Comparative pharmacology of recombinant rat AT_{1A}, AT_{1B} and human AT₁ receptors expressed by transfected COS-M6 cells

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1 Currently available antagonists and agonists cannot distinguish between angiotensin AT₁ receptor subtypes.

2 We synthesized a series of compounds selected on the basis of having the most diverse structural features with respect to losartan (DuP753), the prototype non-peptide AT₁ receptor antagonist. Using a radioligand-receptor binding assay and membranes prepared from COS-M6 cells transfected with individual AT₁ receptor subtypes, we determined whether any of these compounds could distinguish between the receptor subtypes.

3 The diversity of the structural features of this series of compounds was reflected by the wide range of affinities (pIC₅₀ values) displayed towards competing with [¹²⁵I]-Sar¹Ile⁸ angiotensin II for binding to the AT₁ receptors.

4 Direct comparisons of the pIC₅₀ values of individual compounds for rat AT_{1A}, AT_{1B} and human AT₁ receptors revealed only minor differences.

5 It is concluded that compounds based structurally on losartan are unlikely to distinguish between these receptors.

Keywords: Recombinant receptors; radioligand binding; receptor subtypes; non-peptide antagonists; comparative pharmacology; rat AT_{1A} receptor; rat AT_{1B} receptor; human AT₁ receptor; angiotensin II (AII); losartan (DuP753)

Introduction

The existence of multiple receptors for angiotensin peptides, particularly subtypes of the angiotensin II (AII) receptor, has been postulated for some time, based on the findings of numerous studies of the structure-activity relationships of various agonist and antagonist peptide analogues of AII in a variety of tissues (Peach, 1977). Four years ago, the receptors for AII were pharmacologically characterized, by use of radioligand receptor binding, into two types (Whitebread *et al.*, 1989; Chiu *et al.*, 1989) and assigned more recently the nomenclature, AT₁ and AT₂ (Bumpus *et al.*, 1991). This characterization is based on the differential selectivity of the non-peptide AII receptor antagonists, losartan and PD123177 and also the peptides CGP42112A and *p*-aminophenylalanine⁶AII (Timmermans *et al.*, 1991) for these receptors. The AT₁ subtype fully justifies recognition as a receptor, but the status of the AT₂ subtype beyond that of a binding site is less clear.

Whilst receptors have been defined traditionally by their ligand binding characteristics and function, the advent of molecular biology has allowed identification of apparently different subtypes based on their amino acid sequences rather than their binding profiles and function. The receptor designated AT₁ on the basis of its binding properties and linkage through phospholipase C to raise intracellular calcium has been cloned from cultured bovine adrenal glomerulosa cells (Sasaki *et al.*, 1991), rat aortic vascular smooth muscle cells (Murphy *et al.*, 1991), a rat kidney cDNA library (Iwai *et al.*, 1991), a rat liver cDNA library and a human lung cDNA library (K.T. Pun, personal communication), a human lymphocyte genomic library (Furuta *et al.*, 1992) and a human liver cDNA library (Takayanagi *et al.*, 1992). These receptors possess the seven transmembrane domain structure typical of other G-protein coupled receptors. There is an 8% difference in amino acid sequence between the bovine adrenal and rat vascular AT₁ receptors, although the single polypeptide

encoded by the cDNAs are of identical length. These differences may be attributed to either or both the species concerned and perhaps differences in the function of these receptors related to their anatomical location. The cloning of an AT₁ receptor from a rat adrenal cDNA library with an amino acid sequence 96% homologous to the receptor cloned from rat vascular smooth muscle cells but showing substantial difference in the 5' and 3' non-coding regions led to the use of the terms AT_{1A} for the latter and AT_{1B} for the adrenal receptor (Iwai & Inagami, 1992). Further evidence for particular anatomical localization and, therefore, increasing justification for their consideration as potentially functionally important subtypes came from the observation that the AT_{1B} receptor also appeared to be preferentially expressed in the rat pituitary gland (Kakar *et al.*, 1992). In contrast, so far, subtypes of the human AT₁ receptor have not been found.

