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RESEARCH ARTICLE

Synthesis and Biological Evaluation of Novel Antihypertensive Compounds

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Abstract: Hypertension has been associated as a pathogenesis involved in the renin angiotensin system. The most commonly used drug to block the AT₁R, is Losartan which has specific pharmacophore groups such as imidazole and biphenyl. However the development of new selective antagonists would be advantageous to improving the treatment of hypertension. We investigated innovative antihypertensive candidates **1-3** using *in vitro* and *in vivo* assays.

Although only Compound **2** showed low affinity to the AT₁R, it had no effect on blood pressure. Compound **1** produced a reduction in blood pressure and this effect seems to be mediated through ACE inhibition and not the blockage of the AT₁R. Compound **1** was able to inhibit the ACE activity in a similar way to captopril, while Compounds **2** and **3** showed no effect on the enzyme activity.

Further studies need to be conducted to understand the mechanisms involved as well as signaling pathways.

Keywords: ACE, AT₁ Receptor, Binding, Blood Pressure, CHO Cells, Hypertension, Imidazole, Pharmacophore Group, Renin Angiotensin System.

INTRODUCTION

Hypertension is one of the most important causes of premature death worldwide and the problem is growing; in 2025, World Health Organization estimates that 1.56 billion adults will be living with hypertension. In addition, hypertension kills approximately 8 million people every year [1]. Today, globally, approximately one billion people have high blood pressure, and two-thirds of these are in developing countries.

Hypertension and other pathologies such as cardiac failure and renal diseases have been associated with the renin–angiotensin–aldosterone system (RAAS), which is an important regulator of arterial pressure and fluid-electrolyte homeostasis. Drugs that inhibit or antagonize RAAS components are described as targeting both blood pressure and

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related structural and functional abnormalities of heart and blood vessels, thus preventing target-organ damage and related cardiovascular events [2, 3]. These drugs include the Angiotensin II (Ang II) type 1 receptor blocker (AT_1), the selective aldosterone receptor antagonists and angiotensin-converting enzyme inhibitors (ACEi), the last ones blocking the formation of Ang II and preventing the inactivation of bradykinin [4, 5].

Clinical studies have clearly demonstrated that the use of ACEi or AT_1 receptor antagonists reduce albuminuria, delay the progressive loss of renal function and decrease mortality rate [3, 6 - 8].

Ang II, a vasoconstrictor peptide generated by ACE, is the major regulator of blood pressure (BP), electrolyte balance and endocrine functions acting through two distinct subtypes of receptor, AT_1 and AT_2 , both belonging to the G-protein coupled receptor (GPCR) family [9]. Modeling strategy using pharmacophoric group of Ang II in benzylimidazole led to the synthesis of Losartan (Cozaar[®]), which is an oral nonpeptide AT_1 receptor antagonist and the first on the market launched in 1994 [10].

Structure activity relationship (SAR) of various compounds synthesized during the development of Losartan have revealed various binding sites present on the AT_1 receptor [11]. Since then, more efficient drugs using molecular modification based on SAR regarding particular pharmacophore groups such as imidazole and biphenyl have been investigated [12 - 16].

The development of new selective antagonists for the AT_1 receptor would be useful and informative on the role of the RAAS in the pathogenesis of hypertension, stroke, myocardial infarction, heart failure and end-stage renal disease [2, 17, 18]. Concerning the importance of the discovery of new antihypertensive agents our main goal was focused on the development of innovative Compounds **1-3**. The compounds' design strategy was based on structure modification of Losartan which involved hydantoin group moiety linked to the biphenyl-1*H*-tetrazole scaffold known as pharmacophore groups (Fig. 1).

Hydantoin group has demonstrated different pharmacological activities depending on the nature of substitution on the hydantoin ring. A wide range of pharmacological properties from hydration group can be found such as fungicidal, herbicidal, antitumor, antiinflammatory, anti-HIV, hypolipidemic, anticonvulsant and antihypertensive activities [19 - 21].

We expect that the introduction of hydantoin groups could result in a medicine combining therapy and contribute to the identification of a new chemical entity (NCE) as a selective antagonist for AT_1 receptor. In addition these new compounds could be more absorbed more easily than Losartan since hydantoin groups has a higher lipophilicity.

Looking for pharmaceutical application of new compounds 1-3, synthesis were performed using classical methodologies and proof of biological concept was designed *in vitro* to evaluate their binding affinity to the AT_1 receptor, ACE inhibition and *in vivo* to evaluate their antihypertensive actions.

