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Synthesis, binding studies, and in vivo biological evaluation of novel non-peptide antihypertensive analogues

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Abstract—AT_1 antagonists (SARTANs) constitute the last generation of drugs for the treatment of hypertension, designed and synthesized to mimic the C-terminal segment of the vasoconstrictive hormone angiotensin II (AngII). They exert their action by blocking the binding of AngII on the AT_1 receptor. Up to date eight AT_1 antagonists have been approved for the regulation of high blood pressure. Although these molecules share common structural features and are designed to act under the same mechanism, they have differences in their pharmacological profiles and antihypertensive efficacy. Thus, there is still a need for novel analogues with better pharmacological and financial profiles. An example of a novel synthetic non-peptide AT_1 antagonist which devoids the classical template of SARTANs is MM1. In vivo studies showed that MMK molecules, which fall in the same class of MM1, had a significant antihypertensive (40–80% compared to the drug losartan) activity. However, in vitro affinity studies showed that losartan has considerably higher affinity. The theoretical docking studies showed that MM1 acts on the same site of the receptor as losartan. They exert hydrophobic interactions with amino acid Val108 of the third helix of the AT_1 receptor and other hydrophobic amino acids in spatial vicinity. In addition, losartan favors multiple hydrogen bondings between its tetrazole group with Lys199. These additional interactions may in part explain its higher in vitro binding affinity.
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1. Introduction

Angiotensin II (AII) is a vasoconstrictive peptide hormone formed within the renin–angiotensin system (RAS) which plays an important role in regulating cardiovascular homeostasis.[1–3] Research efforts for the control of hypertension have focused on competing Ang II binding to AT_1 receptors. The research efforts of DuPont were crowned by the discovery of the first potent and orally active non-peptide AII antagonist losartan (COZAAR).[4–14] New molecules similar in structure appeared in the pharmaceutical market defining a new class of drugs called SARTANS.[14–24] To comprehend the stereoelectronic requirements for receptor binding, the stereochemical features of angiotensin II, its peptide antagonists sarmesin and sarilesin, synthetic peptide analogues, and AT_1 non-peptide antagonists (commercially available and novel compounds) were explored. AT_1 antagonists are designed to mimic the C-terminal part of Ang II.[25–28,45] Based on superimposition studies of losartan with the model proposed for sarmesin, a new avenue was explored in an attempt to design and synthesize novel AT_1 antagonists. Thus, few years ago we have briefly reported MM1 synthesis which possesses pyrrolidinone instead of biphenyltetrazole template. Its stereoelectronic properties were also analyzed and compared with those of losartan.[29] MM1 was designed to mimic conformational characteristics of His6-Pro7-Phe8 and constitutes the first lead compound. Two other derivative compounds were also synthesized namely MMK2 and MMK3. Detailed synthesis of the three

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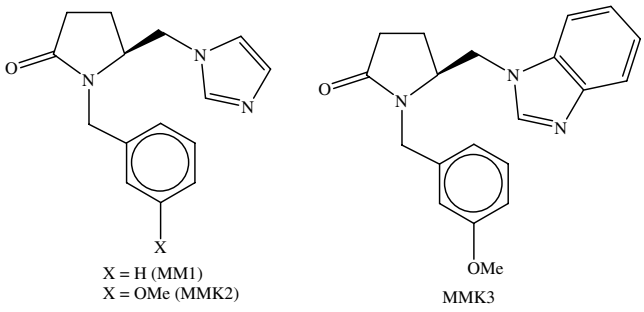


Figure 1. Chemical structures of MM1–MMK3.

compounds is reported in this research activity. The structures of losartan, MM1, MMK2, and MMK3 are shown in Figure 1. In addition, comparative in vitro binding studies with AT_1 and AT_2 receptors are performed, as well as in vivo experiments with adult normotensive male New Zealand White rabbits.

Moreover, theoretical (docking) studies were applied to investigate possible interactions between the prototype of these series MM1 with binding amino acids. Such studies may aid to provide a plausible explanation for the in vivo and in vitro results strengthening the rational design of novel analogs with improved binding affinities.

2. Results

2.1. Synthesis of MM1, MMK2, and MMK3

The synthesis of MM1, MMK2 and MMK3 is shown in Figure 2 and is presented in detail in the Experimental Part. Briefly, *S*-pyroglutamic acid (**1**) was converted to methyl (*S*)-pyroglutamate (**2**) by treatment with $SOCl_2$ in methanol. This ester was subsequently dissolved in dry THF and benzyl bromide (or *m*-methoxy-benzyl bromide) was added. After cooling to 0 °C, NaH was added portionwise to gain compounds **3a** and **3b**, respectively. Reduction of **3a** and **3b** with $LiBH_4$ led to the alcohols **4a** and **4b**. After tosylation, the activated tosyl esters **5a** and **5b** were converted to the target compounds MM1 (**6a**) and MMK2 (**6b**) by treatment with freshly prepared lithium imidazolide and MMK3 (**7**) by treatment with lithium benzimidazolide, freshly prepared as well.