Although these subtypes of AT₁ receptors had structural differences and were distributed differentially, currently available antagonists and agonists cannot distinguish between them (Murphy *et al.*, 1991; Kakar *et al.*, 1992; Takayanagi *et al.*, 1992). The aim of the present study was therefore to determine whether, from a group of chemically diverse selective 'AT₁ receptor antagonists', we could find a compound that displayed selectivity for one of three types of angiotensin AT₁ receptor so far cloned, rat AT_{1A}, AT_{1B} and human AT₁. It was important that all three receptors should be studied under identical conditions if subtle differences were to be detected. Therefore, individual cDNAs encoding specific angiotensin receptor subtypes were transfected into COS-M6 cells which then transiently express the receptor. Competition curves for the different drugs were constructed from a radioligand-receptor binding assay and membranes prepared from transfected COS-M6 cells. COS-M6 cells do not express endogenous angiotensin receptors. Thus, we were able to compare and contrast the pharmacology of these AT₁ receptors under identical experimental conditions.

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Methods

Amplification and purification of plasmid DNA encoding AT₁ receptors

The rat AT_{1A} receptor was isolated by combined PCR/hybridisation screening from a size-selected rat liver cDNA library, constructed in the mammalian expression vector pCDM8 (Invitrogen). The Rat AT_{1B} receptor was cloned from a rat adrenal cDNA library into pCDM8. The human AT₁ receptor was obtained by hybridisation screening of a human lung cDNA library in λ gt11 (Clontech), and subcloned into the vector pCDNA1 (Invitrogen). In all three clones, full length receptor expression is driven from the human cytomegalovirus (CMV) promoter-enhancer. Each clone was transformed into the *E. coli* host strain MC1061/P3. All subsequent amplification and purification of the plasmid DNA from the bacteria was identical for each of the receptor subtypes.

Twenty ml of 2YT medium (containing 50 μ g ml⁻¹ kanamycin, 40 μ g ml⁻¹ ampicillin and 12.5 μ g ml⁻¹ tetracycline) was inoculated with the *E. coli* strain MC1061/P3 containing the relevant plasmid. Bacteria were left to grow at 37°C in an orbital shaker (300 r.p.m.). The growth of the bacteria was monitored until the optical density of the medium was approximately 0.5 at A_{600 nm}; 10 ml of medium was then removed and used to inoculate a further 500 ml of 2YT medium (containing the same antibiotics as outlined above). Bacteria were left to grow overnight. Purification of the plasmid DNA was then carried out with a QIAGEN plasmid kit (Hybaid).

Transfection of the plasmid DNA encoding specific angiotensin receptor subtypes into COS-M6 cells

COS-M6 monkey fibroblast cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% v/v foetal calf serum, 2 mM glutamine, 100 u ml⁻¹ penicillin G, 100 μ g ml⁻¹ streptomycin and 0.25 μ g ml⁻¹ amphotericin at 37°C in a humidified atmosphere of air/CO₂ (19:1). The day prior to transfection, cells were seeded at a density of 8 million cells/800 ml flask, so that they were subconfluent the next day. A transfection solution was prepared by mixing DEAE dextran (6 mg), plasmid DNA (30 μ g) and chloroquine (100 μ M) with 15 ml of serum-free DMEM. After washing the cells twice (25 ml) with serum-free DMEM, the transfection solution was added to the cells and left for 3 h. The transfection solution was then removed and the cells washed again (2 $\times$ 25 ml) with serum-free medium; 15 ml of 'shock-solution' (phosphate buffered saline containing 10% v/v dimethylsulphoxide) was then added to the cells for 2 min. After washing with DMEM containing serum (25 ml), 50 ml of medium was added to the cells and the cells left for 3 days, after which, membranes were prepared.

