzotriazole)/DIPEA (*N,N*-diisopropylethylamine) (3:3:6, eq.) for 1 h. Removal of Fmoc was achieved with 20% piperidine in DMF. On completion of peptide assembly, the resin was washed with DCM (dichloromethane), followed by methanol, and was allowed to dry overnight. The sequence was cleaved from the resin and was deprotected by stirring the dry resin in a TFA solution (95%) containing water (2.5%) and tri-isopropylsilane (2.5%) (10 ml · g^{−1} dry resin) (by vol.) at room temperature (25°C) for 2 h. The reaction mixture was filtered under vacuum, and the solvent was evaporated under reduced pressure. The residue was washed with petroleum ether and precipitated with diethyl ether.

Carboxamides of Glp-Asp-ProNH₂ were prepared by standard solution synthesis, coupling Glp-Asp-ProNH₂ (PolyPeptide Laboratories GmbH, Wolfenbuettel, Germany) to a series of amines: methylamine, dimethylamine, benzylamine, aniline, hydroxylamine and *O*-benzylhydroxylamine. HOBt (1.2 eq.) and DCC (1,3-dicyclohexylcarbodi-imide) (2.0 eq.) were added to a stirring solution of Glp-Asp-ProNH₂ (0.15 mmol) in DMF (560 μ l, 0.26 M), followed by the required amine (1.2 eq.). The reaction mixture was stirred at room temperature for 24 h. The

precipitate was filtered and washed with DCM. The solvent was evaporated under reduced pressure, and the residue was washed with petroleum ether and diethyl ether.

Carboxamides of Glp-Asn-Pro (American Peptide Company, Sunnyvale, CA, U.S.A.) were prepared by standard solution-phase coupling to the appropriate amine. All amines were purchased from Sigma–Aldrich, except for 1,3-benzodioxal-5-amine, 3-(trifluoromethyl)aniline, and 6-amino-1,3-dihydroisobenzofuran-1-one, which were supplied by Maybridge (Tintagel, Cornwall, U.K.). To a stirring solution of Glp-Asn-ProOH (0.15 mmol) in DMF (560 μ l, 0.26 M), HOBt (1.0 eq.) and DCC (1.0 eq.) were added, followed by the required amine (1.2 eq.). The reaction mixture was stirred at room temperature for 24 h and filtered, and the solvent was evaporated under reduced pressure. The residue was washed successively with small volumes of ethyl acetate, petroleum ether and diethyl ether.

Benzyl and methyl esters of Glp-Asn-Pro were prepared by first synthesizing the tripeptide acid on solid phase using H-Pro-2-Cl Trt resin (loading capacity: 0.7 mmol $\cdot$ g $^{-1}$). This was swollen using DCM and washed with DMF. Each amino acid (3 eq.) was coupled on to the resin with HBTU/DIPEA (3:6 eq.) at each step. The reaction time for each step was 1 h. Deprotection, cleavage from the resin and precipitation of the product were carried out as described above. To a stirring solution of Glp-Asn-ProOH (0.1 mmol) in DMF (560 μ l, 0.5 M), DMAP (4-dimethylaminopyridine) (30 %) and DCC (1.2 eq.) were added, followed by benzyl alcohol (1.2 eq.). The reaction mixture was stirred at room temperature for 16 h. The precipitate was filtered and washed with DCM, and the solvent was evaporated under reduced pressure. The residue was washed with petroleum ether and diethyl ether. The methyl ester was prepared similarly, except that methanol (400 μ l) was used as both solvent and reagent.

Glp-Asn-ProNH $_2$, Glp-Asn-Pro-TyrNH $_2$, Glp-Asn-Pro-Trp-Ser-TyrNH $_2$, Glp-Asn-Pro-Trp-TyrNH $_2$, Glp-Asn-Pro-TrpNH $_2$, Glp-Asn-Pro-Tyr-TrpNH $_2$, Glp-Asn-Pro-Tyr-Trp-TrpNH $_2$, Glp-Asn-Pro-AMC, Glp-Asn-Pro-Trp-AMC, Glp-Asn-Pro-Trp-Trp-AMC, Glp-Asn-Pro-Tyr-Trp-AMC and Glp-Asn-Pro-Tyr-Trp-Trp-AMC were custom synthesized either by the American Peptide Company or by PolyPeptide Laboratories GmbH. Glp- β Asn-ProNH $_2$ and Glp-Asn-GlyNH $_2$ were supplied by PolyPeptide Laboratories GmbH. TRH and TRH-AMC were purchased from Sigma–Aldrich and Bachem U.K., St. Helens, U.K. respectively. The homogeneity and identity of each peptide was confirmed by HPLC and mass spectral analysis. All peptides were stored at -20°C .