2.2. Biological studies

The experiments in membranes containing AT_1 receptors and cell cultures were repeated on triplicates. The mean values of the obtained results were identical. MM1 as well as MMK2 and MMK3 showed no considerable activity compared to prototype losartan (IC_{50} value $\sim 10^{-9}$ M) for the AT_1 receptor (Fig. 3). Preliminary experiments indicate that the three molecules, like losartan, do not compete for [^{125}I]Sar1,Ile8-angiotensin II binding at AT_2 receptors. Figure 4 summarizes the antihypertensive effect and the degree of potency of the three compounds in a preparation of anesthetized rabbits made hypertensive by AII infusion. MM1 and MMK2

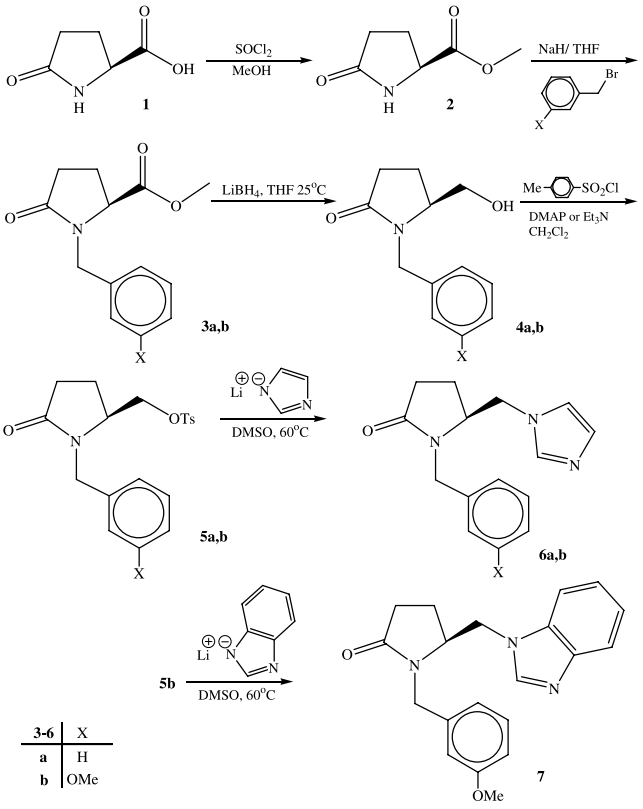


Figure 2. Synthesis of (5*S*)-1-benzyl-(1*H*-imidazol-1-yl-methyl)-2-pyrrolidinone (MM1), (5*S*)-1-(3-methoxybenzyl)-5-(1*H*-imidazol-1-yl-methyl)-2-pyrrolidinone (MMK2), and (5*S*)-1-(3-methoxybenzyl)-5-(1*H*-benzimidazol-1-ylmethyl)-2-pyrrolidinone (MMK3).

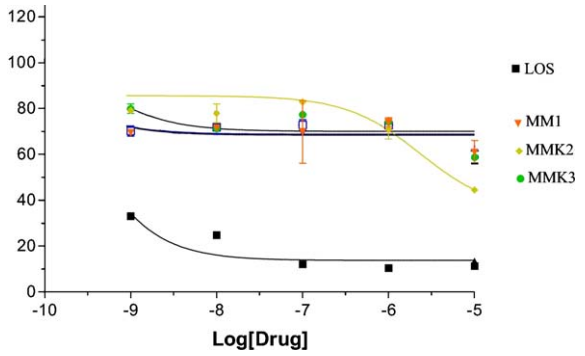
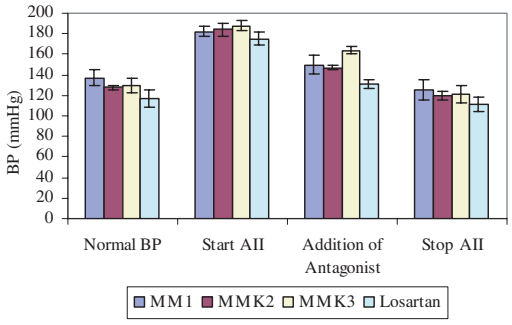


Figure 3. In vitro competition binding studies with membranes containing human AT_1 receptor.

showed high antihypertensive activity (72% and 80% relatively to losartan defined as 100%). MMK3 showed smaller antihypertensive activity (48%).