Preparation of membranes

Cultured COS-M6 cells were washed with ice-cold phosphate buffered saline, harvested with a Costar scraper and collected by centrifugation (1000 g for 10 min). The cells were then homogenised (Polytron; maximal setting, 10 s) in ice-cold 50 mM Tris 5 mM EDTA buffer (pH 7.4) and centrifuged for 10 min at 1000 g. The supernatant was then removed and the remaining pellet subjected to a further homogenization (Polytron; maximal setting, 10 s) and centrifuged for 10 min at 1000 g. The combined supernatants from each homogenisation were then centrifuged at 30,000 g for 35 min. The resultant pellet was resuspended in 4 ml of buffer, an aliquot removed for protein determination and the remainder frozen in 0.25 ml aliquots at -40°C.

Radioligand-receptor binding assay

Total reaction volume was 300 μ l, consisting of 150 μ l membranes (30–50 μ g protein; previously been shown to display 5–20,000 c.p.m. specific binding), 75 μ l [¹²⁵I]-Sar¹Ile⁸ AII (0.4 nM) and 75 μ l of varying concentrations of competitor or assay buffer. All drugs were dissolved in 100 μ l absolute ethanol, 100 μ l 1 M NaOH and made up to 10 ml with H₂O to produce 1 mM stock solutions. Further dilutions of drugs were carried out in assay buffer which consisted of 100 mM NaCl, 50 mM Tris-Base (pH 7.4), 10 mM MgCl₂, 1 mM EDTA, 1 mM bacitracin and 0.1% w/v BSA (protease-free). Membranes and radioligand were also diluted in assay buffer. Nine or ten concentrations of competitor were examined, each concentration in duplicate per assay. Membranes were equilibrated with competitor for 60 min at room temperature prior to the addition of radioligand for a further 90 min. Membrane bound radioligand was separated from free radioligand by vacuum filtration and washing (100 mM NaCl; 10 mM MgCl₂, approx 5 ml per assay tube) over glass fibre filters using a Skatron cell harvester. Filters were pretreated with 0.1% v/v polyethylenimine in order to obtain low filter blanks. After filtration the filters were counted in a γ -counter (LKB, Clinigamma, Sweden).

Protein determination

The method of Lowry *et al.* (1951) was followed, with bovine serum albumin used as standard.

Data analysis

Non-specific binding was measured in the presence of 10 μ M unlabelled AII and did not exceed 10% of the total binding. An iterative curve fitting programme, GRAPHPAD (ISI Software), was used to determine the pIC₅₀ values for the competition curves.

Materials

Ampicillin, angiotensin II, bacitracin, bovine serum albumin (protease-free), chloroquin, DEAE-dextran, kanamycin and tetracycline were purchased from Sigma Chemicals (Poole, Dorset); [¹²⁵I]-Sar¹Ile⁸ angiotensin II from New England Nuclear (DuPont, Stevenage); Dulbecco's modified Eagle's medium was purchased from Flow Laboratories (Irvine, Scotland); 2YT medium, antibiotic/antimycotic mixture and glutamine from Gibco (Paisley, Scotland); foetal calf serum from Sera Lab (Crawley, Sussex). Qiagen plasmid kits were purchased from Hybaid (Teddington, Middlesex). The rat AT_{1A}, and human AT₁ receptor cDNAs were cloned in the Department of Molecular Science, Glaxo Group Research. Drs Kevin Catt and Adrian Clark provided the cDNA clone-encoding the rat AT_{1B} receptor. The mammalian expression vectors pCDM8 and pCDNA1 were obtained from Invitrogen (San Diego, CA, U.S.A.). The following antagonists described in this report were synthesized in the Department of Medicinal Chemistry, Glaxo Group Research, Ware, Hertfordshire: L158809 (5,7-dimethyl-2-ethyl-3-[[2'-(1H-tetrazol-5-yl) [1,1']-biphenyl-4-yl]-methyl]-3H-imidazo[4, 5-b]pyridine), EXP3174 (2-n-butyl-4-chloro-1-[(2'-(1H-tetrazol-5-yl) biphenyl-4-yl) methyl] imidazole-5-carboxylic acid), SKF108566 ((E)-alpha-[(2-butyl-1-[(4-carboxyphenyl)methyl]-1H-imidazol-5-yl) methylene]-2-thiophenepropanoic acid), GR137977 (1-[3-bromo-2-[2-(1H-tetrazol-5-yl) -phenyl]-benzofuran-5-yl-methyl]-2-butyl-4-methyl-1, 4, 6, 7-tetrahydro-imidazo-[4,5-E][1,4]-diazepine-5,8-dione), valsartan (3-methyl-2-[pentanoyl-[2'-(1H-tetrazol-5-yl)-biphenyl-4-ylmethyl]-amino]-2S-butyric acid), losartan (2-propyl-4-pentafluoroethyl-1-[(2'-(1H-tetrazol-5-yl) biphenyl-4-yl) methyl] imidazole-5-carboxylic acid), GR117289 (1-[(3-bromo-2-[2-(1H-tetrazol-5-yl)phenyl]-5-benzofuranyl) methyl]-2-butyl-4-chloro-1H-imidazole-5-carboxylic acid), GR131452 (5-butyl-2-isopropyl-4-[2'-(1H-tetrazol-5-yl)-

