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Cyclic insulin-regulated aminopeptidase (IRAP)/AT_4 receptor ligands

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Abstract: The angiotensin IV receptor (AT_4 receptor) is the insulin-regulated aminopeptidase enzyme (IRAP, EC 3.4.11.3). This membrane-spanning enzyme belongs to the M1 family of zinc-dependent metallo-peptidases. It has been proposed that AT_4 receptor ligands exert their physiological effects by binding to the active site of IRAP and thereby inhibiting the catalytic activity of the enzyme. The biological activity of a large series of linear angiotensin IV analogs was previously disclosed. Herein, the synthesis and biological evaluation of a series of angiotensin IV analogs, encompassing macrocyclic ring systems of different sizes, are presented. It is demonstrated that disulfide cyclizations of angiotensin IV can deliver ligands with high IRAP/AT_4 receptor affinity. One ligand, with an 11-membered ring system (**4**), inhibited human IRAP and aminopeptidase N (AP-N) activity with similar potency as angiotensin IV but was considerably more stable than angiotensin IV toward enzymatic degradation. The compound provides a promising starting point for further optimization toward more drug-like derivatives. The cyclic constrained analogs allowed us to propose a tentative bioactive conformation of angiotensin IV and it seems that the peptide adopts an inverse γ-turn at the *C*-terminal. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: angiotensin IV; insulin-regulated aminopeptidase; IRAP; cystinyl aminopeptidase; aminopeptidase N; disulfide cyclization; structure–activity relationship; peptide synthesis; bioactive conformation

INTRODUCTION

Enzymatic conversions of neuropeptides frequently render bioactive fragments with modified biological profiles as compared to the native peptide from which they are derived [1,2]. Hence, the κ opioid receptor selective opioid peptide dynorphin produces dysphoria and is converted to the δ opioid receptor agonist Leu-enkephalin, with euphoric properties [3]. The tachykinin substance P is processed to the bioactive fragment substance P (1–7), a peptide that mimics some but opposes, other effects of the parent peptide [1]. The hexapeptide angiotensin IV (Val-Tyr-Ile-His-Pro-Phe), a bioactive degradation product in the renin–angiotensin system that has attracted our interest, often exerts different effects than its precursors [4–6]. This peptide seems to be of particular relevance in the CNS, where it has been associated with distinct actions. The most significant, reproducible and probably most interesting effect of angiotensin IV from a pharmaceutical point of view is its ability to facilitate learning and memory, which has been demonstrated in several behavioral models [7–14]. The peptide might at some level be involved in a number of neurobiological actions including neuronal development and neuron survival. It stimulates DNA synthesis and enhances thymidine incorporation [15].

The AT_4 receptor was initially described as the specific high-affinity binding site for angiotensin IV [16]. The receptor was first suspected to be a G protein coupled receptor and was also found to bind with high affinity to LVV-hemorphin-7, a hydrolysis product of the β-chain of hemoglobin [17]. More recently, the AT_4 receptor was identified as a transmembrane enzyme, the insulin-regulated aminopeptidase (IRAP), an abundant protein found in the vesicles with the insulin-sensitive glucose transporter GLUT4 [18]. IRAP (official denomination: CAP, EC 3.4.11.3) belongs to the M1 family of zinc-dependent metallo-peptidases [19]. It has been proposed that AT_4 receptor ligands exert their physiological effects by binding to the active site of IRAP, inhibiting its catalytic activity [20]. Vasopressin [21–23], oxytocin [24], CCK-8 [25–30] and somatostatin [31,32] constitute a few examples of substrates to IRAP that are all known to facilitate learning and memory. It

Abbreviations: AP-N, aminopeptidase N; Boc, *tert*-butyloxycarbonyl; *t*-Bu, *tert*-butyl; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; CAP, cystinyl aminopeptidase; Cha, cyclohexylalanine; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hcy, homocysteine; L-Leu-pNA, L-leucine-*p*-nitroanilide; NMM, N-methylmorpholine; 1,10-Phe, 1,10-phenantroline; PDMS, plasma desorption mass spectrometry; PMSF, phenyl methyl sulfonyl fluoride; RP-HPLC, reversed phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Trt (trityl), triphenylmethyl.

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is proposed that angiotensin IV ligands, at least in part, mediate their actions by prolonging the half-life of one or more of these neuropeptides [4,18].