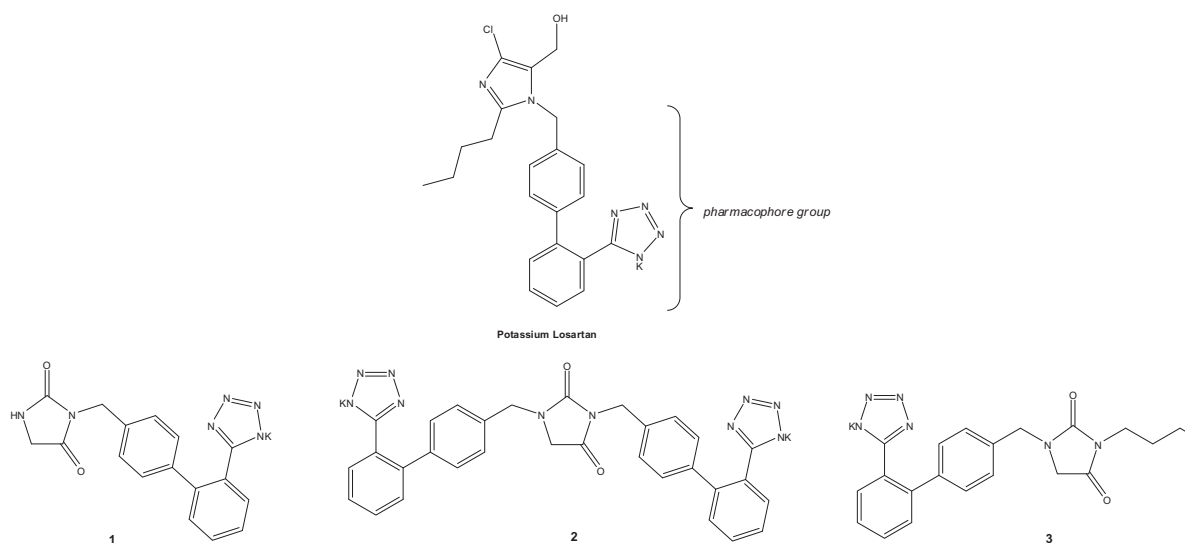


Fig. (1). Structural modification for novel AT_1 receptor antagonists.

EXPERIMENTAL SECTION

Chemical Synthesis

Unless otherwise noted, reactions were performed in predried apparatus under an atmosphere of nitrogen. *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF) and methanol (MeOH) were purchased as anhydrous from Sigma Aldrich Co. (USA). Solvents and reagents were purchased from commercial suppliers and purified by standard techniques. Intermediate 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1*H*-tetrazole (5) was purchased from Zhejiang Tianyu Pharmaceutical Co. Ltda (China). The reactions were monitored by thin layer chromatography on silica gel with fluorescent indicator 254 nm (Fluka). Flash chromatography was performed using Combiflash Companion from Teledyne Isco and a prepacked column (12 gram Flash Column RediSep[®]R_f or 40 gram Flash Column RediSep[®]R_f). Melting points were recorded on a DSC 822 STARE System Mettler Toledo. Infrared (IR) spectra were recorded on a Nicolet 4700 spectrophotometer and measured by cm⁻¹. ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) were measured with a Bruker ARX-400 spectrometer. Chemical shifts are expressed in ppm (δ) as an internal standard. Signal multiplicities were represented by s (singlet), d (doublet), dd (doublet of doublets), dq (doublet of quintets), t (triplet), br (broad) and m (multiplet). *J* values were given in hertz. CDCl₃ or DMSO-*d*₆ was used as solvent. Mass spectra (MS) were determined using an Acquity UltraPerformance Waters and high-resolution mass spectra (HRMS) XEVO Q-TOF from Waters. Purity of compounds **1-3** was determined by high performance liquid chromatography (HPLC) analysis (Lachrom – Merck column C18 - 4,6 x 150 – 5µm, standard gradient: 20 - 55% CH₃CN in formic acid 0.05% (0 - 5 min), 55% CH₃CN (5 - 8 min), 55 - 20% CH₃CN (8 - 13 min), 20% CH₃CN (13 - 15 min), flow rate 1 mL/min, UV detection at 254 nm, purity ≥ 95%).