Enzyme assays

All enzyme assays were carried out at 37°C in 20 mM potassium phosphate buffer, pH 7.5. One kat of enzyme activity was defined as that amount of enzyme that catalyses the conversion of 1 mol of substrate per s. As described previously, PPII activity was measured by HPLC and fluorometric assays using enzyme purified from porcine brain [9,37]. The PPII preparation had a protein concentration of 0.8 mg $\cdot$ ml $^{-1}$ and a specific activity of 2.8×10^{-3} μ kat $\cdot$ mg $^{-1}$ with TRH-AMC (5 μ M) as substrate, while the DPP-IV (dipeptidyl peptidase IV) (EC 3.4.14.5) preparation had a protein concentration of 7.7 mg $\cdot$ ml $^{-1}$ and a specific activity of 0.29 μ kat $\cdot$ mg $^{-1}$ with Gly-Pro-AMC (100 μ M) as substrate [9]. The final concentration of PPII in the assay was 0.53 μ g $\cdot$ ml $^{-1}$. Compounds were judged to be resistant to hydrolysis by PPII if no Glp was detected in the HPLC assay following the incubation of 1 mM compound for 18 h with PPII (0.08 μ g) in a total assay volume of 1 ml. Under these conditions, TRH (1 mM) was completely degraded to yield Glp and His-Pro

Figure 1 Inhibition of PPII hydrolysis of TRH-AMC by Glp-Asn-Pro-Tyr-Trp-AMC

Initial rates were determined using the continuous fluorometric assay. Initial rates against substrate concentration for increasing inhibitor concentration were fitted to a rectangular hyperbola (Michaelis–Menten plot) by non-linear regression analysis as shown in the upper graph. All kinetic parameters stated in the text were determined by non-linear regression analysis of data using the computer program Prism (GraphPad Software). Linear regression analysis was used to fit data to a Lineweaver–Burk plot to illustrate the type of inhibition observed. The results shown for Glp-Asn-Pro-Tyr-Trp-Trp-AMC in the lower graph are typical for all compounds with the sequence Glp-Asn-Pro-Xaa, thus far tested. The goodness of fit (r^2) for both non-linear and linear regression analysis plots was > 0.95 . All $V_{\max}$ values fell within the confidence intervals of the intercept obtained from linear regression analysis, and the slope of a plot of $V_{\max}$ versus inhibitor concentration (not shown) was not found to deviate significantly from 0, supporting the interpretation that Glp-Asn-Pro-Tyr-Trp-Trp-AMC is acting as a classical competitive inhibitor of PPII.

diketopiperazine. Kinetic parameters for compounds that were not hydrolysed by PPII were determined using a continuous coupled fluorometric assay, whereas those for PPII substrates were determined using a discontinuous fluorometric assay [9,37]. All the sequences Glp-Asn-Pro-Xaa tested, thus far, behaved as linear competitive inhibitors as indicated by the constancy of $V_{\max}$ with changing inhibitor concentration (see [9] and Figure 1). Inhibitory potency of all compounds was therefore calculated by using an appropriate equation i.e. $v_i/v_o = [I]/([I] + K_i(1 + [S]/K_m))$, where v_i and v_o are the initial rates measured in the presence and absence of inhibitor respectively, K_m is the Michaelis constant, K_i is the inhibitor constant, and $[I]$ and $[S]$ are the inhibitor and substrate concentrations respectively. Initial rates for the hydrolysis of TRH-AMC (5 μ M) by PPII were determined in triplicate in the presence and absence of each compound, as described previously [9]. Compounds selected as being of high potency were studied in more detail by non-linear regression analysis of initial-rate data collected in duplicate at five different substrate concentrations and four different concentrations of peptide. The reversibility and time-dependence of inhibition produced by Glp-Asn-Pro-Tyr-Trp-Trp-AMC was examined as described previously [9].