The fact that there is convincing experimental support for the hypothesis that IRAP/AT_4 receptor ligands have memory-potentiating effects encouraged us to commence a long-term program aiming at identifying metabolically stable low molecular weight ligands with ability to cross the blood–brain barrier. Such ligands would provide valuable research tools and could serve as starting points in lead optimization programs. Our first objective was to utilize constrained analogs to gain information on the bioactive conformation(s) that angiotensin IV adopts when binding to its receptor. Previously, detailed and informative structure–activity relationships of linear peptidic angiotensin IV analogs have been disclosed [33–36]. In essence, it was demonstrated that the character of the amino acid residues at the *N*-terminal part of the hexapeptide is most critical for binding and that displacement of the individual amino acid residues His^4, Pro^5 and Phe^6 at the *C*-terminal end by Gly or by the corresponding D-amino acids can be performed with no negative impact on the bioactivity [33]. IRAP is a CAP with a potential preference for disulfide-containing substrates. To some extent with that in mind, we have now prepared a series of constrained angiotensin IV analogs encompassing cyclic disulfide ring systems. Our primary objective was to utilize these rigidified analogs to gain information and to explore the bioactive conformation of angiotensin IV.

We herein report that potent IRAP/AT_4 receptor ligands can be obtained after disulfide cyclizations. On the basis of their measured affinities and the conformational preferences of the cyclic moiety, we propose a tentative bioactive conformation of the *C*-terminal of angiotensin IV when it binds to the IRAP/AT_4 receptor protein.

MATERIALS AND METHODS

General

Preparative RP-HPLC was performed on a Vydac 10 μm C18 column (22 × 250 mm) using gradients of CH_3CN in 0.1% aqueous TFA at a flow rate of 3 ml/min and detection at 230 nm. Analytical RP-HPLC was performed on a Vydac 10 μm C18 column (4.6 × 150 mm) using detection at 220 nm. Mass spectroscopy was carried out on an Applied Biosystems (Uppsala, Sweden) BIOION 20 PDMS. Amino acid analyses were performed at the Department of Biochemistry, Uppsala University, Sweden, using an LKB 4151 alpha plus analyzer with ninhydrin detection. Samples (oxidized when appropriate) were hydrolyzed with 6 M HCl at 110 °C for 24 h.

Materials

Wang resins and amino acid derivatives were obtained from Bachem (Bubendorf, Switzerland) or Novabiochem (Läufelfingen, Switzerland). H-Gly-2-Cl-trityl resin and H-Cha-2-Cl-trityl resin were prepared from 2-Cl-tritylchloride resin (Alexis Corporation, Läufelfingen, Switzerland) as described in the literature [37]. HBTU was obtained from Senn Chemicals (Dielsdorf, Switzerland). DMF (analytical grade) was provided by Fischer Chemicals (Loughborough, UK) and was used without further purification. L-Leu-pNA and L-alanine-*p*-nitroanilide were obtained from Sigma-Aldrich (Bornem, Belgium) and *p*-nitroaniline from VWR International (Leuven, Belgium). Tyr^4 of angiotensin IV was iodinated using the Iodogen iodination reagent from Pierce (Erembodegem, Belgium) as described by Lahoutte *et al.* (2003). ^{125}I was obtained from MP Biomedicals (Asse, Belgium). Monoiodinated angiotensin IV was isolated on a GraceVydac C18 monomeric 120A RP-HPLC column and stored at −20 °C in 10 mM KH_2PO_4, pH 6.5, containing 45% ethanol. All other chemicals/reagents were of the highest grade commercially available and were used as received. CHO-K1 cells were kindly obtained from the Pasteur Institute (Brussels, Belgium).

Solid-phase Peptide Synthesis

The peptides were synthesized on a 100 μmol scale with a Symphony instrument (Protein Technologies Inc., Tucson, AZ, USA) using Fmoc/*t*-Bu protection. The starting polymer was Fmoc-Phe Wang resin (0.62 mmol/g), H-Cys(Trt)-2-Cl-trityl resin (0.55 mmol/g), or H-Cha-2-Cl-trityl resin (0.73 mmol/g) and the side chain protection groups were as follows: Tyr(*t*-Bu), Cys(Trt), Hcy(Trt), and His(Trt) or His(Boc). Removal of the Fmoc group was achieved by reaction with 20% piperidine in DMF (2 × 2.5 ml) for 5 + 10 min. Coupling of the amino acids (125 μmol) was performed in DMF (2.5 ml) using HBTU (125 μmol) in the presence of NMM (500 μmol). Double couplings (2 × 30 min) were used for all amino acids except Hcy, which was coupled once for 1 h. After introduction of each amino acid, remaining amino groups were capped by addition of 20% acetic anhydride in DMF (1.25 ml) to the coupling mixture and allowing the reaction to proceed for 5 min. After completion of the synthesis, the Fmoc group was removed and the partially protected peptide resin was washed with several portions of DMF and CH_2Cl_2 and dried in a stream of nitrogen and *in vacuo*.