3-((2'-(1-Trityl-1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl)imidazolidine-2,4-dione (6). To a suspension of hydantoin (4) (1.00 g, 10.00 mmol) and anhydrous potassium carbonate (1.65 g, 12.00 mmol) in dry DMF (10 mL) was added a solution of 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1*H*-tetrazole (5) (5.56 g, 10.00 mmol) in dry DMF (60 mL). The reaction mixture was stirred at room temperature for 18 h. The reaction mixture was poured onto water (50 mL) and extracted with ethyl acetate (100 mL). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate and concentrated in vacuum to afford the crude product which was purified by column chromatography on a Combiflash Companion (ethyl acetate/hexane) giving the compound **6** as an off-white solid (4.32 g, 75%). mp 158 °C. IR 3289, 3058, 1776, 1714, 1492, 1449, 1345, 1266, 1192, 1032. ESI-MS *m/z* 577 [M+H]⁺, 312, 205, 161. ¹H NMR (400MHz, CDCl₃): δ 7.91 (dd, 1H, *J* = 7.2, 1.7 Hz), 7.46 (dq, 2H, *J* = 7.5, 1.7 Hz), 7.36-7.32 (m, 4H), 7.28-7.25 (m, 6H), 7.20 (d, 2H, *J* = 8.3 Hz), 7.08 (d, 2H, *J* = 8.3 Hz), 6.92 (dd, 6H, *J* = 7.2, 1.4Hz), 6.23 (br, 1H), 4.56 (s, 2H), 3.86 (s, 2H). ¹³C NMR (100MHz, CDCl₃) δ 170.8, 164.0, 162.8, 158.0, 141.7, 141.2, 140.9, 134.5, 130.7, 130.2, 129.9, 129.5, 128.3, 128.2, 127.7, 127.6, 126.3, 82.9, 46.4, 41.9.

3-((2'-(1*H*-Tetrazol-5-yl)biphenyl-4-yl)methyl)imidazolidine-2,4-dione (7). Method A: HCl 37% (0.4 mL) was added dropwise to a solution of compound **6** (630.9 mg, 1.09 mmol) in 30 mL of a water/THF mixture (1:4). The mixture was stirred at room temperature for 24 h. THF was removed under reduced pressure and the pH of the solution was made basic with 1N aqueous NaOH and after was washed with ethyl acetate (10 mL). The aqueous phase was acidified with 1N aqueous HCl and the resulting suspension was extracted with ethyl acetate (3 x 25 mL). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate and concentrated in vacuum to afford the crude product which was purified by column chromatography on a Combiflash Companion (methanol/chloroform) to give the compound **7** as colorless solid (298 mg, 82%). Method B: A mixture of the compound **6** (2.64 g, 4.56 mmol) and methanol (80 mL) was refluxed for 16 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on a Combiflash Companion (methanol/chloroform) to give the compound **7** as colorless solid (1.44 g, 95%). mp 204 °C. IR 3470-2500, 3289, 3217, 1772, 1708, 1604, 1452, 1356, 1141, 1066. ESI-MS *m/z* 333[M-H]⁻. ¹H NMR (400MHz, CDCl₃) δ 8.14 (s, 1H), 7.70-7.65 (m, 2H), 7.59-7.53 (m, 2H), 7.20 (d, 2H, *J* = 8.3 Hz), 7.05 (d, 2H, *J* = 8.3 Hz, 2H), 5.76 (s, 1H), 4.53 (s, 2H), 4.00 (s, 2H). ¹³C NMR (100MHz, CDCl₃) δ 172.0, 157.4, 155.0, 141.1, 138.3, 135.0, 131.0, 130.7, 130.6, 128.9, 127.8, 127.2, 123.5, 46.1, 40.6.

Potassium 5-(4'-((2,5-dioxoimidazolidin-1-yl)methyl)biphenyl-2-yl)tetrazol-1-ide (1). To a solution of the compound **7** (1.19 g, 3.56 mmol) in dry MeOH (15 mL) was added anhydrous potassium bicarbonate (356.7 mg, 3.56

mmol). The reaction mixture was refluxed for 18 h. The solvent was removed under reduced pressure and the residue was crystallized under acetone/hexane to obtain a white solid compound **1** (980 mg, 74%). IR 3366, 1758, 1695, 1456, 1414, 1352, 1143, 1006. ¹H NMR (400MHz, DMSO-d₆) δ 7.54-7.52 (m, 1H), 7.39-7.28 (m, 3H), 7.11-7.05 (m, 4H), 4.49 (s, 2H), 3.98 (s, 2H). ¹³C NMR (100MHz, DMSO-d₆) δ 172.0, 160.7, 157.4, 141.1, 104.0, 134.5, 132.5, 130.5, 130.1, 129.2, 127.3, 126.7, 126.6, 46.0, 40.8. ESI-MS *m/z* 373 [M+H]⁺, 331, 291, 133. HRMS calculated for C₁₇H₁₄KN₆O₂ [M+H]⁺ 373.0815, found 373.0846.