2.3. Theoretical docking studies

The early models have been constructed based on 3D structure of bacteriorhodopsin (BR) but it is now commonly accepted that BR is not a good template for modeling GPCRs, since it has very low homology to this family[30] and the relative orientation of transmembrane segments is different. The more recent models are based on the 3D structure of rhodopsin (Rh) that was revealed



Antagonist	% BP reduction	Potency (% losartan)
Losartan	-25±2	100
MM1	-18±4	72
MMK2	-20±3	80
MMK3	-12±1	48

Figure 4. In vivo biological data for compounds MM1–MMK3. The adult normotensive male New Zeland White Rabbits were first become hypertensive with the infusion of angiotensin II. The antihypertensive activity of novel non-peptide molecules was compared with the control AT1 antagonist losartan. All concentration 5 μg/ml provided with 0.8 μg/min. Final concentrations of antagonists were 2 mg/ml.

by electronic microscopy[31] or, in much more detail, by recent X-ray crystallography.[32]

For the definition of the active site, several site-directed mutagenesis studies have been performed in order to examine the residues involved either in ligand binding (agonists or antagonists) or in signal transduction.[33–43] These studies are some times controversial and do not establish unequivocally the active site of the AT_1 receptor.[33,35,39,40,44] The key amino acids for binding of non-peptide AT_1 antagonists are Val108, Asn111, Ala163, Arg167, Lys199, Ser252, His256, Asn295, Leu300, and Phe301. These amino acids have been reported for specific interactions with non-peptide antagonists of the class of SARTANs. Other amino acids mostly in the third and seventh helices are important for the maintenance of the integrity of the pocket. These reported amino acids cover a wide area of the receptor thus cannot determine a unique binding pocket. For this reason theoretical calculations have been applied to identify regions of the receptor, capable of binding.[49] SiteID module of Sybyl6.8 showed that losartan and MM1 can accommodate in thirteen pockets of different sizes (Fig. 5). The pocket selected (Table 1) for further docking studies was the one which included three of the most important amino acids (Val108, Lys199, and His256) for antagonist binding. This pocket is located near the polar head groups and exceeds down to the hydrophobic core of the phospholipid bilayers of the membrane in which the receptor is embedded. This observation is important if we consider that AT_1 antagonists have been found to locate themselves near the mesophase of the phospholipid bilayers. Thus, the specific pocket may be easily accessible by the ligands after their diffusion which confirms the proposed two-step mechanism of action for AT_1 antagonists.

Application of the FlexX algorithm (Sybyl6.8—TRIPOS) showed that MM1 could fit spontaneously in the

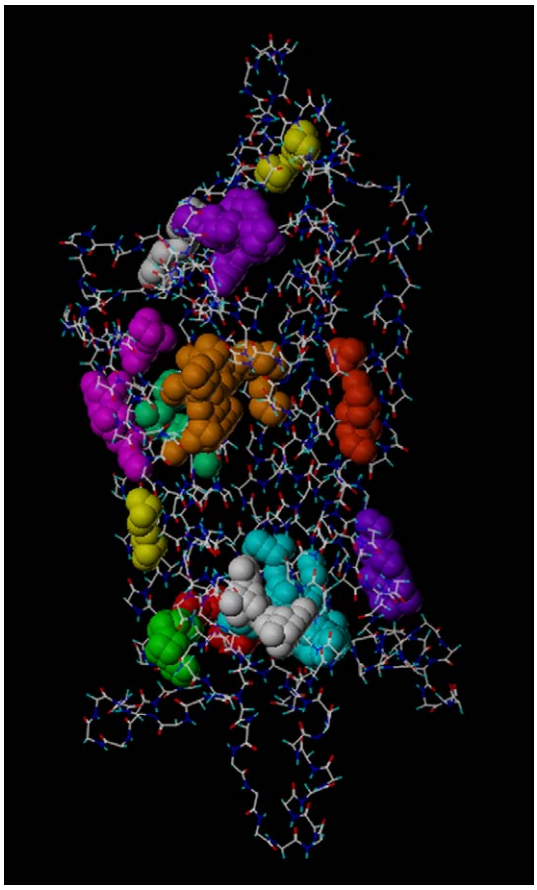


Figure 5. Thirteen resulted theoretical pockets found after applying SiteID software on the model of AT_1 receptor.

putative active site. This algorithm places a base fragment of the ligand in the pocket and then constructs fragment by fragment the remainder while searching for favorable interactions between the ligand and the protein trying to avoid steric overlaps at each step. During this procedure, the conformation of the protein is kept stable. The docking and subsequent scoring were

Table 1. amino acids of the AT_1 receptor which constitute the binding pocket, as found with SiteID module of SYBYL6.8 software

Aminoacid	TM
Val^{108}	3
Leu^{112}	3
Val^{116}	3
Phe^{170}	4 (extracellular)
Phe^{182}	4 (extracellular)
Lys^{199}	5
Asn^{200}	5
Gly^{203}	5
Phe^{204}	5
Pro^{207}	5
Phe^{208}	5
Phe^{249}	6
Trp^{253}	6
His^{256}	6
Gln^{257}	6
Ile^{288}	7
Ala^{291}	7
Tyr^{292}	7