Table 1 Potency of losartan and related structural analogues in competing with [¹²⁵I]-Sar¹Ile⁸ AII for binding to recombinant rat AT_{1A}, AT_{1B} and human AT₁ receptors

Competitor	Rat AT _{1A}		Rat AT _{1B}		Human AT ₁	
	pIC ₅₀	Hill slope	pIC ₅₀	Hill slope	pIC ₅₀	Hill slope
L158809	9.71 ± 0.07	1.03 ± 0.12	9.32 ± 0.11	1.30 ± 0.10	9.68 ± 0.16	1.18 ± 0.24
EXP 3174	8.78 ± 0.06	1.04 ± 0.13	8.72 ± 0.16	1.09 ± 0.10	8.88 ± 0.01	0.93 ± 0.08
SKF108566	8.47 ± 0.08	0.94 ± 0.08	8.67 ± 0.07	1.05 ± 0.07	8.78 ± 0.07	0.88 ± 0.08
GR137977	8.20 ± 0.07	0.80 ± 0.16	8.17 ± 0.13	1.11 ± 0.10	8.22 ± 0.17	1.10 ± 0.10
Valsartan	8.03 ± 0.03	1.20 ± 0.13	8.26 ± 0.01	1.17 ± 0.03	8.30 ± 0.11	1.04 ± 0.20
Losartan	7.93 ± 0.08	0.95 ± 0.09	8.18 ± 0.02	0.97 ± 0.02	8.20 ± 0.06	0.88 ± 0.04
GR117289	8.00 ± 0.05	1.26 ± 0.19	8.08 ± 0.06	0.95 ± 0.11	8.04 ± 0.10	1.20 ± 0.15
GR131452	7.40 ± 0.11	1.16 ± 0.10	7.46 ± 0.01	1.24 ± 0.16	7.53 ± 0.02	0.98 ± 0.09
GR157249	7.14 ± 0.12	0.98 ± 0.02	7.64 ± 0.05	1.06 ± 0.08	7.52 ± 0.05	0.90 ± 0.06
GR127612	6.85 ± 0.06	1.01 ± 0.05	7.11 ± 0.09	1.24 ± 0.13	7.00 ± 0.05	1.01 ± 0.01
GR109793	6.27 ± 0.06	1.01 ± 0.09	6.66 ± 0.03	0.91 ± 0.02	6.78 ± 0.06	0.91 ± 0.02
PD123177	> 5.00		> 5.00		> 5.00	

Membranes were preincubated with the above compounds for 1 h then incubated for a further 1.5 h in the presence of 0.1 nM [¹²⁵I]-Sar¹Ile⁸ AII, prior to separation of bound from free radioligand. Data are expressed as mean pIC₅₀ ± s.e.mean (*n* = 3).