1,3-Bis((2'-(1-trityl-1H-tetrazol-5-yl)biphenyl-4-yl)methyl)imidazolidine-2,4-dione (8). To a suspension of hydantoin (**4**) (400.0 mg, 4.00 mmol) and anhydrous potassium carbonate (1.65 g, 12.00 mmol) in dry DMF (5 mL) was added a solution of 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1H-tetrazole (**5**) (4.45 g, 8.00 mmol) in dry DMF (80 mL). The reaction mixture was stirred at room temperature for 72 h. The reaction mixture was poured onto water (100 mL) and extracted with ethyl acetate (4 x 50 mL). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate and concentrated in vacuum to afford the crude product which was purified by flash column chromatography on a Combiflash Companion (ethyl acetate/hexane) giving the compound **8** as a white solid (3.45 g, 82%). mp 168 °C. IR 3068, 3017, 1770, 1711, 1460, 1216, 1031. ESI-MS *m/z* 1053 [M+H]⁺, 833, 364, 243, 102. ¹H NMR (400MHz, CDCl₃) δ 8.02-8.00 (m, 1H), 7.94-7.91 (m, 1H), 7.50-7.45 (m, 4H), 7.28-7.21 (m, 22H), 7.11 (t, 4H, *J* = 8.5 Hz), 6.99 (d, 2H, *J* = 8.3 Hz, 2H), 6.82 (dd, 12H, *J* = 7.2, 4.4 Hz, 12H), 4.57 (s, 2H), 4.41 (s, 2H), 3.48 (s, 2H). ¹³C NMR (100MHz, CDCl₃) δ 169.2, 164.0, 163.8, 156.5, 156.2, 141.7, 141.4, 141.3, 141.2, 140.1, 134.6, 133.8, 130.5, 130.1, 130.2, 130.0, 129.9, 129.8, 129.5, 128.5, 128.3, 127.8, 127.6, 126.4, 126.2, 82.9, 49.2, 46.6, 42.4.

1,3-Bis((2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl)imidazolidine-2,4-dione (9). A mixture of the compound **8** (2.60 g, 2.47 mmol) and methanol (80 mL) was refluxed for 72 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on a Combiflash Companion (methanol/chloroform) to give the compound **9** as colorless solid (1.05 g, 75%). mp 224 °C. IR 3460-2800, 1767, 1707, 1463, 1239, 1145, 1003. ESI-MS *m/z* 569 [M+H]⁺, 302, 234, 102. ¹H NMR (400MHz, CDCl₃) δ 7.58-7.54 (m, 2H), 7.44-7.36 (m, 6H), 7.16-7.08 (m, 8H), 4.61 (s, 2H), 4.48 (s, 2H), 3.99 (s, 2H). ¹³C NMR (100MHz, CDCl₃) δ 170.6, 156.9, 156.1, 141.4, 139.1, 136.1, 136.0, 131.2, 131.1, 131.0, 129.6, 129.5, 129.4, 128.2, 127.8, 127.7, 124.8, 124.7, 50.2, 46.1, 41.8.

Potassium 5,5'-(4',4''-(2,4-dioximidazolidine-1,3 diyl) bis(methylene) bis(biphenyl-4',2'-diyl))ditetrazol-1-ide (2). To a solution of the compound **9** (391.8 g, 0.69 mmol) in dry MeOH (5 mL) was added anhydrous potassium bicarbonate (138.0 mg, 1.38 mmol). The reaction mixture was refluxed for 18 h. The solvent was removed under reduced pressure and the residue was crystallized under acetone/hexane to obtain the white solid compound **2** (418.3 mg, 94%). IR 2927, 1763, 1702, 1461, 1405, 1352, 1236, 1143, 1011. ESI-MS *m/z* 645 [M+H]⁺, 607, 429, 329, 291. ¹H NMR (400MHz, DMSO-d₆) δ 7.54-7.53 (m, 2H), 7.37-7.31 (m, 6H), 7.15-7.07 (m, 8H), 4.54 (s, 2H), 4.48 (s, 2H), 3.99 (s, 2H). ¹³C NMR (100MHz, DMSO-d₆) δ 170.0, 160.6, 156.3, 141.1, 140.0, 134.2, 134.0, 132.5, 132.4, 130.4, 130.0, 129.9, 129.4, 129.1, 127.2, 126.6, 126.5, 49.6, 45.6, 41.3.