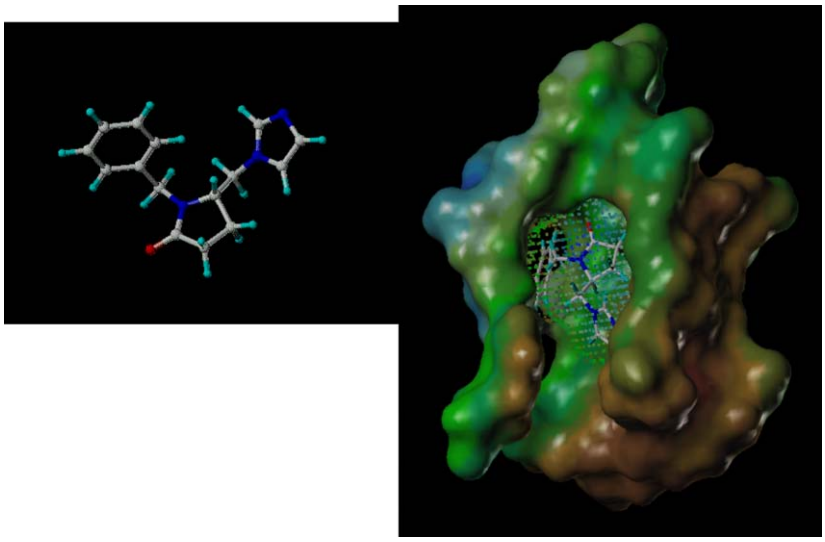


Figure 6. The incorporation of MM1 (left) in the putative pocket of AT_1 receptor (right) as it is revealed using theoretical and experimental data.

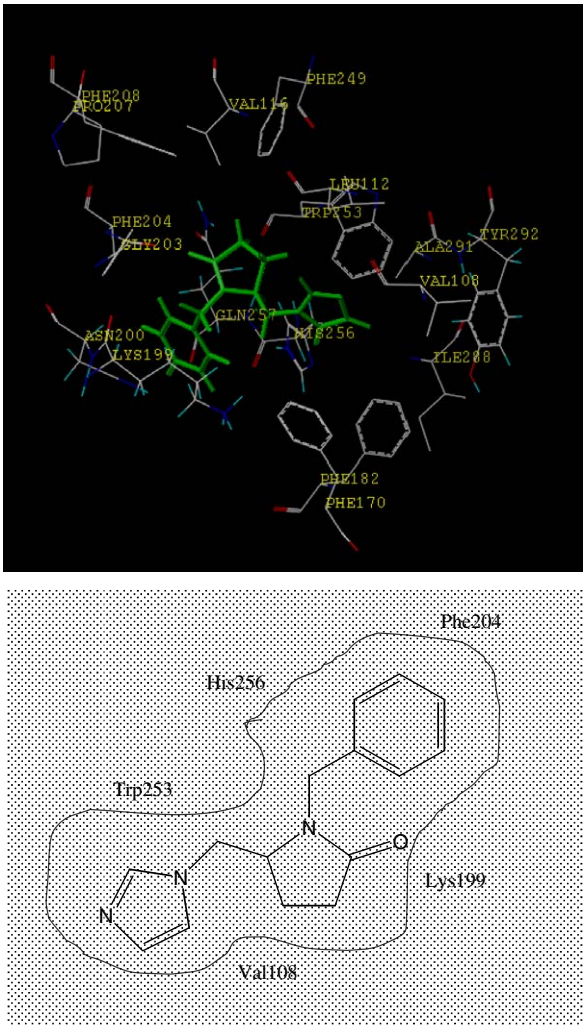


Figure 7. Docking of MM1 into putative pocket of AT_1 receptor.

performed using the formal charges and the default parameters of the FlexX program.

MM1 showed a good fit into the hydrophobic core of the pocket. Starting with the four low energy conformations of MM1 as found elsewhere with NMR studies and molecular modeling,[29] the resulting docked conformation was specific and is presented in (Fig. 6). The interactions between the amino acids located in the cavity of AT_1 receptor and pharmacophore segments of MM1 are mainly hydrophobic in nature and involve phenyl ring of MM1 with Phe204 of AT_1 receptor and pyrrolidinone with amino acids Trp253 and His256 (Fig. 7). MM1 did not show any strong electrostatic interaction or hydrogen bond with the amino acids of the binding site.

3. Discussion

The use of SITE ID algorithm determined putative pockets (active site of AT_1 receptor) for MM1 and losartan molecules. Among those revealed by this algorithm one contained amino acids reported by mutational studies. This pocket, which was supported by experimental and theoretical calculations, was considered for further studies. The application of FlexX algorithm on this pocket showed that MM1 could fit spontaneously in the putative active site. MM1 is docked mainly because of the hydrophobic interactions between its lipophilic segments and hydrophobic amino acids in the core of the pocket. A major difference with losartan is that losartan is proposed to stabilize itself in the pocket with electrostatic interactions and hydrogen bonds.[49] These bonds may be of biological significance and must be considered for the rational design of new AT_1 antagonists using pyrrolidinone template as a scaffold. Docking experiments supported our rational design for MM1.