Compounds identified as PPII inhibitors were tested for their ability to inhibit the activity of DPP-IV, the coupling enzyme in the continuous fluorometric assay for PPII. DPP-IV activity was determined directly using a continuous assay employing Gly-Pro-AMC as the substrate, as described previously [9]. None

of these peptides were found to inhibit DPP-IV activity in this assay, confirming that reduction of AMC production in the continuous coupled assay for PPII was due to inhibition of PPII.

Evaluation of PPII inhibitors in brain slices

Glp-Asn-Pro-AMC and Glp-Asn-Pro-Tyr-Trp-Trp-AMC were tested for their ability to protect endogenous TRH from degradation in hypothalamic slices. Hypothalami were rapidly dissected from male Wistar rats (200–250 g), following decapitation, and slices (300 $\mu\text{m} \times 300 \mu\text{m}$) were cut with a McIlwain tissue chopper. Each incubation contained slices from the hypothalamus of one animal. Slices were initially incubated in a flat-bottomed plastic tube in 0.5 ml Ca^{2+} -free Krebs buffer gassed with 95 % $\text{O}_2/5\%$ CO_2 for 10 min in a shaking water bath at 37 °C. In turn, the Ca^{2+} -free buffer was replaced by Krebs buffer containing Ca^{2+} (2.5 mM), and the incubation was continued for a further 10 min. The tubes were then centrifuged at 2000 *g* for 10 min. The resulting supernatant was removed and taken to denote 'basal' release. Subsequently, the tissue was incubated at 37 °C for 10 min in buffer containing Ca^{2+} (2.5 mM) and KCl (50 mM), and the supernatant arising from this incubation was taken to represent 'stimulated' release. PPII inhibitor at a final concentration of 0.1 mM or vehicle (1 μl saline or DMSO) was present throughout the last two incubations. Supernatant samples were frozen at –80 °C until analysis for TRH content by RIA [38].

Modelling of PPII

The lack of availability of a three-dimensional structure for PPII led us to construct a theoretical homology model of its active site in an attempt to gain further insight into the possible binding modes and mechanism of the inhibitors. PPII shares greatest sequence homology with APA and APN, but there is no crystal structure for either of these enzymes. The crystal structure of LTA_4H , however, has been elucidated [39]. This metalloprotease exhibits approx. 30 % overall sequence identity with residues 130–753 in PPII. The HEXXH zinc-binding motif and active site of these two enzymes are highly conserved, with 51 % sequence identity in the region across residues 436–470 of PPII. The crystal structure of human LTA_4H [PDB (Protein Data Bank) code 1HS6; <http://www.rcsb.org>] [39] was thus used as a template to construct a homology model of human PPII.

The LTA_4H crystal structure 1HS6 was downloaded from the PDB and was read into MOE (Molecular Operating Environment) (Chemical Computing Group), and the sequence was extracted. Subsequently, the 1024-amino-acid sequence for human PPII was read into MOE, and the sequences were aligned using the alignment tools of the homology module. The resulting automated sequence alignment was checked to ensure correct alignment of catalytic core histidine residues within the HEXXH zinc-binding motif, and pre- and post-template non-matched outgap residues were deleted to facilitate construction of a structural model for 624 residues of the PPII query. Co-ordinates were assigned using those residues conserved between both sequences, with 1HS6 serving as a structural template for the procedure. Fine-energy minimization [RMSD (root mean square deviation) 0.0005 Å (1 Å = 0.1 nm)] using the MOE implementation of the AMBER94 force field [40] was utilized for model generation and structure optimization. All zinc-binding residues were constrained during model construction and during the minimization protocol. A total of 300 intermediate models were generated and scored, and the highest scoring final solution (based on packing scores) was utilized in subsequent investigations. Hydrogens were added, assuming a pH of 7.4 and standard amino acid pK_a values. Structures were written out in mol2 format and read into Sybyl

Table 1 Effects of modifying the amide nitrogen of the asparaginyl side chain and the proline residue in Glp-Asn-ProNH₂ on the interactions with PPII

The ability of each compound to be hydrolysed by PPII was assessed using HPLC. Initial rates were measured in triplicate in the presence (v_i) and absence (v_o) of inhibitor (I) using the continuous fluorometric assay with TRH-AMC (5 μM) as substrate (S). K_i values were calculated from initial rates using the relationship $v_i/v_o = [I]/([I] + K_i(1 + [S]/K_m))$. Each value represents the mean $\pm$ S.D. [$n=3$, except when indicated by an asterisk (*), where $n=11$].

