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# N- and C-terminal structure–activity study of angiotensin II on the angiotensin $AT_2$ receptor

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**Abstract**

The predominant angiotensin II receptor expressed in the human myometrium is the angiotensin $AT_2$ receptor. This preparation was used for a structure–activity relationship study on angiotensin II analogues modified in positions 1 and 8. The angiotensin $AT_2$ receptor present on human myometrium membranes displayed a high affinity ($pK_d = 9.18$) and was relatively abundant (53–253 fmol/mg of protein). The pharmacological profile was typical of an angiotensin $AT_2$ receptor with the following order of affinities: (angiotensin III ≥ angiotensin II > angiotensin I > PD123319 > angiotensin-(1–7) > angiotensin-(1–6) ≈ angiotensin IV ≫ Losartan). Modifications of the N-terminal side chain and of the primary amine of angiotensin II were evaluated. Neutralisation of the methylcarboxylate (Asp) to a methylcarboxamide (Asn) or to a hydroxymethyl (Ser) or substitution for a methylsulfonate group (cysteic acid) improved the affinity. Extension from methylcarboxylate (Asp) to ethylcarboxylate (Glu) did not affect the affinity. Introduction of larger side chains such as the bulky *p*-benzoylphenylalanine (*p*-Bpa) or the positively charged Lys did not substantially affect the affinity. Complete removal of the side chain (angiotensin III), however, resulted in a significant affinity increase. Removal or acetylation of the primary amine of angiotensin II did not noticeably influence the affinity. Progressive alkylation of the primary amine significantly increased the affinity, betain structures being the most potent. It appears that quite important differences exist between the angiotensin $AT_1$ and $AT_2$ receptors concerning their pharmacological profile towards analogues of angiotensin II modified in position 1. On position 8 of angiotensin II, a structure–activity relationship on the angiotensin $AT_2$ receptor was quite similar to that observed with angiotensin $AT_1$ receptor. Bulky, hydrophobic aromatic residues displayed affinities similar to or even better than [Sarcosine$^1$]angiotensin II. Aliphatic residues, especially those of reduced size, caused a significant decrease in affinity especially [Sarcosine$^1$, Gly$^8$]angiotensin II who showed a 30-fold decrease. Introduction of a positive charge (Lys) at position 8 reduced the affinity even further. Stereoisomers in position 8 (L → D configuration) also induced lower affinities. The angiotensin $AT_2$ receptor display a structure–activity relationship similar to that observed on the $AT_1$ receptor for the C-terminal position of the peptide hormone. Position 1 structure–activity relationships are however fundamentally different between the angiotensin $AT_1$ and $AT_2$ receptor. © 1998 Elsevier Science B.V.

*Keywords:* Angiotensin II; Angiotensin $AT_1$ receptor; Angiotensin $AT_2$ receptor; Myometrium, human; Uterus

## 1. Introduction

Angiotensin II is the active mediator of the renin–angiotensin system and produces a wide variety of physiological effects, including vascular contraction, aldosterone secretion, catecholamine release, glycogenolysis, decreased renal filtration and myoproliferation in heart and vascular tissue (Peach, 1981; Vallotton, 1987; Powell et al., 1990). The development of selective ligands permitted the identification of two angiotensin II receptor subtypes, designated $AT_1$ and $AT_2$ receptors (Bumpus et al., 1991). The angiotensin $AT_1$ receptor is preferentially recognized by Losartan (DuP753, Chiu et al., 1989), whereas the angiotensin $AT_2$ receptor is preferentially recognized by CGP42112 and PD123177 (Whitebread et al., 1989; Chang and Lotti, 1990). The angiotensin $AT_2$ receptor sequence was deduced by the cloning of its gene from human placenta and uterus (Furuta et al., 1992; Tsuzuki et al., 1994). The angiotensin $AT_2$ receptor is a member of the seven-transmembrane domain receptor family and has a low homology (~32%) in the amino acid sequence with the angiotensin $AT_1$ receptor. In the human myometrium membrane preparation (a non-vascular smooth muscle

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membrane preparation), the angiotensin $AT_2$ receptor constituted the vast majority of angiotensin II receptor population (Whitebread et al., 1989; Criscione et al., 1990; Bottari et al., 1991). It was observed that angiotensin $AT_2$ receptor expression ($AT_2$ mRNA) in adult human uterus is consistent with the angiotensin $AT_2$ population estimated from ligand binding data on the same tissue (Whitebread et al., 1989). The biochemical characterization of the angiotensin $AT_2$ receptor from human myometrium shows a glycoprotein nature with an apparent molecular mass around 66–70 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lazard et al., 1994; Servant et al., 1994).

The physiological roles of angiotensin $AT_2$ receptor are still a matter of debate. The vast majority of the classical pharmacological actions of angiotensin II appear to be mediated via the angiotensin $AT_1$ receptor (Timmermans et al., 1993), although, several recently proposed actions are potentially mediated by the angiotensin $AT_2$ receptor. A current hypothesis proposes that the angiotensin $AT_1$ receptor and the angiotensin $AT_2$ receptors have opposing effects which suggests physiological antagonism between the two receptors (Levy et al., 1992; Nakajima et al., 1995; Höhle et al., 1996). The high level of expression of angiotensin $AT_2$ receptor in wound repair, cardiac hypertrophy and after vascular injury (Viswanathan and Saavedra, 1992; Nio et al., 1995) and its abundant expression during embryonic and neonatal development is suggestive of an involvement in tissue growth and/or differentiation. On the other hand, recent studies suggested the implication of the angiotensin $AT_2$ receptor in programmed cell death (apoptosis), oocyte maturation and ovulation as well as counteracting the growth actions of the angiotensin $AT_1$ receptor (Nakajima et al., 1995; Yamada et al., 1996; Yoshimura et al., 1996). The signaling pathways associated to the angiotensin $AT_2$ receptor are still elusive due to the uncertain efficacy of its selective ligands. Important functional differences exist between angiotensin $AT_2$ receptor selective non-peptide ligands (PD123319 and PD123177) when assessed in vivo, whereas in vitro studies revealed rather similar molecular structure and binding properties (Widdop et al., 1993).