As it is mentioned the in vitro experiments showed that MM1 has considerably lower in vitro activity. The in vivo experiments, as explained in the introduction, show that MM1 is almost equipotent with losartan. Two plausible explanations can be given for this observation: (a) The diffusion of both molecules into the receptor site may be facilitated (i.e., proteins); (b) the receptor site adopts different conformation. It should be pointed out that in the in vivo experiments, the drug is given after rabbits have become hypertensive, while in the in vitro studies receptors were in a 'rest state'.

4. Conclusion

Our studies confirm our rational design for the prototype MM1 and its analogues MMK2 and MMK3. The docking theoretical study will guide the second step in our rational design which includes the synthesis of derivative molecules of MM1 which must contain an acidic group (i.e., carboxylate or an isosteric tetrazole) in order to form electrostatic interactions or hydrogen bonds between MMK derivatives and the AT_1 receptor pocket. This will be a strict requirement for improving the antihypertensive activity not only because it will provide multiple contacts with the receptor but also because it may optimize its partitioning in membrane bilayers.

5. Experimental

5.1. Synthesis of MM1, MMK2, and MMK3

Mps were determined on an mp apparatus (Büchi) and are uncorrected. Specific rotations were measured at 25 °C on an electronic polarimeter Perkin-Elmer 343 using a 10 cm cell. NMR spectra were recorded in $CDCl_3$ on a 200 MHz spectrometer. *S*-Pyroglutamic acid, and 3-methoxy-benzyl bromide were purchased from Aldrich and benzyl bromide from Fluka. TLC plates (silica gel 60 F_{254}) and silica gel 60 (70–230 or 230–400 mesh) for column chromatography were purchased from Merck. THF was dried by standard procedures and stored over molecular sieves. DMSO was purchased from Merck and stored also over molecular sieves. All other solvents and chemicals were of reagent grade and used without further purification. Fast atom bombardment (FAB) mass spectra were recorded using a VG analytical ZAB-SE instrument. Electron spray ionization (ESI) mass spectra were recorded on a Finnigan, Surveyor MSQ Plus spectrometer.

Lithium imidazolide and lithium benzimidazolide were prepared as follows: Imidazole (250 mg, 3.6 mmol) or benzimidazole (425 mg, 3.6 mmol) was dissolved in 10 mL dry toluene and 1.6 mL (2.5 M) of *n*BuLi was added at 0 °C. The mixture was stirred for 2 h at room temperature and evaporated. The resulted solid in both cases was dried under vacuum overnight.

5.1.1. (5*S*)-1-Benzyl-5-carbomethoxy-2-pyrrolidinone (3a). Methyl (*S*)-pyroglutamate (7.2 g, 0.05 mol) was dissolved in dry THF (250 mL) and benzyl bromide (6.53 mL, 0.055 mol) was added under stirring in one portion. The mixture was cooled to 0 °C and NaH[46] (60% in paraffin oil, 3 g, 0.075 mol) was added portionwise. After stirring for 30 min at 0 °C, the mixture was stirred for 30 min at rt. The product was washed twice successively with a saturated aqueous NH_4Cl solution and H_2O, and the aqueous layer was extracted with Et_2O. The combined organic layers were washed with brine, dried with Na_2SO_4, and evaporated. The residue was purified by column chromatography (Silica gel 60) using a petroleum ether/Et_2O (3:7–1:9) mixture and finally only diethyl ether as eluent.

Product was obtained as an oil. Yield 3.6 g (45%) $[\alpha]_D^{25}$ +3.5 (*c* 1, MeOH).

^{1}H NMR ($CDCl_3$): δ = 7.29–7.15 (m, 5H, aromatic), 5.0 (d, J = 14.6 Hz, 1H, CH*H*Ph), 4.0 (d, J = 14.6 Hz, 1H, CH*H*Ph), 3.98 (m, 1H, CH), 3.63 (s, 3H, OCH_3), 2.63–2.27 (m, 4H, 2× CH_2). ^{13}C NMR ($CDCl_3$): δ = 175.2, 172.4, 135.9, 128.9, 128.7, 127.9, 58.9, 52.5, 45.8, 29.7, 23.0.

Compounds (5*S*)-1-benzyl-5-hydroxymethyl-2-pyrrolidinone (**4a**)[47] and (5*S*)-1-benzyl-5-hydroxymethyl-2-pyrrolidinone tosyl ester (**5a**)[48] were prepared according to the literature.