3-Butylimidazolidine-2,4-dione (10). To a suspension of hydantoin (**4**) (2.00 g, 20.00 mmol), anhydrous potassium carbonate (3.45 g, 25.00 mmol) and potassium iodide (33.2 mg, 0.20 mmol) in dry DMF (30 mL) was added 1-bromobutane freshly distilled (3.2 mL, 30.00 mmol). The reaction mixture was stirred at room temperature for 48 h. The reaction mixture was poured onto water (50 mL) and extracted with ethyl acetate (100 mL). The combined organic extracts were washed with brine, dried over magnesium sulfate anhydrous and concentrated in vacuum to afford the crude product as a yellow oil which was purified by column chromatography on a Combiflash Companion (ethyl acetate/hexane) to gave the compound **10** as a colorless solid (1.96 g, 63%). mp 98 °C. IR 3297, 2960, 2871, 1773, 1701, 1457, 1344, 1309, 1203, 1116. ESI-MS *m/z* 157 [M+H]⁺, 102. ¹H NMR (400MHz, CDCl₃) δ 6.74 (sl, 1H), 3.97 (s, 2H), 3.51 (t, 2H, *J* = 7.4 Hz), 1.64-1.56 (m, 2H), 1.39-1.30 (m, 2H), 0.94 (t, 3H, *J* = 14.7 Hz, 3H).

3-Butyl-1-((2'-(1-trityl-1H-tetrazol-5-yl)biphenyl-4-yl)methyl)imidazolidine-2,4-dione (11). To a suspension of compound **10** (1.93 g, 12.36 mmol), anhydrous potassium carbonate (2.56 g, 18.54 mmol) and potassium iodide (103 mg, 0.62 mmol) in dry DMF (10 mL) was added a solution of 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1H-tetrazole (**5**) (7.56 g, 13.60 mmol) in dry DMF (60 mL). The reaction mixture was stirred at room temperature for 72 h. The reaction mixture was poured onto water (50 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate and concentrated in vacuum to afford the crude product which was purified by column chromatography on a Combiflash Companion (ethyl acetate/hexane) to afford the yellow solid compound **11** (7.19 g, 92%). mp 185 °C. IR 3064, 3035, 2958, 1768, 1708, 1464, 1360, 1234,

1031. ESI-MS m/z 413 $[M+Na]^+$, 243, 102. 1H NMR (400MHz, $CDCl_3$) δ 7.99-7.97 (m, 1H), 7.50-7.48 (m, 2H), 7.36-7.30 (m, 4H), 7.27-7.23 (m, 6H), 7.13 (d, 2H, $J = 8.3$ Hz), 7.00 (d, 2H, $J = 8.3$ Hz), 6.88-6.86 (m, 6H), 4.44 (s, 2H), 3.52 (t, 2H, $J = 7.28$ Hz), 3.50 (s, 2H), 1.64-1.59 (m, 2H), 1.44-1.32 (m, 2H), 0.95 (t, 3H, $J = 7.32$ Hz). ^{13}C NMR (100MHz, $CDCl_3$) δ 170.8, 164.9, 157.9, 147.8, 142.4, 142.1, 134.8, 131.6, 130.7, 130.2, 129.9, 128.7, 128.3, 127.9, 127.6, 127.2, 126.2, 83.4, 49.4, 46.4, 39.0, 30.2, 20.0, 13.6.