Despite the rising interest for the angiotensin $AT_2$ receptor, no systematic structure–activity relationship (SAR) study has yet been reported for this receptor. Recent studies showed that position 1 of angiotensin II, which was previously considered important exclusively for the binding affinity, could also influence the selectivity of angiotensin II receptors from different species including Mycoplasma and chicken (Whitebread et al., 1993; Kempf et al., 1996; Bouley et al., 1997). Furthermore, it has been shown that many different modifications of the phenylalanine residue in position 8 of angiotensin II yield antagonists of the angiotensin $AT_1$ receptor (Regoli et al., 1974). Previous studies have used many peptide and non-peptide analogues of angiotensin II such as Losartan, PD123319, Sarile ([Sarcosine$^1$, Ile$^8$]angiotensin II), Saralasin ([Sarcosine$^1$, Ala$^8$]angiotensin II) and CGP42112 to characterize receptor populations on membrane preparations. Ligand–receptor interaction studies using photolabile angiotensin II analogues have recently shown that neither the N-terminus nor the C-terminus of angiotensin II share the same contact sites on the two angiotensin II receptors (Servant et al., 1997, manuscript in preparation), therefore considerable differences between the structure–activity relationship of the two receptors are expected. Considering that little is known about the structure–activity relationship of angiotensin II analogues on the angiotensin $AT_2$ receptor, we were interested to explore the effects of modifications in positions 1 and 8 of angiotensin II on angiotensin $AT_2$ receptor recognition. We present an in-depth structure–activity study with angiotensin II analogues modified in positions 1 and 8 interacting with the angiotensin $AT_2$ receptor of human myometrium.

## 2. Materials and methods

### 2.1. Materials [1]

Bovine serum albumin, bacitracin, soybean trypsin inhibitor, angiotensin II (human angiotensin II; Asp–Arg–Val–Tyr–Ile–His–Pro–Phe) and [Asn$^1$,Val$^5$]angiotensin II were purchased from Sigma Chemical Co. (St. Louis, MO). Angiotensin I (human angiotensin I; Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu), angiotensin III (Arg–Val–Tyr–Ile–His–Pro–Phe), angiotensin IV (Val–Tyr–Ile–His–Pro–Phe), angiotensin (1–7) (Asp–Arg–Val–Tyr–Ile–His–Pro) and angiotensin (1–6) (Asp–Arg–Val–Tyr–Ile–His) were purchased from Bachem (Torrance, CA). L-163017 (6-[benzoylamino]-7-methyl-2-propyl-3-[[2′-[*N*-[3-methyl-1-butoxylcarbonylaminosulfonyl][1,1′]-biphenyl-4-yl]methyl]-3H-imidazol[4,5-bipyridine]) were generous gifts from Merck, PD123319 (1-[4(dimethyl-amino)3-methylphenyl]methyl-5-(diphenylacetyl)4, 5,6,7-tetrahydro-1H-imidazo [4,5-c] pyridine-6-carboxylic acid) was a generous gift from Parke-Davis Warner-Lambert. Losartan (or DuP753, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole potassium salt) was provided by DuPont Pharmaceuticals and CGP42112 (*N*-α-nicotinoyl–Tyr–(*N*-α-Cbz–Arg)–Lys–His–Pro–Ile) was provided by Novartis (Switzerland). All other angiotensin II analogues modified in positions 1 or 8 were synthesized in our laboratory. Solid phase peptide synthesis was carried out using the Boc-TFA-HF protection strategy as reported earlier (Escher

[1] Abbreviations are according to the IUPAC-IUB Commission for biochemical nomenclature; additional abbreviations are explained in the text. All optically active amino acids are of the L-configuration unless stated otherwise.

et al., 1978b). The crude peptides were filtered on Sephadex G-15 with 2 M acetic acid. The fractions containing the peptides were pooled and purified by low pressure gel chromatography (reversed phase C-18) using a linear gradient of acetonitrile. The purified peptides were characterized by analytical high pressure liquid chromatography and by mass spectrometry (FAB-MS, Hewlett Packard 5988A instr.). The radioligand, $^{125}$I-angiotensin II ([Asp$^1$, $^{125}$I-Tyr$^4$, Ile$^5$]angiotensin II), was prepared with iodogen as described by Fraker and Speck (1978). The product was purified to apparent homogeneity by high pressure liquid chromatography (reversed phase C-18) and its specific radioactivity ($\approx$ 1000 Ci/mmol) was determined by self-displacement in the binding system.

### 2.2. Tissue samples

Human myometrial tissue was obtained immediately after surgery from 54 women who underwent hysterectomy for different benign conditions (e.g. genital prolapse or uterine fibroids). The age of the patients ranged from 35 to 60 years old. Their informed consent was obtained and the study was approved by the local ethics committee of the faculty of Medicine (University of Sherbrooke, Sherbrooke, Canada). Myometrial tissues were collected from the uterine corpus outside the endometrium and serosa in areas free of macroscopically visible anomalies (e.g. myomas). They were then cut into small pieces, flash-frozen in liquid nitrogen and stored at $-80$°C.

### 2.3. Preparation of human myometrium membranes

Human myometrium membranes were prepared by using the modified method of Servant et al. (1994). For each preparation, 3 to 7 tissue samples were thawed in ice-cold (4°C) medium containing 20 mM sodium bicarbonate, minced and homogenized for 2 min with a Sorvall Omni-Mixer set at force 10. The material was rehomogenized for three periods of 10 s with a Polytron homogenizer set at force 11. The homogenate was centrifuged at $500 \times g$ for 20 min, then the supernate was recentrifuged at $35\,000 \times g$ for 20 min. The pellet was resuspended in a medium containing 25 mM Tris · HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml bacitracin and 50 $\mu$g/ml soybean trypsin inhibitor to a final concentration of 2–5 mg of protein/ml. Aliquots were flash-frozen and stored at $-80$°C. The amount of protein was determined by the method of Lowry et al. (1951).