5.1.2. (5*S*)-1-Benzyl-5-(1H-imidazol-1-ylmethyl)-2-pyrrolidinone (6a). Freshly prepared lithium imidazolide (0.22 g, 3 mmol) was added in a solution of the tosyl ester **5a** (0.54 g, 1.5 mmol) in DMSO (4 mL) at room temperature. The resulting mixture was stirred for 24 h at 60 °C under argon atmosphere. After addition of water (2 mL), the mixture was extracted with ethyl acetate. The organic layer was washed with brine to neutral pH, dried with Na_2SO_4, and evaporated under reduced pressure and the residue was purified by column chromatography (silica gel, elution system: AcOEt/MeOH 9:1). The product (0.24 g) was obtained as an oil. Yield 62%.

$[\alpha]_D^{25}$ +8.3 (*c* 1, MeOH) R_f = 0.58 ($CHCl_3$–MeOH 7:3).

^{1}H NMR ($CDCl_3$): δ = 7.41–7.11 (m, 7H, aromatic), 6.80 (s, 1H, aromatic), 5.10 (d, J = 15.1 Hz, 1H, CH*H*Ph), 4.05 (d, 2H, CH_2N), 3.96 (d, J = 15.1 Hz, 1H, CH*H*Ph), 3.78 (m, 1H, CH), 2.35–1.78 (m, 4H, 2× CH_2). ^{13}C NMR ($CDCl_3$): δ = 175.0, 137.5, 135.9, 130.2, 128.9, 128.0, 119.2, 56.8, 48.7, 44.9, 29.1, 22.4.

Anal. Calcd for $C_{15}H_{17}N_3O$: C, 70.56; H, 6.71; N, 16.46. Found: C, 70.42; H, 6.88; N, 16.40. MS (ESI): *m*/*z* = 278 (17%) $(M+Na)^+$, 256 (100%) $(M+H)^+$.

5.1.3. (5*S*)-1-(3-Methoxybenzyl)-5-carbomethoxy-2-pyrrolidinone (3b). Methyl (*S*)-pyroglutamate (7.2 g, 0.05 mol) was reacted with *m*-methoxybenzyl bromide (11.06 g, 0.055 mol) under the same conditions as for compound **3a**, described above. The residue was purified by column chromatography (Silica gel 60) using a petroleum ether–AcOEt (3:7 to 1:9) mixture as eluent.

The product is obtained as an oil. Yield: 5.2 g (40%).

$[\alpha]_D^{25}$ +4.70 (*c* 1.5, MeOH), $R_f = 0.45$ (AcOEt/petroleum ether 4:1).

^{1}H NMR ($CDCl_3$): 7.23 (m, 1H, aromatic), 6.79 (m, 3H, aromatic), 5.01 (d, $J = 14.8$ Hz, 1H, CH*H*Ph), 4.06 (m, 1H, CH), 3.94 (d, $J = 14.8$ Hz, 1H, CH*H*Ph), 3.78 (s, 3H, OCH_3), 3.70 (s, 3H, $COOCH_3$), 2.62–2.00 (m, 4H, 2× CH_2).

^{13}C NMR ($CDCl_3$): 175.0, 172.2, 159.7, 137.2, 129.6, 120.6, 113.8, 113.1, 58.5, 55.1, 52.3, 45.4, 29.4, 22.7.

5.1.4. (5*S*)-1-(3-Methoxybenzyl)-5-hydroxymethyl-2-pyrrolidinone (4b). $LiBH_4$ (0.1 g, 5 mmol) was added in dry tetrahydrofuran (THF) (2 mL) under argon atmosphere. Afterwards, a solution of the aforementioned substance **3 b** (1.1 g, 4.55 mmol) in dry THF (2.5 mL) was added dropwise at room temperature. The reaction was quenched 45 min later, with the addition of an aqueous acetic acid solution (20%) at 0 °C. Then the mixture was neutralized with solid Na_2CO_3. The mixture was extracted with ethyl acetate and the organic layer was washed with water. After drying with sodium sulfate and evaporation, the product was obtained as a pure oil.

Yield 0.94 g (97%).

$[\alpha]_D^{25}$ −81.2 (*c* 1.1, MeOH), $R_f = 0.51$ ($CHCl_3$/MeOH 9:1).

^{1}H NMR ($CDCl_3$): $\delta = 7.23$ (m, 1H, aromatic), 6.81 (m, 3H, aromatic), 4.82 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 4.16 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 3.77 (s, 3H, OCH_3), 3.76 (m, 1H, CH), 3.53 (m, 2H, C*H*$_2$OH), 2.80 (b, 1H, OH), 2.42–2.01 (m, 4H, 2× CH_2).

^{13}C NMR ($CDCl_3$): $\delta = 176.1$, 159.7, 138.1, 129.7, 120.0, 113.5, 112.7, 61.9, 58.6, 55.1, 44.2, 30.5, 20.8.

MS (FAB) *m*/*z*: 236 (100%) $(M+H)^+$, 204 (39%).