1-((2'-(1*H*-Tetrazol-5-yl)biphenyl-4-yl)methyl)-3-butylimidazolidine-2,4-dione (12). A mixture of the compound **11** (2.24 g, 3.54 mmol) and methanol (75 mL) was refluxed for 28 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on a Combiflash Companion (methanol/chloroform) to give the yellow oil compound **12** (1.38 g, 48%). IR 3400-2800, 1766, 1702, 1467, 1382, 1237, 1114, 1060. ESI-MS m/z 391 $[M+H]^+$. 1H NMR (400MHz, $CDCl_3$) δ 7.67-7.64 (m, 2H), 7.57-7.51 (m, 2H), 7.20 (d, 2H, $J = 8.3$ Hz), 7.08 (d, 2H, $J = 8.3$ Hz), 4.49 (s, 2H), 3.89 (s, 2H), 3.38 (t, 2H, $J = 7.1$ Hz), 1.52-1.50 (m, 2H), 1.28-1.25 (m, 2H), 0.86 (t, 3H, $J = 7.32$ Hz). ^{13}C NMR (100MHz, $CDCl_3$) δ 170.0, 156.6, 155.6, 140.9, 138.6, 135.6, 130.6, 130.5, 130.4, 129.0, 127.6, 127.2, 124.3, 49.4, 45.4, 37.8, 29.6, 19.3, 13.4.

Potassium 5-(4'-((3-butyl-2,4-dioximidazolidin-1-yl)methyl)biphenyl-2-yl)tetrazol-1-ide (3). To a solution of the compound **12** (570.1 mg, 1.46 mmol) in dry MeOH (5 mL) was added anhydrous potassium bicarbonate (146.1 mg, 1.46 mmol). The reaction mixture was refluxed by or during 18 h. The solvent was removed under reduced pressure and the residue was crystallized under acetone/hexane to obtain the white solid compound **3** (488.0 mg, 78%). IR 1760, 1699, 1464, 1424, 1008. ESI-MS m/z 429 $[M+H]^+$, 391, 102. 1H NMR (400MHz, $DMSO-d_6$) δ 7.57-7.52 (m, 2H), 7.40-7.30 (m, 2H), 7.09 (s, 4H), 4.45 (s, 2H), 3.89 (s, 2H), 3.36 (t, 2H, $J = 7.08$ Hz), 1.58-1.44 (m, 2H), 1.34-1.21 (m, 2H), 0.85 (t, 3H, $J = 7.32$ Hz). ^{13}C NMR (100MHz, $DMSO-d_6$) δ 170.4, 161.0, 156.9, 141.4, 140.2, 134.5, 132.5, 130.8, 130.2, 129.7, 127.5, 126.9, 126.8, 49.8, 45.8, 38.2, 29.9, 19.6, 13.8. HRMS calculated for $C_{21}H_{22}KN_6O_2$ $[M+H]^+$ 429.1441, found 429.1465.

Biological Evaluation

Proof of concept was designed *in vitro* and *in vivo* to evaluate selectivity of these compounds for the AT1 receptor.

In Vitro Studies

Competition Binding Assays

CHO cells were purchased from American Type Culture Collection (ATCC). 3H -AngII was obtained from GE Healthcare (USA). AngII was from Sigma Aldrich (USA). Cell culture supplies were obtained from Invitrogen (USA).

CHO cells stably expressing the AT₁ receptor were transferred to 12-well culture plates 24h before binding assays. One day after plating, cells were washed briefly in 25mM Tris-HCl buffer, pH 7.4 containing 140mM NaCl, 5mM $MgCl_2$ and 0.1% bovine serum albumin (BSA). Binding experiments were performed at 4°C to avoid any functional interference such as receptor activation, phosphorylation, internalization and were initiated by the addition of 3H -AngII 4pM and different concentrations (10^{-12} to 10^{-6} M) of Losartan, compounds **1-3** as a competitor in a 0,5mL final volume assay. The binding buffer consisted of 25mM Tris-HCl, pH 7.4, including 5mM $MgCl_2$, 0.1% BSA and 100 (g/mL bacitracin (Sigma Aldrich, USA).

In Vivo Studies

Animal Model

Experiments were performed on 250 – 300 g male Spontaneously Hypertensive Rats (SHR). Animals used in the present experiments were obtained from the colony of Centro de Desenvolvimento de Modelos Experimentais (CEDEME), Escola Paulista de Medicina/Universidade Federal de São Paulo, Brazil. This project was approved by the Ethic Committee on Animal Experimentation (Universidade Federal de São Paulo) (CEP 1825/08).

Experimental Groups

The different groups of animals analyzed in this study are described in Table 1. These animals had their BP measured at times: zero time (before administration of the compounds) and 15, 30, 60, 120 and 240 min after intravenous (i.v.) administration of the compounds.