Y	Z	W	K_i (μM)	Hydrolysed
H	H	ProNH ₂	$16.1 \pm 1.6^*$	—
H	H	HomoProNH ₂	19.3 ± 0.8	—
Benzyl	H	ProNH ₂	99.6 ± 8.0	+
Hydroxyl	H	ProNH ₂	115 ± 11	—
Phenyl	H	ProNH ₂	133 ± 20	—
Methyl	H	ProNH ₂	103 ± 13	+
Benzoyloxy	H	ProNH ₂	> 200	—
Methyl	Methyl	ProNH ₂	> 200	—
H	H	AlaNH ₂	> 200	—
H	H	GlyNH ₂	No inhibition	—
H	H	AsnNH ₂	No inhibition	—
H	H	TrpNH ₂	50 ± 4	—
H	H	ProOH	No inhibition	—

6.91 (Tripos), using the appropriate metal parameters for the treatment of the zinc heteroatom in subsequent modelling.

To better understand the SARs observed for the compounds, as well as the determinants of molecular recognition by PPII, computational docking studies were undertaken. Docking was carried out using the FlexX utility as implemented in Sybyl 6.91 [41]. A binding subsite of radius 10 Å from the zinc atom was described, and the zinc atom was explicitly included in the active site consideration. FlexX was run using default settings to obtain up to 100 binding modes for each inhibitor ligand screened, and 300 solutions for the TRH substrate, each ranked by drug score [42]. The top 20 solutions for each ligand were imported, visualized in MOE and common binding modes were identified manually.

Using FlexX with metal parameters assigned for zinc, each of Glp-Asn-ProNH₂, Glp-Asn-Pro-AMC and Glp-Asn-Pro-Tyr-Trp-AMC were docked, resulting in up to 100 ranked poses for each ligand examined. FlexX has previously demonstrated its utility in the simulation of metalloprotease ligand docking [43]. Each set of resultant docked poses was examined to identify conserved binding modes, and a representative of each 'distinct' binding mode was retained. The distinct binding modes identified for each individual ligand were compared with those of every other ligand, and a conserved binding mode for the Glp-Asn-Pro region in all active compounds emerged. A similar docking protocol was employed for the substrate, TRH, in which 300 docked poses were examined, revealing a clear conserved ligand-docked orientation within the model active site.

RESULTS

Effects of modifications to the amide side chain of asparagine and C-terminal proline on binding and inhibition

The results presented in Table 1 show that chemical alterations to the amide nitrogen of the asparagine side chain in

Glp-Asn-ProNH₂ have a significant effect on binding affinity and the susceptibility of the Glp-Xaa peptide bond to hydrolysis. Replacement of one of the amide hydrogen atoms with an aliphatic, aromatic or hydroxy group can be seen to reduce binding affinity considerably. A further decrease in inhibitory potency was caused by dimethylation of the amide nitrogen. Binding was abolished by replacing asparagine with either β Asn or Dab. HPLC analysis revealed that Glp-Asn(*N*-benzyl)-ProNH₂ and Glp-Asn(*N*-methyl)-ProNH₂ were hydrolysed by PPII, although the rates of hydrolysis for these compounds, as measured by Glp formation, were considerably lower (5.7- and 15.1-fold respectively) than that for TRH, each under essentially saturating conditions. It can be seen from Table 1 that deamidation of the C-terminal proline residue in Glp-Asn-ProNH₂ resulted in a loss of inhibition. Elimination of a C-terminal ring through replacing the proline residue with alanine or glycine led to a reduction and loss of inhibition respectively, whereas replacement of the proline residue with tryptophan and expansion of the proline ring did not significantly alter potency.