5.1.5. (5*S*)-1-(3-Methoxybenzyl)-5-hydroxymethyl-2-pyrrolidinone tosyl ester (5b). A solution of alcohol **4b** (0.76 g, 3.3 mmol) in methylene chloride (10 mL) was cooled at 0 °C. Triethylamine (0.51 mL) and tosyl chloride (0.685 g, 3.3 mmol) were added in three portions, successively, under cooling. The solution was stirred at room temperature overnight. The mixture was washed with a cooled 1 N HCl solution (2 mL), a saturated $NaHCO_3$ solution (1.5 mL), and brine. Then it was dried with Na_2SO_4 and evaporated. The residue was purified by column chromatography (Silica gel 60) using AcOEt/petroleum ether (9:1) as eluent. Product was obtained as an oil. Yield 0.83 g (65%).

$[\alpha]_D^{25}$ +23.7 (*c* 1, $CHCl_3$), $R_f = 0.49$ (AcOEt/petroleum ether 9:1).

^{1}H NMR ($CDCl_3$): $\delta = 7.70$ (m, 2H, aromatic), 7.38 (m, 2H, aromatic), 7.21 (m, 1H, aromatic), 6.71 (m, 3H, aromatic), 4.82 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 3.98 (m, 2H, CH_2OS), 3.80 (s, 3H, OCH_3), 3.78 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 3.62 (m, 1H, CH), 2.42–1.94 (m, 7H, CH_3, 2× CH_2).

^{13}C NMR ($CDCl_3$): $\delta = 174.9$, 159.9, 145.3, 137.5, 132.2, 129.6, 129.7, 127.8, 120.0, 113.5, 113.1, 68.6, 55.3, 55.1, 44.3, 29.6, 21.6, 21.0.

5.1.6. (5*S*)-1-(3-Methoxybenzyl)-5-(1H-imidazol-1-ylmethyl)-2-pyrrolidinone (6b). Lithium imidazolide (0.22 g, 3 mmol) was added in a solution of the tosyl ester **5b** (0.58 g, 1.5 mmol) in dimethylsulfoxide, under the same conditions as for compound **6a**, described above. The residue was purified by column chromatography (Silica gel 60, elution system: AcOEt/MeOH 1:1). The product (0.23 g) was obtained as an oil. Yield 55%.

$[\alpha]_D^{25}$ +10.9 (*c* 0.78, MeOH), $R_f = 0.39$ (AcOEt/MeOH 1:1).

^{1}H NMR ($CDCl_3$): $\delta = 7.39$–6.81 (m, 7H, aromatic), 5.01 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 4.02 (d, 2H, CH_2N), 3.87 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 3.80 (s, 3H, OCH_3), 3.75 (m, 1H, CH), 2.14–1.78 (m, 4H, 2× CH_2).

^{13}C NMR ($CDCl_3$): $\delta = 175.0$, 160.0, 137.5, 130.2, 130.0, 120.2, 119.2, 113.8, 113.2, 56.9, 55.3, 48.7, 44.9, 29.1, 22.4.

Anal. Calcd for $C_{16}H_{19}N_3O_2$: C, 67.35; H, 6.71; N, 14.73. Found: C, 67.29; H, 6.79; N, 14.68. MS (FAB) *m*/*z*: 286 (100%) $(M+H)^+$, 204 (10%).

5.1.7. (5*S*)-1-(3-Methoxybenzyl)-5-(1H-benzimidazol-1-ylmethyl)-2-pyrrolidinone (7). Lithium benzimidazolide (0.372 g, 3 mmol) was added in a solution of the tosyl ester **5b** (0.58 g, 1.5 mmol) in dimethylsulfoxide, under the same conditions as for compound **6a**, described above. The residue was purified by column chromatography (Silica gel 60, elution system: $CHCl_3$/MeOH 9:1). The product (0.38 g) was obtained as an oil, which was solidified during storage at 4 °C. Yield 75%.

$[\alpha]_D^{25}$ +22.3 (*c* 0.7, $CHCl_3$), $R_f = 0.92$ (AcOEt/MeOH 1:1).

^{1}H NMR ($CDCl_3$): $\delta = 7.74$–6.60 (m, 9H, aromatic), 4.98 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 4.01 (d, 2H, CH_2N), 3.80 (s, 3H, OCH_3), 3.75 (d, $J = 15.2$ Hz, 1H, CH*H*Ph), 3.62 (m, 1H, CH), 2.45–1.80 (m, 4H, 2× CH_2).

^{13}C NMR ($CDCl_3$): $\delta = 175.0$, 159.9, 145.3, 137.5, 130.0, 129.8, 127.9, 120.2, 113.5, 113.2, 68.9, 55.3, 55.2, 44.4, 29.7, 21.7.

Anal. Calcd for $C_{20}H_{21}N_3O_2$: C, 71.62; H, 6.31; N, 12.53. Found: C, 71.47; H, 6.35; N, 12.48. MS (ESI): *m*/*z* = 358 (20%) $(M+Na)^+$, 336 (100%) $(M+H)^+$.