### 2.4. Binding assays

Human myometrium membrane samples (75 $\mu$g of protein) were incubated in a medium containing 25 mM Tris · HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 1 mM EDTA, 2 mg/ml bacitracin and 50 $\mu$g/ml soybean trypsin inhibitor for 3 h at 37°C in a final volume of 500 $\mu$l with $^{125}$I-angiotensin II (0.12 nM), 1 $\mu$M of Losartan and selected concentrations of angiotensin II analogues. Incubations were terminated by adding 3.0 ml of ice-cold 25 mM Tris · HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, followed immediately by filtration under vacuum through Gelman A/E glass fiber filters that had been presoaked in binding medium. The receptor-bound radioactivity was analysed by gamma counting.

### 2.5. Analysis of binding data

The concentration necessary to inhibit 50% of specifically bound radioligand ($IC_{50}$) varied somewhat with the radioligand concentration [L] and the $K_d$ of the radioligand. $pK_d$ at equilibrium was determined using the Cheng–Prussof equation (Cheng and Prusoff, 1978):

$$pK_d = \frac{-\log IC_{50}}{1 + [L]/K_d^1}$$

where $K_d^1$ is the dissociation constant of the radiolabel $^{125}$I-angiotensin II, $K_d^1 = 0.81$ nM.

Statistical analyses were made by using paired or unpaired Student *t*-tests when applicable to determine the statistical significance of the results. Differences were considered significant at $P < 0.05$.

## 3. Results

In saturation studies, membranes were incubated with increasing concentrations of $^{125}$I-angiotensin II (Fig. 1). Scatchard analysis (Fig. 1, inset) of these data revealed

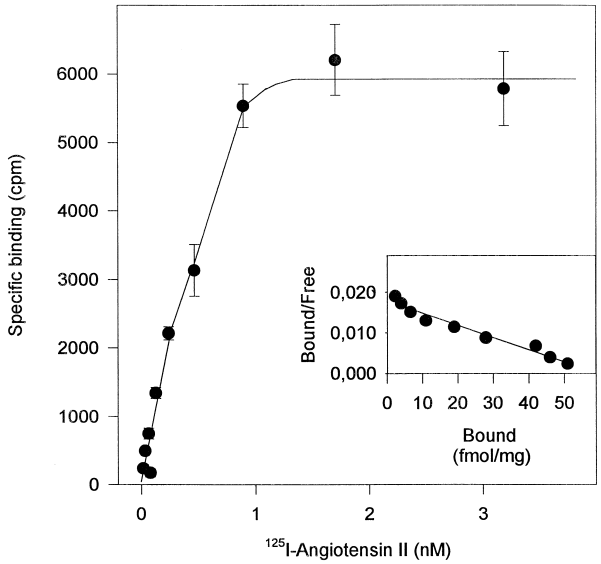


Fig. 1. Saturation of $^{125}$I-angiotensin II binding sites on human myometrium membranes. Membranes (75 $\mu$g of protein) were incubated at 37°C for 3 h in the presence of increasing concentration of $^{125}$I-angiotensin II. Non-specific binding was determined in the presence of 1 $\mu$M angiotensin II. The Scatchard transformation of the same data is shown in the inset. Each point represents the mean ± S.D. of triplicate determinations. Similar data were obtained in two further separate experiments.

only one class of high affinity binding sites with a $pK_d$ of $9.20 \pm 0.24$ and a maximal binding capacity of $76 \pm 23$ fmol/mg of protein (3 experiments). When $^{125}$I-angiotensin II (0.12 nM) was incubated with human myometrium membranes for 3 h at 37°C, approximately 3% of the radioactivity remained specifically bound to the subcellular particles. The addition of increasing concentrations of unlabeled angiotensin II diminished $^{125}$I-angiotensin II binding in a dose-dependent manner (Fig. 2). Scatchard analysis of binding data revealed only one class of high affinity binding sites with a $pK_d$ of $9.09 \pm 0.11$ and a maximal binding capacity of $174 \pm 66$ fmol/mg of protein (5 experiments).

The specificity of angiotensin II binding sites on these membranes was analyzed by dose-displacement experiments using angiotensin II and other ligands. In Table 1, both CGP42112 and PD123319 (selective angiotensin $AT_2$ receptor antagonists) showed respective $pK_d$ of 10.55 and 7.35 whereas Losartan (selective angiotensin $AT_1$ receptor antagonist) showed no significant inhibitory effect on the binding of $^{125}$I-angiotensin II. Angiotensin I, the precursor of angiotensin II, showed a weak affinity about 40 times lower than that of angiotensin II in the presence of 1 $\mu$M Cilazapril, an angiotensin II converting enzyme inhibitor. Angiotensin III showed an affinity similar to that of angiotensin II ($pK_d \approx 9.59$). Other degradation products of angiotensin II, including angiotensin IV, angiotensin (1–7) and angiotensin (1–6), showed affinities 2373, 934 and 2374 times lower respectively that of angiotensin II. Substitution of Ile$^5$ by Val$^5$ decreased the affinity of angiotensin II from a $pK_d$ of 9.59 to 9.18.

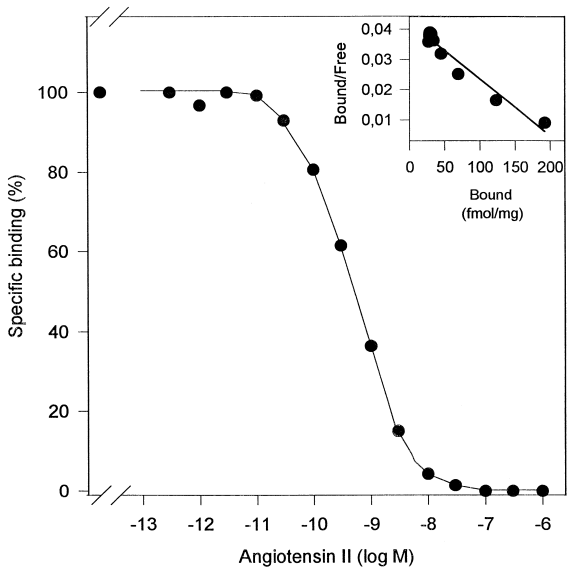


Fig. 2. Effect of increasing concentrations of angiotensin II on $^{125}$I-angiotensin II specific binding to human myometrium membranes. Membranes (75 $\mu$g of protein) were incubated at 37°C for 3 h in medium containing $^{125}$I-angiotensin II (100 000 cpm) and increasing concentrations of unlabeled angiotensin II. After 3 h, the incubation was stopped by filtration. The Scatchard transformation of the same data is shown in the inset. Each point is the mean of duplicate which had less than 10% of variation. These experiments are representatives of three separate experiments.