Kinetic analysis of a series of carboxamide analogues of Glp-Asn-ProNH₂

Earlier studies showed that replacement of the C-terminal amino group of Glp-Asn-ProNH₂ by AMC resulted in a > 10-fold enhancement of binding affinity to PPII, thus indicating the possible existence of a binding region for hydrophobic moieties in the vicinity of the S₂' subsite on the enzyme. In order to investigate further the enzyme–ligand binding interactions in this region, we synthesized a diverse series of carboxamide analogues of Glp-Asn-ProNH₂, containing both unsubstituted and substituted aromatic rings (Table 2). There was no detectable hydrolysis of any of these compounds by PPII, as indicated by absence of Glp formation in the discontinuous HPLC assay. Overall, members of this series did not show wide variations in inhibitory potency, with *K_i* values generally in the range 1–20 μ M (Table 2). Although all of the carboxamides of Glp-Asn-Pro tested exhibited greater affinity for PPII than TRH itself, none had a *K_i* value less than that of Glp-Asn-Pro-AMC.

Kinetic properties of C-terminally extended analogues of Glp-Asn-ProNH₂

The kinetic properties of a set of analogues of Glp-Asn-ProNH₂ extended at the C-terminus were analysed to provide information regarding binding capacity and recognition elements distal to the active site. None of the C-terminally extended analogues were PPII substrates. It can be seen from the data presented in Table 3 that C-terminal extension of Glp-Asn-Pro with hydrophobic amino acids resulted in substantial increases in binding affinity. The most potent inhibitor of this set, Glp-Asn-Pro-Tyr-Trp-Trp-AMC, displayed an approx. 500-fold increase in affinity, as measured by its *K_i* value, compared with Glp-Asn-Pro-AMC (Table 3), which hitherto had been the most potent competitive PPII inhibitor reported. Inhibition by Glp-Asn-Pro-AMC and Glp-Asn-Pro-NH₂ was shown previously to be competitive and fully reversible [9]. In the present study, non-linear regression analysis of kinetic data indicated that Glp-Asn-Pro-Tyr-Trp-Trp-AMC and Glp-Asn-Pro-Tyr-Trp-AMC were also classical competitive inhibitors of PPII; data for Glp-Asn-Pro-Tyr-Trp-Trp-AMC are shown in Figure 1. Inhibition by Glp-Asn-Pro-Tyr-Trp-Trp-AMC was found to be fully reversible and not time-dependent.

Effects of PPII inhibitors on the recovery of TRH released from hypothalamic slices

It can be seen from Figure 2 that the recovery of TRH released from rat brain slices under basal and depolarizing conditions

Table 2 Inhibitory effects of compounds in which Glp-Asn-Pro is coupled to an amine or alcohol

HPLC analysis revealed that none of these compounds were PPII substrates. The ability of compounds to inhibit PPII purified from porcine brain was screened using the continuous fluorometric assay with TRH-AMC (5 μ M) as substrate. *K_i* values were calculated from determinations of initial rates in the absence and presence of inhibitor. Results are means $\pm$ S.D. (*n* = 3).

Compound	<i>K_i</i> (μ M)
Amine	
AMC	0.47 $\pm$ 0.05
5-Aminoindan	0.84 $\pm$ 0.08
3,5-Dimethoxyaniline	0.85 $\pm$ 0.13
4-(Trifluoromethyl)aniline	0.97 $\pm$ 0.18
1,3-Benzodioxol-5-amine	1.24 $\pm$ 0.07
3-(Trifluoromethoxy)aniline	1.25 $\pm$ 0.13
<i>m</i> -Anisidine	1.27 $\pm$ 0.06
5-Amino-2-chloropyridine	1.63 $\pm$ 0.16
<i>m</i> -Phenetidine	1.67 $\pm$ 0.14
6-Amino-3,4-benzocoumarin	1.90 $\pm$ 0.40
5-Amino-2-methoxyphenol	2.54 $\pm$ 0.00
6-Amino-1,3-dihydroisobenzofuran-1-one	2.78 $\pm$ 0.37
Aniline	2.80 $\pm$ 0.22
3,4-Dimethoxyaniline	3.18 $\pm$ 0.07
4-Phenylbutylamine	3.45 $\pm$ 0.58
6-Amino-2-mercaptobenzothiazole	3.62 $\pm$ 0.12
2-Phenylethylamine	3.98 $\pm$ 0.28
<i>p</i> -Anisidine	4.07 $\pm$ 0.02
3-Phenyl-1-propylamine	4.89 $\pm$ 0.69
2-Hydroxyaniline	7.42 $\pm$ 0.27
4-Amino- <i>N</i> -methylphthalamide	8.90 $\pm$ 0.56
5,6,7,8-Tetrahydro-1-naphthylamine	10.14 $\pm$ 0.00
Methylamine	10.84 $\pm$ 1.94
5-Chloro-2-methoxyaniline	10.85 $\pm$ 0.03
<i>o</i> -Anisidine	12.63 $\pm$ 0.76
Benzylamine	12.81 $\pm$ 0.76
2-Aminobenzyl alcohol	21.87 $\pm$ 0.62
Dimethylamine	> 200
Piperidine	> 200
Alcohol	
Benzyl alcohol	15.29 $\pm$ 0.43
Methyl alcohol	84.16 $\pm$ 1.46