5.2. In vitro binding studies

Membranes containing human AT_1 receptors were purchased from PerkinElmer Life Sciences, Inc., Boston,

MA, USA, and membranes containing either AT_1 or AT_2 receptors were kindly provided by Prof. A. Balmforth, Biomedical Sciences, University of Leeds. (3-[^{125}I]iodotyrosyl4) Sar1 Ile8, Angiotensin II was purchased from Amersham Biosciences, Little Chalfont, Bucks, UK, and all other general reagents were from Sigma, Poole, Dorset, UK.

5.3. In vitro binding experiments

The binding buffer solution comprised 20 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, and 0.1% (w/v) BSA, and was adjusted to pH 7.4. Competitor drug concentrations ranged between 10^{-5} and 10^{-9} M and triplicate determinations made. All drugs were DMSO soluble and when diluted the DMSO concentration did not exceed 1% (v/v). The radioligand used for the experiments was [^{125}I]Sar1,Ile8-angiotensin II, a non-specific peptide appropriate for both AT_1 and AT_2 receptors. A constant concentration of radioligand of 0.1 nM (≈40,000 cpm) was maintained in all samples. The total binding is defined as the binding in the absence of competitive compounds. Non-specific binding, in the presence of 10^{-5} M losartan, was about 300 cpm. Two kinds of membranes were used for the binding experiments containing human AT_1 or AT_2 receptors. 23.5 μg of membrane protein was used in each binding assay. Binding assay comprised: 25 μL radioligand, 25 μL test compound or buffer, and 50 μL membrane sample. Incubations were carried out at room temperature for at least 1.5 h. The samples were harvested using a Brandel Cell Harvester on GF/B filters presoaked in 1% (v/v) polyethylimine and washed with chilled binding buffer. The radioactivity retained on the filters was determined on a Packard RiasStar 5405 gamma counter.

Binding assays with intact cells were carried out in a similar manner to that given above except that the buffer used was made isotonic by inclusion of 150 mM NaCl. One hundred and five cells in 50 μl buffer replaced the use of membranes. Washing of filters was also carried out using isotonic buffer.

5.4. In vivo experiments

Adult normotensive male New Zealand White rabbits weighing between 2.5 and 3.3 kg were used in the study following previously described methods.[50,51] In brief, animals were anesthetized by pentobarbitone (30 mg/kg), intubated, and mechanically ventilated with 100% oxygen using a respirator for small animals (MD Industries, Mobile, Al, USA). The tidal volume was 15 mL and the rate was adjusted to keep blood gases within normal rage. Two polyethylene catheters were inserted, one in the carotid artery for continuous blood pressure monitoring via a transducer attached to a multichannel recorder (Nihon-Kohden, Model 6000, Japan) and the other one in the jugular vein for the administrations of solution made by diluting angiotensin II (AII) (Hypertensin, CIBA) in 5% dextrose at a final concentration of 5 μg/mL. Based on previous testing with this rabbit preparation submaximal angiotensin II-dependent hypertension was induced by infusing angiotensin II via a syringe pump (Harvard Apparatus Pump 22, Harvard Apparatus, Natick. MA, USA) at a constant rate of 0.2 mL/min (1 μg/min). Each compound including losartan was initially dissolved with 0.05 mL DMSO and then diluted in dextrose 5% at a final concentration of 2 mg/mL. Five minutes after the establishment of hypertension, three equal boluses of each compound were given via an ear vein 20 min apart. The blood pressure was monitored for 20 min after the third bolus and then angiotensin infusion was halted and blood pressure was recorded for an additional period of 20 min until the end of the experiment. Boluses of the same dose of losartan, as positive control, were used in the same experimental model.

5.5. Theoretical calculations for AT_1 binding

A homology model based on the X-ray structure of rhodopsin human AT_1 receptor developed by James Ellis and Dan Donnelly (University of Leeds, UK) was used for these studies. Determination of the active site: SiteID module of the SYBYL6.8 (TRIPOS) was used to scan the protein and provide the theoretical binding sites located in the seven helices of AT_1 receptor. Default parameters of the SiteID program were used.

Docking. FlexX algorithm of SYBYL6.8 (TRIPOS) was conducted for the docking analysis between the ligand (MM1) and the selected site of the receptor.

Ligand and receptor preparation. The structure of the ligand was prepared in MOL2 format using the 2D sketcher module of Sybyl6.8 and Gasteiger–Huckel charges were assigned to the ligand atoms. The ligand was subjected to minimization until converged to a maximum derivative of 0.001 kcal^{-1} Å^{-1}. MM1 conformation derived from NMR studies and molecular modelling was used as the initial reference structure,[29] after the removal of the hydrogen atoms and the atom charges. Possible bound waters and ligands were removed from the protein and hydrogen atoms were added subsequently.

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