Table 1
Specificity of $^{125}$I-angiotensin II binding to human myometrium membranes

| Abbreviation | $pK_d$ | R.A. | *n* |
|---|---|---|---|
| Angiotensin I | $7.95 \pm 0.18$ | 2.33 | 4 |
| Angiotensin II | $9.59 \pm 0.16$ | 100 | 7 |
| Angiotensin III | $9.89 \pm 0.31$ | 200 | 6 |
| Angiotensin IV | $6.15 \pm 0.11$ | 0.04 | 3 |
| Angiotensin (1–7) | $6.67 \pm 0.12$ | 0.12 | 3 |
| Angiotensin (1–6) | $6.16 \pm 0.13$ | 0.04 | 3 |
| Losartan | $< 4$ | 0.0003 | 3 |
| PD123319 | $7.35 \pm 0.04$ | 0.58 | 5 |
| CGP42112 | $10.55 \pm 0.20$ | 929 | 4 |
| L-163017 | $8.51 \pm 0.13$ | 8.50 | 3 |

$pK_d$ is the negative log of dissociation constant.
R.A. is the relative affinity of the analogue to angiotensin II (100%).
*n* is the number of experiments in duplicate.
Values are means ± SD.

In Fig. 3, typical dose–displacement curves illustrate the inhibition of $^{125}$I-angiotensin II binding by angiotensin II analogues modified in position 1 (top) or position 8 (bottom). Table 2 shows the relative affinities of the analogues modified in position 1. The effects of substituting the natural methylcarboxy group (Asp) for the methylcarboxamide (Asn) or for the ethylcarboxy group (Glu) are shown by peptides **1**–**3**. The capacity of [Gly$^1$]angiotensin II (peptide **4**) to inhibit $^{125}$I-angiotensin II binding illustrates the effect of modifications of the N-terminal primary amine (peptides **5**–**8**) and of the side chain at position 1 (peptides **16**–**23**). Peptides **5**–**7** show the effect of mono-, di- and trialkylation of the terminal primary amine [$N^\alpha$-MeGly (Sarcosine), $N^\alpha,N^\alpha$-$Me_2$Gly ($Me_2$Gly) and $N^\alpha,N^\alpha,N^\alpha$-$Me_3$Gly ($Me_3$Gly)] whereas peptide **8** shows the effect of acylation [$N^\alpha$-acetylGly (AcGly)] at this position. Glycine, which possesses only a hydrogen atom as a lateral chain, amino acids containing non-carboxylic polar residues (serine, cysteine, cysteic acid, peptides **16**–**18**), aminoisobutyryl (Aib), lysine (peptide **19** and **20**) and amino acids containing photoreactive moieties (peptides **21**–**23**), all showed relatively high affinities for the angiotensin $AT_2$ receptor. Peptides **9** and **10** illustrate the same effects of trimethylation on acylation of the terminal primary amine of the serine residue ($Me_3$Ser and AcSer) as has been observed on Gly$^1$ (**7**, **8**). Peptides **11**–**13** and **15** ([Ac$^1$]angiotensin II, [Suc$^1$]angiotensin II, [Lac$^1$]angiotensin II and [$N_3$-phenylacetyl$^1$]angiotensin II) are the respective deamino forms of the following angiotensin II analogues: [Gly$^1$]angiotensin II (**4**), [Asp$^1$]angiotensin II (angiotensin II, **1**), [Ser$^1$]angiotensin II (**16**) and [$N_3$-Phe$^1$]angiotensin II (**22**), showing the non-influence of deamination.

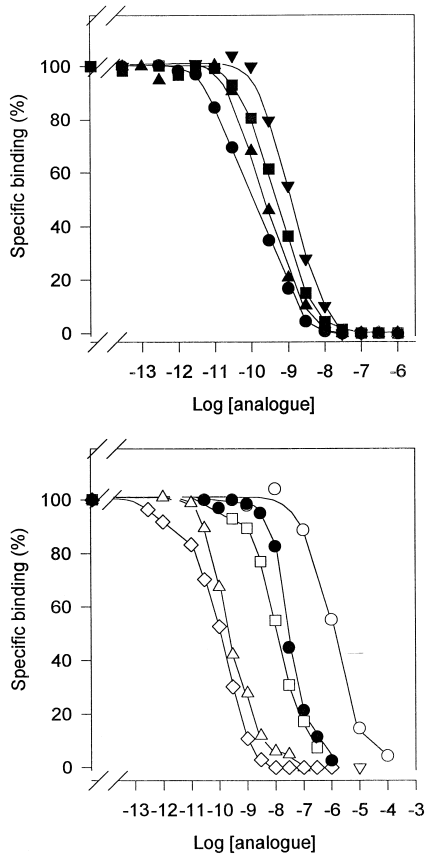


Fig. 3. Concentration-dependent inhibition of specific $^{125}$I-angiotensin II binding on human myometrium membranes by unlabeled analogues modified in position 1 (upper panel) or position 8 (lower panel). Membranes were incubated as in Fig. 1. Legend: (upper panel) [Asp$^1$]angiotensin II (1, ●), [Gly$^1$]angiotensin II (4, ■), [Me$_3$Gly$^1$]angiotensin II (7, ▲), [$N_3$-Phe$^1$]angiotensin II (22, ▼) and (lower panel) [Sarcosine$^1$, Phe$^8$]angiotensin II (31, ◇), [Sarcosine$^1$, Gly $^8$]angiotensin II (25, ●), [Sarcosine$^1$, Trp$^8$]angiotensin II (33, □), [Sarcosine$^1$, Tyr$^8$]angiotensin II (38, ▽) and [Sarcosine$^1$, Lys$^8$]angiotensin II (30, ○). Each point represents the mean of duplicate determinations. The variation between duplicates was less than 10%. The data are expressed as values relative to the total binding observed in the absence of unlabeled ligand (3795 ± 578 cpm) and corrected for non-specific binding (364 ± 17 cpm). These experiments are representatives of three to seven separate experiments.