Table 3 Inhibition of PPII by C-terminally extended analogues of Glp-Asn-ProNH₂

HPLC analysis indicated that all of the compounds were resistant to PPII hydrolysis. *K_i* values were calculated either from initial rates measured in the absence and presence of inhibitor, using the continuous fluorometric assay with TRH-AMC (5 μ M) as substrate, or by non-linear regression analysis of initial-rate data collected in duplicate at five different substrate concentrations and four different concentrations of peptide. Results are means $\pm$ S.D. [*n* = 3, except when indicated by an asterisk (*), where *n* = 11, or by a dagger (†), where *n* = 4].

Peptide							<i>K_i</i> (μ M)
P ₁	P ₁ '	P ₂ '	P ₃ '	P ₄ '	P ₅ '	P ₆ '	
Glp	Asn	Pro	Tyr	Trp	Trp	AMC	0.001 $\pm$ 0.000†
Glp	Asn	Pro	Tyr	Trp	AMC		0.038 $\pm$ 0.006†
Glp	Asn	Pro	Trp	Trp	AMC		0.072 $\pm$ 0.009
Glp	Asn	Pro	Trp	AMC			0.083 $\pm$ 0.016
Glp	Asn	Pro	AMC				0.458 $\pm$ 0.057*
Glp	Asn	Pro	Tyr	TrpNH ₂			0.639 $\pm$ 0.018
Glp	Asn	Pro	Tyr	Trp	TrpNH ₂		0.734 $\pm$ 0.073
Glp	Asn	Pro	TrpNH ₂				5.17 $\pm$ 0.20
Glp	Asn	Pro	Trp	TyrNH ₂			5.91 $\pm$ 0.85
Glp	Asn	Pro	Trp	Ser	TrpNH ₂		8.11 $\pm$ 0.55
Glp	Asn	Pro	TyrNH ₂				10.3 $\pm$ 0.55
Glp	Asn	ProNH ₂					16.1 $\pm$ 1.6*

Figure 2 Effects of (a) Glp-Asn-Pro-AMC and (b) Glp-Asn-Pro-Tyr-Trp-Trp-AMC on the release of TRH from rat brain hypothalamic slices

TRH release was measured under basal and depolarizing conditions in the presence of vehicle (saline or DMSO) or (a) Glp-Asn-Pro-AMC (0.1 mM in saline) or (b) Glp-Asn-Pro-Tyr-Trp-Trp-AMC (0.1 mM in DMSO). Results are means $\pm$ S.E.M. ($n = 6$). * $P < 0.05$; ** $P < 0.01$ compared with corresponding basal; *** $P < 0.001$ compared with corresponding conditions in the absence of inhibitor (Student's t test).

was increased significantly in the presence of Glp-Asn-Pro-AMC or Glp-Asn-Pro-Tyr-Trp-Trp-AMC ($P < 0.001$, $n = 6$; Student's t test).