Peptides were cleaved and deprotected by treating with TFA–H_2O–triethylsilane (90 : 5 : 5; *ca* 2 ml/100 mg resin) for 1.5–2 h. The resin was filtered off and washed with TFA. The combined filtrates, collected in a centrifuge tube, were concentrated and the product was precipitated by the addition of cold ether. The crude peptide was then collected by centrifugation, washed with several portions of ether, and dried. The crude peptide (*ca* 25 mg) was dissolved in 0.1% TFA-10% CH_3CN–H_2O (2.2 ml), filtered through a 0.45-μm nylon membrane, and purified by RP-HPLC. Selected fractions were analyzed by RP-HPLC and/or PDMS. Those containing pure material were pooled, lyophilized and dissolved in water (occasionally supplemented with CH_3CN). The peptide content, usually 70–80% on a weight basis, was then determined by amino acid analysis of a withdrawn aliquot. Yields and analytical data are presented in Table 1.

General Procedure for S-S Oxidation

The reduced peptide, purified by RP-HPLC and lyophilized, was dissolved in 95% aqueous TFA at a concentration of

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Table 1 Amino acid analysis of the cyclic insulic-regulated aminopeptidase (IRAP)/AT_4 receptor ligands

No	Amino acid analysis	Yield (%)	MW	PDMS ($M + H^+$)
1	Cys, 0.99; Tyr, 0.86; His,1.00; Pro, 1.01; Phe, 1.00	40.0	766.9	767.6
2	Val, 1.01; Cys, 1.75; Ile, 1.01; Pro, 1.00; Phe, 0.99	34.0	678.9	679.7
3	Val, 1.01; Tyr, 0.97; Cys, 1.29; His, 1.01; Phe, 1.02	23.9	768.9	770.0
4	Val, 1.00; Tyr, 1.02; Ile, 1.02; Cys, 1.45; Pro, 0.97	66.1	694.9	696.0
5	Val, 1.02; Tyr, 0.93; Cys, 2.00; His, 1.02; Pro, 1.04	40.7	718.9	719.4
6	Hcy, 1.93; Tyr, 0.90; His, 1.08; Pro, 1.02; Phe, 1.07	50.7	794.9	795.8
7	Cys, 0.97; Tyr, 0.90; Hcy, n.d.; His 0.91; Pro, 1.00; Phe, 1.00	26.2	780.9	781.9
8	Hcy, n.d.; Tyr, 0.78; Cys, 1.24; His, 1.00; Pro, 1.01; Phe, 1.00	33.9	780.9	782.3
9	Val, 1.00; Cha, 1.01; Ile, 0.98; His, 1.00; Pro, 1.00; Phe, 1.01	86.3	764.9	766.4
10	Val, 1.00; Tyr, 0.99; Cha, 1.01; His, 0.99; Pro, 1.01; Phe, 1.01	61.3	814.9	815.4
11	Val, 1.01; Tyr, 0.99; Ile, 1.00; His, 1.00; Pro, 0.99; Cha, 1.01	51.4	780.9	781.5

1–2 mg/ml. The solution was cooled in an ice-bath, and DMSO was added to give a final concentration of 10% (v/v). The reaction mixture was then kept at room temperature until HPLC analysis showed the oxidation to be complete, usually for 6–20 h. The residue after evaporation of the TFA was diluted with water (*ca* 10 volumes) and purified by RP-HPLC before amino acid analysis and determination of the peptide content.

Cell Culture, Transient Transfection and Membrane Preparation

CHO-K1 and HEK293 cell lines were cultured in 75 and 500 cm^3 culture flasks in Dulbecco's modified essential medium (DMEM) supplemented with L-glutamine (2 mM), 2% (v/v) of a stock solution containing 5000 IU/ml penicillin and 5000 μg/ml streptomycin (Invitrogen, Merelbeke, Belgium), 1% (v/v) of a stock solution containing non essential amino acids, 1 mM sodium pyruvate and 10% (v/v) fetal bovine serum (Invitrogen, Merelbeke, Belgium). The cells were grown in 5% CO_2 at 37 °C until confluent.

HEK293 cells were transiently transfected with plasmid DNA, pCIneo containing the gene of human IRAP (CAP kindly obtained from Prof. M. Tsujimoto, Lab of Cellular Biochemistry, Saitama, Japan) or pTEJ4 [38] carrying the complete human AP-N cDNA [39]. The transient transfection was performed as described previously with 8 μl/ml LipofectAMINE (Invitrogen, Merelbeke, Belgium) and 1 μg/ml plasmid DNA [40]. After transfection, the cells were cultured for two more days. CAP and AP-N transfected HEK293 cells displayed a ten and eight times higher enzyme activity than non transfected cells.