Tables 3 and 4 show the relative affinities of the compounds modified in position 8. Peptides **1**, **31** and **40** ([Asp$^1$, Phe$^8$]angiotensin II, [Sarcosine$^1$, Phe$^8$]angiotensin II and [Sarcosine$^1$, $N_3$-Phe$^8$]angiotensin II) are compared to their respective diastereomers: [Asp$^1$, D-Phe$^8$]angiotensin II (**24**), [Sarcosine$^1$, D-Phe$^8$]angiotensin II (**32**) and [Sarcosine$^1$, $N_3$-D-Phe$^8$]angiotensin II (**41**). Peptides **25**–**31** (Table 3) demonstrate the effect of an increase in the hydrophobic character of the lateral chain on $^{125}$I-angiotensin II binding to the human angiotensin AT$_2$ receptor. [Sarcosine$^1$, Gly$^8$]angiotensin II (peptide **25**) has been used as reference due to the absence of a side chain. Table 4 shows the effect of different substitutions on the Phe$^8$ aromatic ring on $^{125}$I-angiotensin II binding to AT$_2$. Peptides **33** and **34** ([Sarcosine$^1$, Trp$^8$]angiotensin II and [Sarcosine$^1$, Pyr$^8$]angiotensin II (Pyr = pyrenylalanine) are, respectively, heterocyclic and polycyclic aromatic compounds. Peptides **36** to **42** are angiotensin II analogues

Table 2
Binding properties of modified angiotensin II analogues in position 1 on angiotensin AT$_2$ receptor

| No. | Abbreviation | p$K_d$ | R.A. | *n* |
|---|---|---|---|---|
| 1 | [Asp$^1$]angiotensin II | 9.18 ± 0.20 | 100 | 7 |
| 2 | [Asn$^1$]angiotensin II | 9.84 ± 0.16 | 457 | 3 |
| 3 | [Glu$^1$]angiotensin II | 9.36 ± 0.27 | 149 | 4 |
| 4 | [Gly$^1$]angiotensin II | 9.84 ± 0.20 | 454 | 7 |
| 5 | [Sar$^1$]angiotensin II | 10.07 ± 0.02 | 769 | 6 |
| 6 | [Me$_2$Gly$^1$]angiotensin II | 10.22 ± 0.12 | 1072 | 4 |
| 7 | [Me$_3$Gly$^1$]angiotensin II | 10.31 ± 0.27 | 1335 | 4 |
| 8 | [AcGly$^1$]angiotensin II | 9.81 ± 0.16 | 419 | 5 |
| 9 | [Me$_3$Ser$^1$]angiotensin II | 10.37 ± 0.13 | 1521 | 3 |
| 10 | [AcSer$^1$]angiotensin II | 9.60 ± 0.22 | 258 | 4 |
| 11 | [Ac$^1$]angiotensin II | 9.82 ± 0.09 | 436 | 4 |
| 12 | [Suc$^1$]angiotensin II | 9.25 ± 0.14 | 117 | 4 |
| 13 | [Lac$^1$]angiotensin II | 9.64 ± 0.31 | 287 | 5 |
| 14 | [Mpr(*S*-Acm)$^1$]angiotensin II | 8.85 ± 0.04 | 46 | 3 |
| 15 | [$N_3$-phenylacetyl$^1$]angiotensin II | 9.07 ± 0.12 | 77 | 3 |
| 16 | [Ser$^1$]angiotensin II | 9.60 ± 0.22 | 258 | 4 |
| 17 | [Cys(*S*-Acm)$^1$]angiotensin II | 9.38 ± 0.15 | 156 | 4 |
| 18 | [cysteic acid$^1$]angiotensin II | 10.09 ± 0.07 | 807 | 4 |
| 19 | [Aib$^1$]angiotensin II | 9.71 ± 0.15 | 337 | 3 |
| 20 | [Lys$^1$]angiotensin II | 9.46 ± 0.20 | 187 | 3 |
| 21 | [L-BPA$^1$]angiotensin II | 9.54 ± 0.09 | 226 | 4 |
| 22 | [$N_3$-Phe$^1$]angiotensin II | 8.92 ± 0.27 | 54 | 5 |
| 23 | [$N_3$-benzoyl$^1$]angiotensin II | 9.34 ± 0.26 | 143 | 3 |

Abbreviations: Sarcosine (Sar), lactyl (Lac), 3-mercaptopropionyl (Mpr), acetamidomethylthio (*S*-Acm), acetyl (Ac), aminoisobutyryl (Aib), *ρ*-benzoylphenylalanine (Bpa).
All angiotensin II analogues have a valine in position 5.
p$K_d$ is the negative log of dissociation constant.
R.A. is the relative affinity of the analogue compared to angiotensin II (100%).
*n* is the number of experiments in duplicate.
Values are means ± SD.

Table 3
Binding properties of modified angiotensin II analogues in position 8 on the angiotensin AT$_2$ receptor

| No. | Abbreviation | p$K_d$ | R.A$^a$. | R.A$^b$. | *n* |
|---|---|---|---|---|---|
| 1 | [Asp$^1$, Phe$^8$]angiotensin II | 9.18 ± 0.20 | 100 | 4466 | 7 |
| 24 | [Asp$^1$, D-Phe$^8$]angiotensin II | 8.98 ± 0.11 | 64 | 2818 | 4 |
| 25 | [Sar$^1$, Gly$^8$]angiotensin II | 7.53 ± 0.03 | 2.34 | 100 | 4 |
| 26 | [Sar$^1$, Ala$^8$]angiotensin II | 8.64 ± 0.07 | 29 | 1286 | 4 |
| 27 | [Sar$^1$, Ile$^8$]angiotensin II | 9.75 ± 0.10 | 372 | 16386 | 3 |
| 28 | [Sar$^1$, Leu$^8$]angiotensin II | 9.01 ± 0.05 | 68 | 3024 | 5 |
| 29 | [Sar$^1$, Met$^8$]angiotensin II | 9.80 ± 0.04 | 418 | 18331 | 3 |
| 30 | [Sar$^1$, Lys$^8$]angiotensin II | 5.88 ± 0.06 | 0.05 | 2.22 | 3 |
| 31 | [Sar$^1$, Phe$^8$]angiotensin II | 10.07 ± 0.01 | 776 | 34506 | 4 |

All angiotensin II analogues have a valine in position 5.
Abbreviations: sarcosine (Sar).
p$K_d$ is the negative log of dissociation constant.
R.A$^a$. is the relative affinity of the analogue compared to angiotensin II (100%).
R.A$^b$. is the relative affinity of the analogue compared to [Sar$^1$, Gly$^8$]angiotensin II (100%).
*n* is the number of experiments in duplicate.
Values are means ± SD.