biphenyl-4-ylmethyl] -2H-pyrazole-3-carboxylic acid), GR-157249 (N-[2-[3-bromo-5-(2-ethyl-4, 5-dimethyl-6-oxo-6H-pyrimidin-1-ylmethyl)-benzofuran-2-yl]-phenyl]-C, C, C-trifluoro-methanesulphonamide), GR127612 (1-[3-bromo-2-[2-(2H-tetrazol-5-yl)-phenyl]-benzofuran-5-ylmethyl] -5-butyl-1H-pyrazole-3-carboxylic acid), GR109793 (4'-(2-butyl-benzolimidazol-1-ylmethyl)-biphenyl-2-carboxylic acid), PD123177 (1-[(4-amino-3-methylphenyl)methyl]-5-(diphenylacetyl)-4, 5, 6, 7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid).

Results

Each competitor was examined three times versus each receptor subtype. The diversity of the structural features of this series of compounds was reflected by the wide range of affinities (pIC₅₀ values) displayed towards competing for binding to the AT₁ receptors (Table 1). The potency series L158809 > EXP3174 = SKF108566 > GR137977 = valsartan = losartan = GR117289 > GR131452 = GR157249 > GR127612 > GR109793 >>> PD123177 was observed for all three AT₁ receptors screened. Direct comparisons of the pIC₅₀ values of individual compounds for the rat AT_{1A}, AT_{1B} and human AT₁ receptors revealed only minor differences; affinities for any given antagonist varied by no more than three fold between the receptor subtypes.

Discussion

Many of the physiological actions of AII appear to be mediated through AT₁ receptors coupled to phospholipase C. Recently, two subtypes of the rat AT₁ receptor have been cloned and termed AT_{1A} and AT_{1B} receptors (Murphy *et al.*, 1991; Iwai & Inagami, 1992). The encoded amino acid sequences of both receptors are 359 amino acids in length and exhibit a high degree of homology (96%). There are 17 amino acid differences in the amino acid sequences between the two rat AT₁ receptors which are dispersed throughout the whole sequence, seven in the extracellular, three in the transmembrane and seven in the intracellular domains. At present only one human AT₁ receptor has been cloned. It is also 359 amino acids in length and exhibits a high degree of amino acid sequence homology (94%) to the rat AT_{1A} receptor. Again, differences in amino acids relative to the rat AT_{1A} receptor, 20 in all, are dispersed throughout the whole receptor, nine in the extracellular, six in the transmembrane and five in the intracellular domains. The amino acid differences in the intracellular domains of these receptors, particularly serine and threonine residues in the carboxy terminal, may reflect the potential to undergo differential phosphorylation

which may lead to differences in the regulation and coupling of these receptors.

The transmembrane domains of G-protein coupled receptors have been associated with formation of the ligand binding site for some time (for review see Kobilka, 1992). Recently, it has been reported that the extracellular domains may also contribute to the binding of ligands (Watling & Krause, 1993). Differences in amino acids in these regions of the AT₁ receptors therefore raise the possibility that they may be pharmacologically distinguishable. Such a possibility is illustrated by the finding that a single amino acid difference in the seventh transmembrane domain between rat and human 5-HT_{1B} receptors is responsible for a distinct difference in the affinities of several ligands for these two receptors (Oksenberg *et al.*, 1992).

The recent development of non-peptide AII receptor antagonists has unequivocally demonstrated the existence of at least two AII receptor subtypes in various tissues, previously unidentifiable by use of peptide analogues of AII (Timmermans *et al.*, 1991). Losartan is regarded as the prototype non-peptide angiotensin AT₁ receptor antagonist. Structurally this compound is a functionalised imidazole linked to an acidic tetrazole moiety by a biphenyl spacer. Since the advent of losartan many non-peptide antagonists of angiotensin II have been described. In an attempt to identify a compound (ligand) which has selectivity for one of the AT₁ receptor subtypes, we have evaluated a series of compounds selected on the basis of having the most diverse structural features with respect to losartan. The compounds chosen have one or more of the following features: a different heterocycle from the imidazole, an alicyclic replacement for the imidazole, different functional groups in the imidazole or its replacement, an alternative spacer to a biphenyl, a different acidic group from the tetrazole. The AT₂ receptor antagonist PD123177 was also included in the set for comparison (for structures see Figure 1).