CHO-K1 cell and transfected HEK293 cell membranes were prepared as described previously [41]. In short, the cells were harvested with 0.2% EDTA (w/v) (in PBS, pH 7.4) and centrifuged for 5 min at 500 *g* at room temperature. After resuspending in PBS, the number of cells were counted and washed. The cells were then homogenized in 50 mM tris-HCl (at pH 7.4) using a Polytron (10 s at maximum speed) and Potter homogenizer (30 strokes at 1000 rpm) and then centrifuged for 30 min (30 000 *g* at 4 °C). The pellet was resuspended in 50 mM tris-HCl, centrifuged (30 min 30 000 *g* at 4 °C) and the supernatant removed. The resulting pellets were stored at −20 °C until use.

For the preparation of porcine frontal cortex membranes, cortical tissue was homogenized in 40 volumes of 50 mM tris-HCl, 5 mM EDTA buffer (pH 7.4) using a Polytron homogenizer. The homogenate was centrifuged (500 *g* at 4 °C) for 10 min and the supernatant was recentrifuged (40 000 *g* at 4 °C) for 20 min. The pellet was dissolved in 40 volumes of 50 mM tris-HCl, 5 mM EDTA buffer and subsequently homogenized and recentrifuged (40 000 *g*) for another 20 min. The final pellet was dissolved in 25 volumes of 50 mM tris-HCl, 5 mM EDTA buffer, homogenized, aliquoted and kept at −80 °C until further use.

Enzyme Assay

Determination of the aminopeptidase catalytic activity was based on the cleavage of the substrate L-Leu-pNA [41] into L-leucine and *p*-nitroaniline. This latter compound displays a characteristic light absorption maximum at 405 nm. Pellets, prepared as described above, were thawed and resuspended using a Polytron homogenizer in enzyme assay buffer (i.e. 50 mM tris-HCl (pH 7.4), 140 mM NaCl, 0.1% (w/v) BSA and 100 μM PMSF). The incubation mixture comprised 50 μl membrane homogenate, 200 μl L-Leu-pNA (1.5 mM) and 50 μl enzyme assay buffer alone or with test compound. The amount of membrane homogenate corresponded to 4×10^5 CHO-K1 cells and 1.5×10^5 transfected HEK293 cells in each well. Assays were carried out at 37 °C in 96-well plates (Medisch Labo Service, Menen, Belgium) and the formation of *p*-nitroaniline was followed by measuring the absorption at 405 nm every 5 min between 10 and 50 min in a Bio-Whittaker ELISA reader. The enzymatic activities were calculated by linear regression analysis of the time-wise increase of the absorption.

Radioligand Binding Assays

The binding affinities of angiotensin IV and of compounds **1**–**11** (Table 2) were assessed, essentially as described previously [17,33], using porcine frontal cortex membranes. The binding of [^{125}I]angiotensin IV to frontal cortex membranes (60 μg of protein) was conducted in a final volume of 500 μl binding buffer containing 50 mM tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% BSA(w/v), 100 μM PMSF,

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Table 2 Prepared cyclic insulin-regulated aminopeptidase (IRAP)/AT_4 receptor ligands

Substance	Sequence	K_i (nM)
Angiotensin IV	Val-Tyr-Ile-His-Pro-Phe	4.6 (IC_{50})
1	*Cys*-Tyr-*Cys*-His-Pro-Phe	5000
2	Val-Cys-Ile-Cys-Pro-Phe	15 700
3	Val-Tyr-*Cys*-His-*Cys*-Phe	640
4	Val-Tyr-Ile-*Cys*-Pro-*Cys*	6.5
5	Val-Tyr-*Cys*-His-Pro-*Cys*	913
6	*Hcy*-Tyr-*Hcy*-His-Pro-Phe	56.7
7	*Cys*-Tyr-*Hcy*-His-Pro-Phe	1150
8	*Hcy*-Tyr-*Cys*-His-Pro-Phe	66.4
9	Val-*Cha*-Ile-His-Pro-Phe	1700
10	Val-Tyr-*Cha*-His-Pro-Phe	23.4
11	Val-Tyr-Ile-His-Pro-*Cha*	7.9

20 μM bestatin, 0.05 nM [^{125}I]angiotensin IV and a variable concentration of test substance. The samples were incubated for 2 h (37 °C) and the binding was terminated by filtration through Whatman GF/B glass fiber filters (presoaked in 0.3% polyethylenimine for 20 h) using a Brandel cell harvester. The filters were washed with 4 × 2 ml of 50 mM tris-HCl (pH 7.4), containing 150 mM NaCl and 5 mM EDTA. The radioactivity was measured in a gamma-counter. Nonspecific binding was determined in the presence of 1 μM unlabeled angiotensin IV. All experiments were performed in triplicate.