Table 4
Binding properties of modified angiotensin II analogues on the aromatic ring in position 8 on the angiotensin $AT_2$ receptor

| No. | Abbreviation | $pK_d$ | R.A. | *n* |
|---|---|---|---|---|
| 31 | [Sar$^1$, Phe$^8$]angiotensin II | $10.07 \pm 0.01$ | 100 | 4 |
| 32 | [Sar$^1$, D-Phe$^8$]angiotensin II | $9.35 \pm 0.22$ | 19 | 3 |
| 33 | [Sar$^1$, Trp$^8$]angiotensin II | $7.83 \pm 0.04$ | 0.57 | 4 |
| 34 | [Sar$^1$, Pyr$^8$]angiotensin II | $10.75 \pm 0.15$ | 472 | 4 |
| 35 | [Sar$^1$, Bpa$^8$]angiotensin II | $10.08 \pm 0.05$ | 100 | 3 |
| 36 | [Sar$^1$, Cl-Phe$^8$]angiotensin II | $10.52 \pm 0.05$ | 283 | 4 |
| 37 | [Sar$^1$, Br-Phe$^8$]angiotensin II | $10.68 \pm 0.03$ | 405 | 3 |
| 38 | [Sar$^1$, Tyr$^8$]angiotensin II | $9.82 \pm 0.13$ | 56 | 4 |
| 39 | [Sar$^1$, Phe-*S*-Acm$^8$]angiotensin II | $8.97 \pm 0.16$ | 7.87 | 4 |
| 40 | [Sar$^1$, $N_3$-Phe$^8$]angiotensin II | $10.44 \pm 0.11$ | 236 | 4 |
| 41 | [Sar$^1$, $N_3$-D-Phe$^8$]angiotensin II | $9.31 \pm 0.06$ | 17 | 3 |
| 42 | [Sar$^1$, $NO_2$-Phe$^8$]angiotensin II | $10.15 \pm 0.04$ | 119 | 3 |
| 43 | [Sar$^1$, $Me_5$Phe$^8$]angiotensin II | $10.77 \pm 0.08$ | 500 | 5 |
| 44 | [Sar$^1$, $Br_5$Phe$^8$]angiotensin II | $9.40 \pm 0.17$ | 21 | 4 |
| 45 | [Sar$^1$, $Cl_5$Phe$^8$]angiotensin II | $10.30 \pm 0.19$ | 170 | 4 |
| 46 | [Sar$^1$, $F_5$Phe$^8$]angiotensin II | $10.16 \pm 0.07$ | 123 | 4 |

Abbreviations: Sarcosine (Sar), acetamidomethylthio (*S*-Acm), pyrenylalanine (Pyr), *ρ*-benzoylphenylalanine (Bpa).
[Sar$^1$, $Br_5$Phe$^8$]angiotensin II is a mixture of D- and L-enantiomer.
All angiotensin II analogues have a valine in position 5.
$pK_d$ is the negative log of dissociation constant.
R.A. is the relative affinity of the analogue compared to [Sar$^1$]angiotensin II (100%).
*n* is the number of experiments in duplicate.
Values are means ± SD.

containing various substituents in the *para*-position of the aromatic side chain of L-Phe. Fig. 4 shows the influence of hydrophobicity of monosubstitution at the para position of the aromatic ring of the C-terminal amino acid on analogue affinity. Peptides **43** to **46** show the influence of polysubstitution of the aromatic hydrogens with atoms of increasing hydrophobicity (i.e. F, Cl, Me, Br). This analogue series was analyzed for even final correlation between receptor affinity and the properties of the various aromatic substituents.

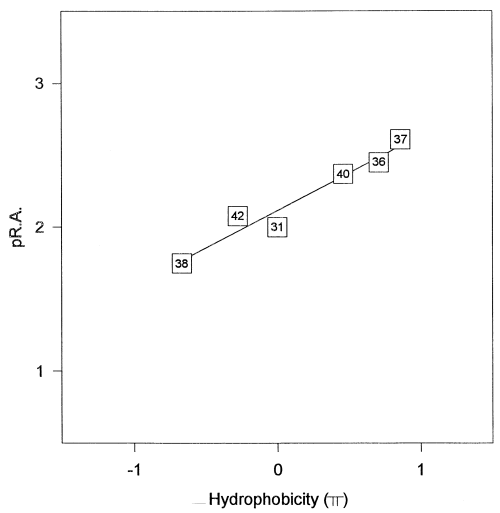


Fig. 4. Correlation between log relative affinity (pR.A.) versus hydrophobicity ($\pi$) of para-position modified analogue of L-Phe$^8$ in modified [Sarcosine$^1$]angiotensin II analogues (**36**–**38**, **40** and **42**). The hydrophobic factor ($\pi$) of the aromatic side-chain substituent of position 8 was calculated according to Hansch et al. (1973). The correlation coefficient is 0.91. Relative affinity ([Sarcosine$^1$]angiotensin II (**31**) = 100%, pR.A. = 2).

## 4. Discussion

We have used a membrane preparation of human myometrium, known to contain almost exclusively the type 2 receptor for angiotensin II (Servant et al., 1994). We have found that the order of affinity of angiotensin I and its degradations products is as follows: angiotensin III ≥ angiotensin II > angiotensin I > angiotensin (1–7) > angiotensin (1–6) ≈ angiotensin IV (Table 1). A similar order of affinity has previously been shown on human myometrium preparations (Whitebread et al., 1989; Cox et al., 1990), $R_3T_3$ cells (Dudley et al., 1991), rat pheochromocytoma PC12W cells (Leung et al., 1992) and ovarian granulosa cells (Pucell et al., 1991). Photolabelling experiments with [Sarcosine$^1$, $^{125}$I-Tyr$^4$, Bpa$^8$]angiotensin II on our preparation revealed a protein of 66 kDa which was not labeled in the presence of an excess of PD123319, as previously reported by Servant et al. (1994) and Lazard et al. (1994) (data not shown).