Model of PPII

Figure 3 illustrates the active site in the homology model of human PPII and highlights the common binding mode observed

for the inhibitors (illustrated by Glp-Asn-Pro-NH₂, green) and predicted docked pose for the substrate (TRH, yellow). The orientation of the Glp-Asn-Pro portion for all our docked active species is conserved in this model. In each, asparagine was found to be oriented so as to facilitate direct hydrogen bonding to Glu⁴⁰⁷. The significance of bound water in molecular design is sometimes underestimated; however, recent work has highlighted the utility of its consideration in mechanistic explanations [44]. When the location of a catalytic water molecule is considered in the homology model of PPII (its location derived from the works of Rozenfeld et al. [27], Thunnissen et al. [39] and Rudberg et al. [45]), a hydrogen-bonding network involving asparagine, water, Glu⁴⁰⁷ and Glu⁴⁴¹ is predicted. Comparison of the positioning of the inhibitor Glp-Asn-Pro motif within the active site to that of the substrate, TRH, shows that (i) the orientation of the recognition residue, Glp, is conserved and (ii) the pendant His in TRH projects into the region occupied by asparagine in the inhibitors.

DISCUSSION

Our previous observation that replacement of the central histidine in TRH with asparagine produces an analogue that inhibits PPII activity [9] provided a starting point for the present studies. To gain a greater understanding of the structural parameters that influence ligand binding and inhibition, as well as to explore the potential for improving inhibitory potency, we have now investigated the effects of structural modifications to Glp-Asn-ProNH₂ on the kinetics of PPII activity.

The results show that the presence of the amide side chain of asparagine in the P₁' position confers resistance to hydrolysis by PPII and appears to be critical for inhibitory binding interactions. Asparagine can be regarded as an isostere of histidine [46]; its side chain, however, occupies less space and offers different possibilities for the formation of inter- and/or intra-molecular hydrogen bonds than the imidazole ring of histidine. These differences may account for the inhibitory characteristics of P₁' asparagine. Notably, all modifications to the asparagine side chain that were

Figure 3 Predicted conserved binding mode for Glp-Asn-Pro inhibitory motif (green), compared with that obtained for TRH (yellow)

The Figure shows the active-site zinc (pink) and key active-site residues of human PPII (labelled in black). Broken lines highlight possible hydrogen-bond interactions that arise in inhibitory peptides that contain a P₁' asparagine residue. Such interactions are not indicated for the substrate, TRH, and suggest a possible mechanism of action for the inhibitor series shown in the present study.

examined led to marked reduction in inhibitory potency. Thus any change to the amide group of asparagine is deleterious to inhibitory properties. Indeed, all the modifications reduced the capability to form hydrogen bonding, reinforcing the idea that participation in such bonding by the asparagine side chain may be crucial for inhibition. Consistent with this, the model shown in Figure 3 predicts that the asparagine side chain participates in a hydrogen-bonding network, involving Glu⁴⁰⁷ and Glu⁴⁴¹, thus appropriating these two residues, which have been shown previously, by site-directed mutagenesis, to be important for the catalytic activity of rat PPII [26]. The amino acid sequences for human and rat PPII display a remarkably high degree of conservation (96 % identical residues), but they differ in length by one amino acid [47]. Thus, when comparing human and rat PPII, it should be noted that residues Glu⁴⁰⁷, Glu⁴⁴¹ and Tyr⁵²⁷ of human PPII correspond to Glu⁴⁰⁸, Glu⁴⁴² and Tyr⁵²⁸ respectively of rat PPII. The model places the recognition residue (Glp) of both substrate and inhibitor in close proximity and in a similar orientation within the active site of PPII. Binding modes predicted for both TRH and the inhibitors by this model agree with the general model for aminopeptidase activity put forward by Rudberg et al. [45] and includes a predicted interaction of Tyr⁵²⁷ with the N of the scissile peptide bond. A key difference between TRH and the inhibitors, however, is the ability of the asparagine-containing peptides to participate in the hydrogen-bonding network noted above.