The stability of angiotensin IV and compound **4** was compared in the presence of CHO-K1 cell membranes. Membrane pellets were thawed and resuspended using a Polytron homogenizer in 50 mM tris-HCl (pH 7.4) enzyme assay buffer and the assays were carried out in polyethylene 24-well plates (Elscolab, Kruibeke, Belgium). Pre incubations were carried out for 40 min at 37 °C in 250 μl containing 150 μl membrane homogenate, 50 μl enzyme assay buffer without or with 30 mM EDTA/600 μM 1,10-Phe and 50 μl enzyme assay buffer without or with compound **4** or unlabeled angiotensin IV (60 μM for nonspecific binding). Then the binding assay was initiated by adding 50 μl enzyme assay buffer containing [^{125}I]angiotensin IV (without or with 30 mM EDTA/600 μM 1,10-Phe) and the mixture was further incubated for 30 min at 37 °C. Final membrane concentrations were the same as for the enzyme assays, final chelator concentrations (when present) were 5 mM EDTA and 100 μM 1,10-Phe, and the final [^{125}I]angiotensin IV concentration was 1 nM, and the final unlabeled ligand concentrations are indicated in Figure 2. After incubation, the mixture was vacuum filtered using a Inotech 24-well cell-harvester through GF/B glass fiber filters (Whatman) pre soaked in 1% (w/v) BSA. After drying, the radioactivity retained in the filters was measured using a Perkin-Elmer γ-counter.

Data Analysis

All the experiments were performed in duplicate and repeated at least three times. The calculation of IC_{50} values from competition-binding (or enzyme inhibition) experiments was performed by nonlinear regression analysis using GraphPad Prism 4.0. The equilibrium dissociation constants (K_i values) of the tested compounds in the binding and enzyme assays were calculated using the equation $K_i = \{IC_{50}/(1 + [L]/K)\}$ in which [L] is the concentration of free [^{125}I]angiotensin IV (binding) or free substrate (enzyme assay) and K the equilibrium dissociation constant (K_D) of [^{125}I]angiotensin IV (from saturation binding experiments) or the Michaelis–Menten constant (K_m) for substrate cleavage [42].

RESULTS

Eight cyclized angiotensin IV analogs were prepared and assayed. Their equilibrium dissociation constants (K_i values) for the IRAP/AT_4 receptor on porcine frontal cortex membranes are summarized in Table 2. The four oxidative disulfide formations starting from two cysteine residues lead to the peptides **1**, **2**, **3** and **4** encompassing 11-membered ring systems and the peptide **5** with a 14-membered ring system. Peptide **4**, where His^4 and Phe^6 had been displaced, was found to be the most potent ligand with a K_i of 6.5 nM, while peptide **3** exhibited an approximately 100 times weaker affinity to the receptor. Formation of the 14-membered ring by cyclization between positions 3 and 6 rendered ligand **5** with an intermediate affinity. Replacement of Tyr^2 and His^4 by two cysteine residues and subsequent cyclization delivered the weakest ligand (**2**) in the series. Cyclization between position 1 and position 3, where Tyr^2 is part of the ring system, resulted likewise in a poor ligand (**1**) with a K_i of 5000 nM. Since the affinity to the IRAP/AT_4 receptor is to a large part determined by the amino acid residues in the *N*-terminal of angiotensin IV [33–36] we decided to map the receptor and the topography of bioactive ligands, to expand the ring size of **1** and induce a higher conformational flexibility of the ring system. Thus, by cyclization of two homocysteine rather than two cysteine residues, peptide **6** containing a 13-membered ring system was formed. This peptide was 100-fold more potent than **1** suggesting that a bioactive conformation can be approached by allowing more flexibility in the ring system. Fine-tuning by oxidative cyclization of [Cys^1, Hcy^3]angiotensin IV and [Hcy^1, Cys^3]angiotensin IV provided ligands **7** and **8**, both encompassing 12-membered ring systems. Despite the same size of the ring system, these ligands displayed a 20-fold difference in affinity for the IRAP/AT_4 receptor (i.e. K_i = 1150 nM for compound **6** and 66 nM for compound **7**, respectively). The binding affinities of the angiotensin IV analogs modified at position 2 that were previously assessed suggested that a six-membered aromatic ring system is strongly preferred in this position [35]. However, Cha was never employed. Therefore, as a reference compound we incorporated Cha, the saturated counterpart of phenylalanine, in position 2. This peptide (**9**), as might have been expected [35], was found to be a very weak binder. However, when Ile in position 3 or Phe in

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position 6 was replaced by the bulky Cha, most of the binding affinity was retained. Peptides **10** and **11** exhibited K_i values of 23 and 7.9 nM, respectively.