We have studied in detail the effects of modifications of the primary amine and of the lateral chain of Asp$^1$ on the affinity of angiotensin II analogues on our angiotensin $AT_2$ preparation. Peptides containing a residue which is ionizable at physiological pH, such as methcarboxyl ([Asp$^1$]angiotensin II) and ethcarboxyl ([Glu$^1$]angiotensin II, **3**) showed similar affinities ($pK_d \approx 9.27$). Deletion of the methcarboxy side-chain ([Gly$^1$]angiotensin II, **4**) or its substitution by a methcarboxamide (uncharged) ([Asn$^1$]angiotensin II, **2**) caused a 4-fold increase in affinity ($P > 0.05$). Further substitutions of the carboxylate by other polar groups, such as a hydroxyl ([Ser$^1$]angiotensin II, **16**) or an acetamidomethylthio group ([Cys(*S*-Acm)$^1$]angiotensin II, **17**), also increased the affinity of the angiotensin II analogues for angiotensin $AT_2$ receptor. Bis-alkylation (dimethylation) of the $C^\alpha$ of [Gly$^1$]angiotensin II to produce aminoisobutyryl–angiotensin II ([Aib$^1$]angiotensin II, **19**), did not affect the affinity. Introduction of an aminobutyryl residue at the same position ([Lys$^1$]angiotensin II, **20**), however, resulted in a 2.5-fold decrease in affinity ($P > 0.05$). This reduction of affinity could be due to the $\epsilon$-amino function which may not partition well into the putative hydrophobic interior of the binding pocket (Pérodin et al., 1996). Substitution by hydrophobic and bulky aromatic residues, as found in photolabelling amino groups (i.e. [Bpa$^1$]angiotensin II (**21**), [$N_3$-Phe$^1$]angiotensin II (**22**), [$N_3$-benzoyl$^1$]angiotensin II (**23**)) caused, respectively, 2.3 to 8.4-fold decrease in

affinity ($P > 0.05$). It appears that the bulkiness of the side chain in position 1 of angiotensin II analogues does not modify significantly its affinity for the angiotensin $AT_2$ receptor. The influence of the terminal primary amine on the affinity of angiotensin II analogues for angiotensin $AT_2$ receptor has also been explored: (Table 2, peptide **11**–**15**). [$Ac^1$]angiotensin II, [$Suc^1$]angiotensin II, [$Lac^1$]angiotensin II, [Mpr(*S*-Acm)$^1$]angiotensin II and [$N_3$-phenylacetyl$^1$]angiotensin II which are the respective desamino analogues of Gly, Asp, Ser, Cys(*S*-Acm) and $N_3$-Phe, all displayed similar if not identical affinity compared to their amino analogues for the angiotensin $AT_2$ receptor e.g. $Lac^1$ (**13**) versus $Ser^1$ (**16**). Progressive alkylation of the amino group of $Gly^1$ (**4**) to a mono, di- or even trimethylated state increased the affinity by 2.3 and 2.9-fold ($P > 0.05$) (**4** to **5** to **6** and **7**). Similar acetylation (**10**) or trimethylation (**9**) of $Ser^1$ instead of Gly showed an affinity profile similar to that observed with $Gly^1$ (**4**). The affinity increases observed with the Gly *N*-methylation series are not easily explainable. Hydrogen bonding with one of the ammonium protons can be excluded due to the maximal affinity observed with the quaternary ammonium of [*N*-$Me_3Gly^1$]angiotensin II (**7**) that cannot form such proton-sharing connections. Several negatively charged residues are present in this segment of the angiotensin $AT_2$ receptor such as Asp at positions 3 and 21 and Glu in position 30. Salt-bridge formation between the receptor and the peptide N-terminus could result from this combination. Considering the order of potency $Me_3Gly^1 > Me_2Gly^1 > Sarcosine^1 > Gly^1 > Asp^1$ with the quaternary ammonium ion displaying the best affinity, this salt bridge formation requires removal of the hydrated shell. This is much easier to achieve on the quaternary ammonium function than on a primary amine. This part of the peptide may directly interact with the hydrophilic and glycosylated extracellular part of the elongated N-terminus of the angiotensin $AT_2$ receptor. This suggestion is consistent with the photolabeling results obtained with [$Bpa^1$]angiotensin II (Servant et al., 1997). On the other hand, the indifference observed between the amino- (**4**, **1**, **16**, **17**, **22**) and the corresponding desamino analogues (**11**–**15**) could indicate that the removal of such a hydrate shell requests an energy similar to the energy produced through the formation of the salt bridge, hence an affinity indifference between amino and the corresponding desamino analogues.

The structure–activity relationship observed in position 1 of angiotensin II is quite different when the $AT_1$ and the angiotensin $AT_2$ receptors are compared. Taking into account that the N-terminus of angiotensin II closely interacts with extracellular portions of both the angiotensin $AT_1$ and $AT_2$ receptors in an amphiphilic environment according to the photoaffinity labeling experiments performed on angiotensin $AT_1$ receptor (manuscript in preparation) and angiotensin $AT_2$ receptor (Servant et al., 1997). However, important sequence differences are observed in those parts of the respective receptors. This approach led to the identification of angiotensin II analogues having important receptors selectivities, e.g. peptide 7 that displays a 500-fold selectivity towards angiotensin $AT_2$ receptor (angiotensin $AT_2$ $pK_d = 10.37$; $AT_1$ $pK_d = 7.63$) despite this minor difference with angiotensin II.