The C-terminal prolineamide of Glp-Asn-ProNH₂ is important, but not crucial, for binding to the S₂' subsite on PPII. Thus deamidation or replacement of the C-terminal ProNH₂ with amides of glycine, alanine or asparagine causes a loss or substantial reduction of inhibition. The enzyme, nevertheless, does appear to be tolerant to replacement of proline with tryptophan and the expanded (six-membered) ring of homoprolineamide. The complete loss of inhibition observed for Glp-Asn-ProOH mirrors the poor affinity displayed by the TRH analogue Glp-His-ProOH [9]. It seems that this is most likely to be due to the negative charge at the C-terminus being unfavourable. Comparison of *K_i* values for Glp-Asn-ProNHBzl (where Bzl is benzyl), Glp-Asn-ProOBzl, Glp-Asn-ProNHMe (where Me is methyl) and Glp-Asn-ProOMe indicates aromaticity and/or hydrogen bonding to be important for binding to the enzyme. This is supported by the observation that Glp-Asn-Pro-piperidine and Glp-Asn-Pro-dimethylamide, where the C-terminal groups do not possess such properties, are poor PPII inhibitors. Exploration of this region using Glp-Asn-ProNH₂Y (where Y is a substituted aryl group) reinforced the importance of an aromatic substituent, since all of these compounds showed good inhibitory potency, with a slight preference for oxygen-containing substituents in the meta position. Consistent with this, AMC, which has an oxygen meta to its amino group, was the most favourable C-terminal group of those tested.

The advantage conferred by aromatic substitution at the C-terminus with respect to inhibitory potency was exemplified further by the C-terminal extension of Glp-Asn-ProNH₂ with aromatic amino acids. These elongations can be regarded as probes for additional hydrophobic binding interactions with the target enzyme. The results suggest that such interactions do occur. The most potent inhibitor identified in this set, Glp-Asn-Pro-Tyr-Trp-AMC, was approx. 16000 and 500 times more potent than Glp-Asn-ProNH₂ and Glp-Asn-Pro-AMC respectively, making it the most potent competitive PPII inhibitor thus far reported.

The increased potency of the extended analogues may simply reflect additional sites of interactions with the PPII molecule. It is possible, though, that they may also affect local conformational changes in the enzyme necessary for catalysis. Members of the

MA (E) clan of metalloproteases (zinc metalloproteases in which zinc is ligated to two histidine residues and a glutamate residue, see <http://merops.sanger.ac.uk>), which includes thermolysin (EC 3.4.24.27), share similar structural and catalytic features with PPII, although the specific details of their catalytic mechanisms may differ [48]. A conformational change on substrate binding, resulting in transition from an open to a closed conformation, has been shown to be necessary for thermolysin to catalyse substrate hydrolysis [49]. If that were also the case for PPII, the effectiveness of the more bulky analogues as inhibitors may result from their preventing this closure. This might also account for the inhibitory activity displayed by LHRH. Modifications to the surface charge of the enzyme [50], as a result of inhibitor binding, may also affect affinity.

The increased recovery of TRH released from brain slices observed in the presence of PPII inhibitors, agrees with the findings of Charli et al. [29] and provides further support for the critical involvement of PPII in the metabolism of neuronally released TRH. Importantly, these results also indicate that the asparagine-containing PPII inhibitors tested are capable of increasing TRH levels in a more physiologically intact environment.

In conclusion, these SAR studies have revealed that, despite PPII having very high substrate specificity for tri- and tetrapeptides, there appears to be an extended binding region contiguous with the active site. The results also demonstrate that it is possible to exploit this in the design of highly potent inhibitors. The SAR for inhibition may be summarized as follows: (i) the N-terminal Glp confers specificity for PPII [10], as well as resistance to hydrolysis by aminopeptidases [1,5]; (ii) the presence of asparagine in position 2 combines resistance to hydrolysis with effective inhibition of the enzyme; (iii) the prolineamide is important for interaction with the S₂' subsite on PPII, but the enzyme appears to be tolerant to a variety of modifications to the C-terminal carboxy function; and (iv) hydrophobic extensions to Glp-Asn-ProNH₂ can produce substantial improvement in inhibitory potency. Notably, the data presented provide critical information for the design of PPII inhibitors, which should be of value as tools for studying the biological functions of TRH and PPII and may provide a basis for the development of new therapeutic agents.

This work was supported by a grant from the Wellcome Trust to J. A. K. G. A. S. and A. A. C. were supported by a grant from the Health Research Board, Ireland. D. G. L. acknowledges the Irish Higher Education Authority's PRTL (Programme for Research in Third Level Institutions) IITAC (Institute for Information Technology and Advanced Computation) programme for computational infrastructure support.

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Received 12 October 2004/2 March 2005; accepted 31 March 2005

Published as BJ Immediate Publication 31 March 2005, DOI 10.1042/BJ20041722