Enzyme Activity

The cystine derivative **4** was tested for its ability to inhibit the catalytic activity of recombinant human IRAP and AP-N, transiently transfected in HEK293 cells; and of IRAP natively expressed in CHO-K1 cells. As described previously, the enzymatic activity was assessed spectrophotometrically by measuring the absorption of *p*-nitroaniline (i.e. the cleavage product of the synthetic substrate L-Leu-pNA) at 405 nm over time at 37°C [41]. The corresponding rate constants (further denoted as enzymatic activities) were calculated by linear regression analysis of the time-wise increase of the absorption.

Compound **4** was competitive with the substrate (data not shown) and displayed a monophasic inhibition curve for all three enzyme preparations (Figure 1). Its K_i values are listed in Table 3. Compound **4** inhibited the activity of recombinant and endogenous IRAP more potently than the activity of recombinant AP-N.

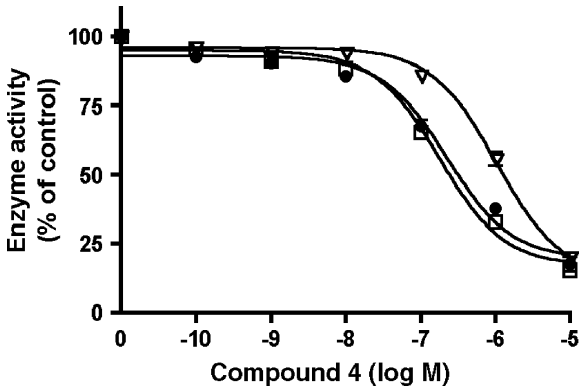

Figure 1 Inhibition of the enzymatic activity by compound **4**. Membranes of HEK293 cells transfected with human IRAP (□) or human AP-N (Δ) (corresponding to 1.5×10^5 cells/incubation) and CHO-K1 cells (●) (corresponding to 4×10^5 cells/incubation) were incubated at 37°C with 1.5 mM L-Leu-pNA in the absence (control activity) or the presence of increasing concentrations of the compound. The rate constants of L-Leu-pNA cleavage (v, corresponding to enzyme activity and expressed as percentage of control) were calculated by linear regression analysis of the absorption (at 405 nm) *versus* time curves with measurements made every 5 min (between 10 and 50 min). The K_i values of the peptide is given in Table 3.

Stability Experiments with (^{125}I)Angiotensin IV Binding

Compound **4** displayed a monophasic [^{125}I]angiotensin IV competition-binding curve for CHO-K1 cell membranes (30 min incubation at 37°C with 1 nM radioligand, data not shown). To detect binding activity, these studies needed to be performed in the presence of the EDTA and 1.10-phenantrolin, chelators that are known to effectively prevent metalloprotease activity [44]. To compare the susceptibility of the cyclic derivative **4** and angiotensin IV to metalloprotease-dependent breakdown, CHO-K1 cell membranes were preincubated with increasing concentrations of each compound for 40 min at 37°C, either in the presence or absence of EDTA and 1.10-Phe before the binding assay. EDTA and 1.10-Phe preincubation produced a marked leftward shift of the angiotensin IV inhibition curve (Figure 2(B), Table 4), suggesting that this compound was rapidly degraded by IRAP and/or other metalloproteases present on the CHO-K1 cell membranes. In contrast, a much smaller shift was observed for the cysteine derivative **4** (Figure 2(A), and Table 4), indicating that this cyclized peptide is more stable than angiotensin IV.

DISCUSSION

The IRAP/AT_4 receptor has emerged as a new potential therapeutic target. While the ultimate goal of our

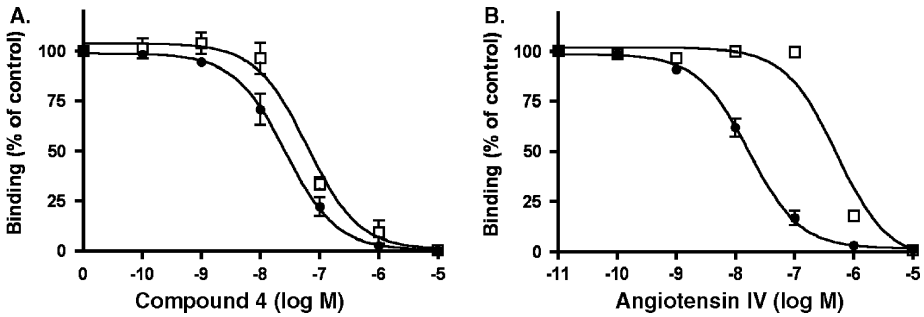

Figure 2 Stability of compound **4**, and angiotensin IV toward breakdown by metalloproteases present in CHO-K1 cell membranes. Membranes were preincubated for 40 min at 37°C with increasing concentrations of compound **4** (A) and angiotensin IV (B) in the presence (●) or absence (□) of EDTA and 1.10-phenantrolin before 30 min incubation with 1 nM [^{125}I]angiotensin IV. Data refer to specific binding of [^{125}I]angiotensin IV (expressed as percent of control binding in the absence of unlabeled compound), calculated by subtracting nonspecific binding in the presence of 10 μM unlabeled angiotensin IV from total binding. Concentrations are the initial concentrations. The corresponding IC_{50} values of these compounds are given in Table 4.