To further study the structure–activity relationship of angiotensin II on the angiotensin $AT_2$ receptor, many analogues of angiotensin II modified in position 8 have been synthesized. The angiotensin $AT_2$ receptor binding pocket appears to be sensitive to the configuration of the residues in position 8. [$Sarcosine^1$, $Phe^8$]angiotensin II (**31**), [$Sarcosine^1$, $N_3$-$Phe^8$]angiotensin II (**40**) and [$Asp^1$, $Phe^8$]angiotensin II (**1**) angiotensin II exhibited higher affinities for the angiotensin $AT_2$ receptor than their respective enantiomers ([$Sarcosine^1$, D-$Phe^8$]angiotensin II (**32**), [$Sarcosine^1$, $N_3$-D-$Phe^8$]angiotensin II (**41**) and [$Asp^1$, D-$Phe^8$]angiotensin II (**24**)) ($P > 0.05$). Deletion of the $Phe^8$ (angiotensin (1–7)) decreased by 830-fold the affinity for the angiotensin $AT_2$ receptor. [$Sarcosine^1$, $Gly^8$]angiotensin II (**25**) showed a 345-fold lower affinity for the angiotensin $AT_2$ receptor than angiotensin II. When the side of the chain was increased from Gly (H) to Ala ($CH_3$) (**26**) and then to Leu (**28**), Ile (**27**) and Met (**29**), the affinities also increased by 13, 30, 164 and 183-fold respectively. Interestingly, substitution with a lysine ([$Sarcosine^1$, $Lys^8$]angiotensin II) (**30**) decreased the affinity by about 10 000-fold when compared to a residue of similar length (Met) but not charged. This decrease in affinity could be explained by the presence of the charged $\epsilon$-amino group, which may not partition favorably within the hydrophobic binding pocket of the angiotensin $AT_2$ receptor (Servant et al., 1997). Introduction of a bulky protecting group on this $\epsilon$-Lys function (*p*-nitrobenzyloxycarbonyl, or $NO_2$-Z which abolishes the charge) increased the affinity by 1000-fold ([$Asp^1$, Lys ($NO_2$-Z)$^8$]angiotensin II) ($pK_d = 8.70$) (data not shown).

Despite relatively good affinities of most position 8 analogues of angiotensin II, none of them exhibited a higher affinity than **31** ([$Sarcosine^1$, $Phe^8$]angiotensin II). Earlier studies on the angiotensin $AT_1$ receptor, a linear correlation between the substituent electronegativity and analogue affinity was observed for analogues modified in position 4 but not with analogues modified in position 8 (Escher et al., 1978a; Guillemette et al., 1984). In this study, we observed that no significant correlation could be found, between the obtained affinity and the electronegativity ($\sigma$) ($r = 0.13$) nor the molecular refractory index (MR) ($r = 0.53$). A direct correlation between substituent hydrophobicity ($\pi$) and analogue affinity for the angiotensin $AT_2$ receptor ($r = 0.91$) is however now reported as show in Fig. 4. A similar effect was observed with the pentasubstitution analogues series (peptide **43**–**46**) which contain substantial ring enlargement by replacing the five aromatic ring hydrogens with other residues, which maintain the normal aromatic thickness and planarity. The capacity of these analogues to inhibit the binding of $^{125}$I-

angiotensin II to angiotensin $AT_2$ is better than that of angiotensin II. Although the molecular diameters of fluorine and hydrogen are similar, with fluorine being somewhat larger, a weak but significant increase of affinity is observed with the [Sarcosine$^1$, $F_5$Phe$^8$]angiotensin II (**46**). The most remarkable effect was obtained however with [Sarcosine$^1$, $Me_5$Phe$^8$]angiotensin II (**43**), which is the bulkiest of all the pentasubstituted ring series and whose ring size was roughly equal to the one of pyrene (**34**). Both analogues displayed better affinities for the angiotensin $AT_2$ receptor than angiotensin II itself. The lower affinity of [Sarcosine$^1$, $Br_5$Phe$^8$]angiotensin II (**44**), is somewhat disappointing since it is lower than peptide **43** and even that of the chlorinated analogue **45**. The expected value would have been around $pK_d$ 10.9. It is important however to mention that this particular peptide was a diastereoisomer mixture of [Sarcosine$^1$, L-$Br_5$Phe$^8$]angiotensin II and [Sarcosine$^1$, D-$Br_5$Phe$^8$]angiotensin II. As shown above, D-amino acids in position 8 confer lower affinities to the respective angiotensin II analogues. Similarly to angiotensin $AT_1$ receptor, binding of $^{125}$I-angiotensin II to angiotensin $AT_2$ receptor appears to be stereospecific in regard to position 8 of angiotensin II (Hsieh and Marshall, 1986). On the other hand and similarly to angiotensin $AT_1$ receptor, the electronegativity does not play an important role in position 8 (Phe), where hydrophobicity and steric parameters are more important (Escher et al., 1980). As a simple rule, the more the substituent character is hydrophobic, the better is its affinity for angiotensin $AT_2$ receptor. From our angiotensin II-receptor contact point investigation with photoaffinity labeling peptides, it appears that the N-terminal part of angiotensin II interacts with the exodomain of angiotensin $AT_2$ receptor while the C-terminal end of the angiotensin II molecule interacts with transmembrane domain 3 (Servant et al., 1997). The increased affinity due to increasingly hydrophobic residues is highly suggestive of a predominantly hydrophobic environment around the side chain of the C-terminal amino acid. The difference in affinity between Phe$^8$ and Ile$^8$, (according to hydrophobic parameters quite similar) is probably due to an aromatic–aromatic interaction as already proposed (Nikiforovich and Marshall, 1993).

In conclusion, we have explored in detail the structure–affinity relationship of angiotensin II on the angiotensin $AT_2$ receptor, focusing on positions 1 and 8 of angiotensin II. We have observed that alkylation and deletion of the primary terminal amine increases the affinity of ligands for the angiotensin $AT_2$ receptor. This is in contrast with the structure–activity relationship on the angiotensin $AT_1$ receptor. Position 1 of angiotensin II is important to maintain a higher affinity but it can be exploited to produce more selective analogues for the angiotensin $AT_2$ receptor. Furthermore, we demonstrated that, in regard to the position 8 of angiotensin II, structure–activity relationship on the angiotensin $AT_2$ receptor is similar to that of the angiotensin $AT_1$ receptor.

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