The diversity of the structural features of this series of compounds was reflected by the wide range of affinities (pIC₅₀ values) displayed towards competing for binding to the AT₁ receptors. The potency series L158809 > EXP3174 = SKF108566 > GR137977 = valsartan = losartan = GR117289 > GR131452 = GR157249 > GR127612 > GR109793 >>> PD123177 was observed for all three AT₁ receptors screened. Direct comparisons of the pIC₅₀ values of individual compounds for the rat AT_{1A}, AT_{1B} and human AT₁ receptors revealed only minor differences, indicating that none of the compounds screened displaced differential selectivity. As a cautionary note, it should be appreciated that the pIC₅₀ values given here may not be a good measure of the absolute affinities of these compounds, due to the presence of 0.1% w/v BSA in the binding assay. This is particularly

Figure 1 Structures of the AT₁ receptor non-peptide antagonists synthesized.

relevant to the diacidic non-peptide compounds, for example EXP3174 and GR117289, where it has been observed that removal (Chiu *et al.*, 1991) or significant reduction of BSA to 0.001% w/v (Robertson *et al.*, 1992) in the binding assay markedly increased their apparent affinities. This has been attributed to a direct interaction of diacidic non-peptide compounds with BSA (Chiu *et al.*, 1991).

Previously, using a limited number of conventional angiotensin II peptide analogues and the two prototype non-peptide antagonists losartan and PD123177, several groups have reported the pharmacological profile of an individual cloned AT₁ receptor (Murphy *et al.*, 1991; Kakar *et al.*, 1992; Takayanagi *et al.*, 1992). A comparison of these limited data

reveals that all three clones appear to display essentially indistinguishable affinities for these compounds. Our studies, using a much wider range of non-peptide compounds, but this time under identical experimental conditions, indicated that compounds based structurally on losartan are unlikely to distinguish between these receptors.

However, further compounds could be synthesized which would discriminate either between these receptors already cloned or others yet to be identified. There are many precedents with G-protein coupled receptors for multiple subtypes detected using molecular biology techniques prior to establishing compounds able to distinguish them. The cloned AT₁ receptors studied here appear to couple only to mobil-

ization of intracellular calcium. The observation of a losartan-sensitive receptor (that is an AT₁ receptor) subtype, coupled to inhibition of adenylate cyclase in rat liver, already suggests at least one further subtype should be identifiable although it may not necessarily differ at the ligand binding site (Bauer *et al.*, 1991). A final and important point relevant to the role of these receptors in man is that since these compounds bind with equal affinity to both human and rat

angiotensin AT₁ receptors, experimental data obtained using these compounds in the rat may predict their effects in man.