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Table 3 Inhibition of enzyme activity by compound **4**

	K_i (nM)
CHO-K1	50.6 ± 14.7
HEK293 + IRAP	25.8 ± 3.2
HEK293 + AP-N	221.9 ± 28.1

The K_i values are calculated according to the Cheng and Prusoff equation using IC_{50} values and the K_m values for L-Leu-pNA from recombinant IRAP (0.25 ± 0.05 mM), recombinant AP-N (0.41 ± 0.02 mM), and endogenous IRAP in CHO-K1 cells (0.39 ± 0.04 mM) [43]. Data are expressed as mean ± S.E.M. of three independent experiments (illustrated in Figure 1).

Table 4 [^{125}I]Angiotensin IV competition-binding in CHO-K1 membranes after preincubation in the absence or presence of EDTA and 1,10-phenantrolin (chelators)

IC_{50} (nM)	No chelators	Chelators
Compound **4**	203.5 ± 144.9	28.7 ± 10.1
Angiotensin IV	1079.3 ± 606.6	17.6 ± 4.5

Data are expressed as mean ± S.E.M. of three independent experiments (illustrated in Figure 2).

research was to discover small drug-like molecules with capacity to cross the blood–brain barrier, we believed that this could be achieved by first developing knowledge of the conformational requirements for high affinity to the IRAP/AT_4 receptor, that is a CAP. One method of probing the receptor-bound conformation of a ligand is via conformational restriction. It has been known for a long time [45,46] that connecting two residues of an acyclic peptide via a disulfide bond can afford a conformationally restricted cyclic analog, thus providing valuable structural information. We have synthesized a series of angiotensin IV analogs having a cystine moiety bridging various positions. As can be seen from Table 2, several of these conformationally restrained analogs maintained good IRAP/AT_4 receptor binding activity.

Already in the 1970s it was proposed that the lipophilic amino acid residues in position 3 and 5 of the hypertensive octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) exert conformationally stabilizing roles [47]. Spear *et al.* reported a series of disulfide cyclizations involving Cys and Hcy in the 3-, 5- and 7-positions of the peptide [46]. It is notable that the cyclized $Hcy^{3,5}$ analog was equipotent to angiotensin II while the cyclized $Cys^{3,5}$ analog was found to be a 20-fold less effective binder. The tyrosine residue constituted a central part in these macrocycles. These studies dealt with the angiotensin II receptor of the AT_1 subtype, a G protein coupled receptor with seven membrane-spanning α-helical domains [48]. In contrast, the present study is focused on the IRAP/AT_4 receptor, which has recently been recognized to be the major cellular target for angiotensin IV. In contrast to the AT_1 receptor, it contains only a single membrane-spanning α-helical domain and a large exodomain containing a zinc-associated catalytic site [49]. Besides the lack of general structural resemblance between the AT_1 receptor and the IRAP/AT_4 receptor, both display large differences in their ligand binding profile and, hence, differences in the structure of their ligand binding site. Hence, it is of no surprise that, in the angiotensin IV series, the disulfide cyclizations in the same region as the one in the earlier studies

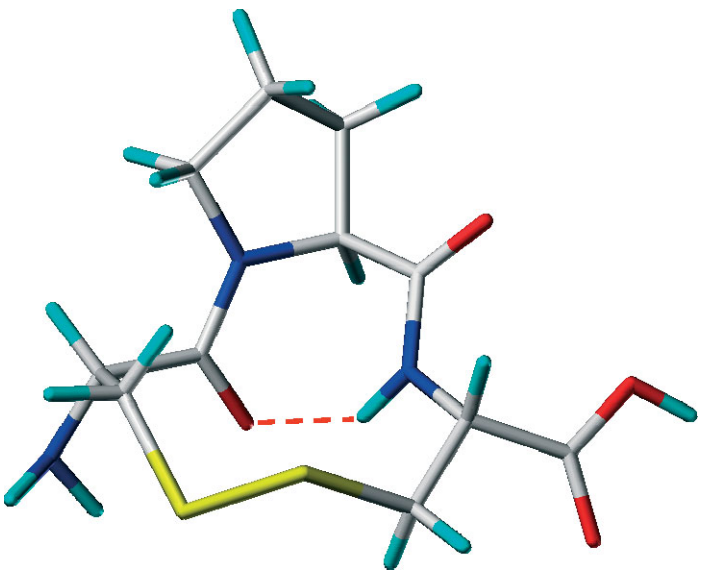

Figure 3 The Cys-Pro-Cys moiety in compound **4** adopting an inverse γ-turn conformation.

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with angiotensin II delivered ligands considerably less potent than angiotensin IV itself. Thus, the cyclized [$Hcy^{1,3}$]angiotensin IV derivative **6** was a ten times weaker binder than angiotensin IV and the cyclized [$Cys^{1,3}$]angiotensin IV derivative **1** lacked activity. Apparently, reducing the ring size by two atoms had a dramatic effect on the bioactivity. Surprising were the results obtained with **7** and **8**, both containing 12-membered ring systems. Although **8** is ten times less potent than angiotensin IV and is equipotent with the homocysteine derivative, it is still a significantly better ligand than **7** encompassing a very similar ring system. We conclude that **6** and **8** can adopt conformations resembling the bioactive conformation of angiotensin IV, while **1** and **7** cannot.

Both experimental and theoretical conformational analysis of model compounds with the general formula Ac-X-Ala-Y-NHMe where X and Y represent Cys or Hcy were performed by others and us [50–52]. These studies also included an analysis of the β- and γ-turn inducing properties of the cyclic peptides. Calculations showed that all four possible combinations of Cys/Hcy containing peptides preferentially adopted the inverse γ-turn conformation, while the classical γ-turn or β-turn conformation was less preferred [50]. Interestingly, the γ-turn inducing capacity was especially pronounced in the Cys/Cys cyclized compound (Figure 3). Among the Cys/Cys cylized compounds, only compound **4** had a similar affinity as compared to angiotensin IV. We therefore hypothesize that angiotensin IV adopts a γ-turn like conformation centered on Pro in its bioactive conformation. In this conformation the *C*-terminal carboxyl group is closer to the *N*-terminal end, which might provide a rationale for the fact that also the truncated angiotensin IV (1–5), lacking the *C*-terminal Phe residue, retains some activity [33]. The fact that compounds **1**–**3** exhibit low affinity suggests that a γ-turn-like conformation is not adopted by angiotensin IV in this region. The reason for the low activity of **1**–**3** may, for example, be due to conformational effects induced by the cyclic moiety or that a key amino acid residue involved in binding has been substituted.

The cystine derivative **4**, with its 11-membered ring system inhibited the catalytic activity of recombinant human IRAP and AP-N, transiently transfected in HEK293 cells; and of IRAP natively expressed in CHO-K1 cells (Table 1). The K_i value of compound **4** for inhibiting the IRAP activity in those experimental systems is very similar to those previously determined for angiotensin IV (recombinant IRAP: $K_i = 62.4 \pm 17.5$ nM, endogenous IRAP in CHO-K1 cells: $K_i = 161.2 \pm 35.0$ nM). Compound **4** displayed five to ninefold lower potency for inhibiting recombinant AP-N activity. Similarly, angiotensin IV also displayed lower potency for inhibiting recombinant AP-N activity ($K_i = 841.4 \pm 37.8$ nM) [43].

To compare the potential breakdown of the disulfide derivative **4** and angiotensin IV by metalloproteases, CHO-K1 cell membranes were preincubated with increasing concentrations of each compound either in the presence or absence of the chelators EDTA and 1.10-phenantrolin before the binding assay with [^{125}I]angiotensin IV (in the presence of EDTA and 1.10-phenantrolin). EDTA and 1.10-phenantrolin are well known for their ability to block IRAP, AP-N and other metalloproteinase activity [53] and, when present in the preincubation, they produced a marked leftward shift of the angiotensin IV inhibition curve (Figure 2(B), Table 4). This suggests that, in the absence of such chelators, angiotensin IV is rapidly degraded by metalloproteases present in the CHO-K1 cell membranes. By contrast, a much smaller shift was observed for compound **4**, indicating that this peptide is more stable than angiotensin IV.

CONCLUSIONS

In summary, we have demonstrated that disulfide cyclizations of angiotensin IV can deliver ligands with high IRAP/AT_4 receptor affinity. One ligand that encompasses an 11-membered ring system (**4**) was found to be equipotent to angiotensin IV. Similar to angiotensin IV, enzyme activity inhibition studies revealed that this compound displays five to ninefold higher potency for human IRAP than for AP-N. In contrast, compound **4** was proven to be considerably more stable than angiotensin IV toward degradation. The compound provides a promising starting point for further optimization toward more drug-like derivatives and we tentatively propose that the *C*-terminal of angiotensin IV adopts an inverse γ-turn in its bioactive conformation.

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