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References

- BAUER, P.H., CHIU, A.T. & GARRISON, J.C. (1991). DuP753 can antagonize the effects of angiotensin II in rat liver. *Mol. Pharmacol.*, **39**, 579–585.
- BUMPUS, F.M., CATT, K.J., CHIU, A.T., DE GASPARO, M., GOOD-FRIEND, T., HUSAIN, A., PEACH, M.J., TAYLOR, D.C. & TIMMERMANS, P.B.M.W.M. (1991). Nomenclature for angiotensin receptors: a report of the nomenclature committee of the Council for High Blood Pressure Research. *Hypertension*, **17**, 720–721.
- CHIU, A.T., CARINI, D.J., DUNCIA, J.V., LEUNG, K.H., MCCALL, D.E., PRICE, W.A.Jr., WONG, P.C., SMITH, R.D., WEXLER, R.R. & TIMMERMANS, P.B.M.W.M. (1991). DuP532: A second generation of nonpeptide angiotensin II receptor antagonists. *Biochem. Biophys. Res. Commun.*, **177**, 209–211.
- CHIU, A.T., HERBLIN, W.F., MCCALL, D.E., ARDECKY, R.J., CARINI, D.J., DUNCIA, J.V., PEASE, L.J., WONG, P.C., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1989). Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **165**, 196–203.
- FURUTA, H., GUO, D.F. & INAGAMI, T. (1992). Molecular cloning and sequencing of the gene encoding human angiotensin II type-1 receptor. *Biochem. Biophys. Res. Commun.*, **183**, 8–13.
- IWAI, N. & INAGAMI, T. (1992). Identification of two subtypes in the rat type I angiotensin II receptor. *FEBS Lett.*, **298**, 257–260.
- IWAI, N., YAMAMO, Y., CHAKI, S., KONISHI, F., BARDHAN, S., TIBBETTS, C., SASAKI, K., HASEGAWA, M., MATSUDA, Y. & INAGAMI, T. (1991). Rat angiotensin II receptor: cDNA sequence and regulation of the gene expression. *Biochem. Biophys. Res. Commun.*, **177**, 299–304.
- KAKAR, S.S., SELLARS, J.C., DEVOR, D.C., MUSGROVE, L.C. & NEILL, J.D. (1992). Angiotensin II type-1 receptor subtype cDNAs: Differential tissue expression and hormonal regulation. *Biochem. Biophys. Res. Commun.*, **183**, 1090–1096.
- KOBILKA, B. (1992). Adrenergic receptors as models for G protein-coupled receptors. *Annu. Rev. Neurosci.*, **15**, 87–114.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- MURPHY, T.J., ALEXANDER, R.W., GRIENDLING, K.K., RUNGE, M.S. & BERNSTEIN, K.E. (1991). Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature*, **351**, 233–236.
- OKSENBERG, D., MARSTERS, S.A., O'DOWD, B.F., JIN, H., HAVLIK, S., PEROUTKA, S.J. & ASHKENAZI, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT_{1B} receptors. *Nature*, **360**, 161–163.
- PEACH, M.J. (1977). Renin-angiotensin system: Biochemistry and mechanism of action. *Physiol. Rev.*, **57**, 313–370.
- ROBERTSON, M.J., BARNES, J.C., DREW, G.M., CLARK, K.L., MARSHALL, F.H., MICHEL, A., MIDDLEMISS, D., ROSS, B.C., SCOPES, D. & DOWLE, M.D. (1992). Pharmacological profile of GR117289 in vitro: a novel, potent and specific non-peptide angiotensin AT₁ receptor antagonist. *Br. J. Pharmacol.*, **107**, 1173–1180.
- SASAKI, K., YAMANO, Y., BARDHAN, S., IWAI, N., MURRAY, J.J., HASEGAWA, M., MATSUDA, Y. & INAGAMI, T. (1991). Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature*, **351**, 230–233.
- TAKAYANAGI, R., OHNAKA, K., SAKAI, Y., NAKAO, R., YANASE, T., HAJI, M., INAGAMI, T., FURATA, H., GUO, D.F., NAKAMUTA, M. & NAWATA, H. (1992). Molecular cloning, sequence analysis and expression of a cDNA encoding human type-1 angiotensin II receptor. *Biochem. Biophys. Res. Commun.*, **183**, 910–917.
- TIMMERMANS, P.B.M.W.M., WONG, P.C., CHIU, A.T. & HERBLIN, W.F. (1991). Nonpeptide angiotensin II receptor antagonists. *Trends Pharmacol. Sci.*, **12**, 55–62.
- WATLING, K.J. & KRAUSE, J.E. (1993). The rising sun shines on substance P and related peptides. *Trends Pharmacol. Sci.*, **14**, 81–84.
- WHITEBREAD, S., MELE, M., KAMBER, B. & DE GASPARO, M. (1989). Preliminary biochemical characterization of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **163**, 284–291.

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