Measurements of the Systolic Blood Pressure and Body Weight

Measurements of the systolic BP and body weight were made in all groups at different times during the day. The systolic BP was measured before and after the drugs administration.

Systolic BP was determined by tail-cuff method (AdInstruments PowerLab 2/25, model ML825) and the Ohaus scale (Union, N. J, USA) was used to determine the body weight.

Enzymatic Activity Assay

ACE catalytic activity was determined fluorimetrically as described by Friedland and Silverstein [22]. Briefly, an aliquot of animal serum (5 μ L) was incubated with 200 L assay buffer (solution containing 1 mmol/L ZPhe-HisLeu (Z-PHL), in 100 mmol/L sodium borohydride buffer, pH 8.3, 300 mmol/L NaCl and 0.1 mmol/L ZnSO₄) for 10 min at 37°C.

Table 1. Groups analyzed in this study.

	Experimental Groups	A single dose (i.v.)
SHR	Control (n=12)	Saline
	Losartan (n=11)	50 mg/kg
	Captopril (n=12)	
	Compound 1 (n=12)	
	Compound 2 (n=12)	
Compound 3 (n=12)		

The enzymatic reaction was stopped by the addition of 1.5 mL 280 mmol/L NaOH following incubation with 100 L o-phthalaldehyde (20 mg/mL methanol) for 10 min. The fluorescent reaction was stopped by the addition of 200 L 3N HCl. The liberated dipeptide, H-L, was measured fluorimetrically (360 nm excitation and 465 nm emission) using Tecan Infinif F200 equipment (USA).

The standard curve was obtained using varying concentrations of L-HL in the blank reaction mixture and it showed a linear relation between relative fluorescence and H-L concentration.

Statistical Analysis

Data are reported as mean \pm SD. The systolic blood pressure was analyzed by One Way Repeated Measures analysis of variance (One Way RM ANOVA) and ACE activity was analyzed by One Way analysis of variance (One Way ANOVA). Both tests were followed by post hoc Tukey's test when needed. The level of significance was set at $p < 0.05$.

RESULTS

Chemistry

Compounds **1-3** were prepared to increase water solubility from NCE in the same way as Losartan. The syntheses of the target compounds are outlined in Schemes **1 - 3**.

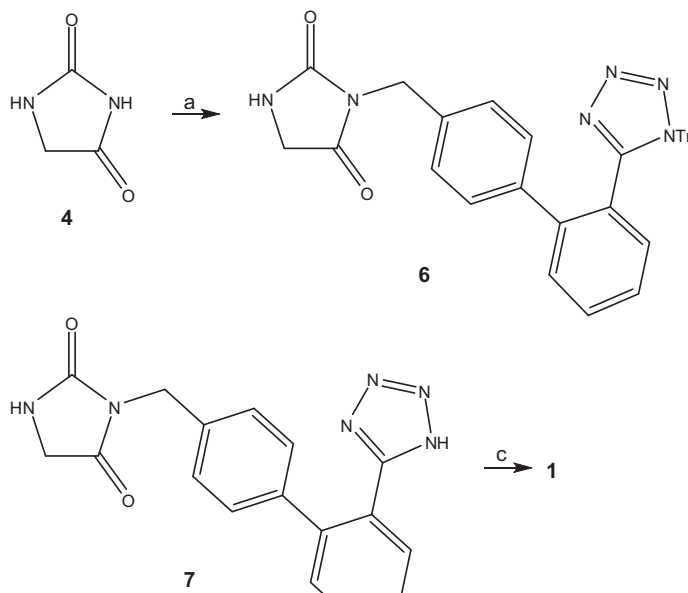
Alkylation of hydantoin (**4**) with 5-(4'-bromomethyl-biphenyl-1-yl)-1-trityl-1*H*-tetrazole (**5**) in DMF in the presence of 1.2 equivalents of potassium carbonate yielded the desired *N*-alkylated product **6** in 75% yield (Scheme **1**) [23]. The triphenylmethyl group was subsequently cleaved with HCl in water/tetrahydrofuran furnishing the compound **7** in 82% yield.

Usifoh [21] reported the removal of the triphenylmethyl using methanol under reflux. We used this protocol to improve the yield in the deprotection step, and compound **7** were obtained in 95% yield. Compound **1** was prepared by treatment of compound **7** with 1.0 equivalent of potassium bicarbonate giving 74% yield.

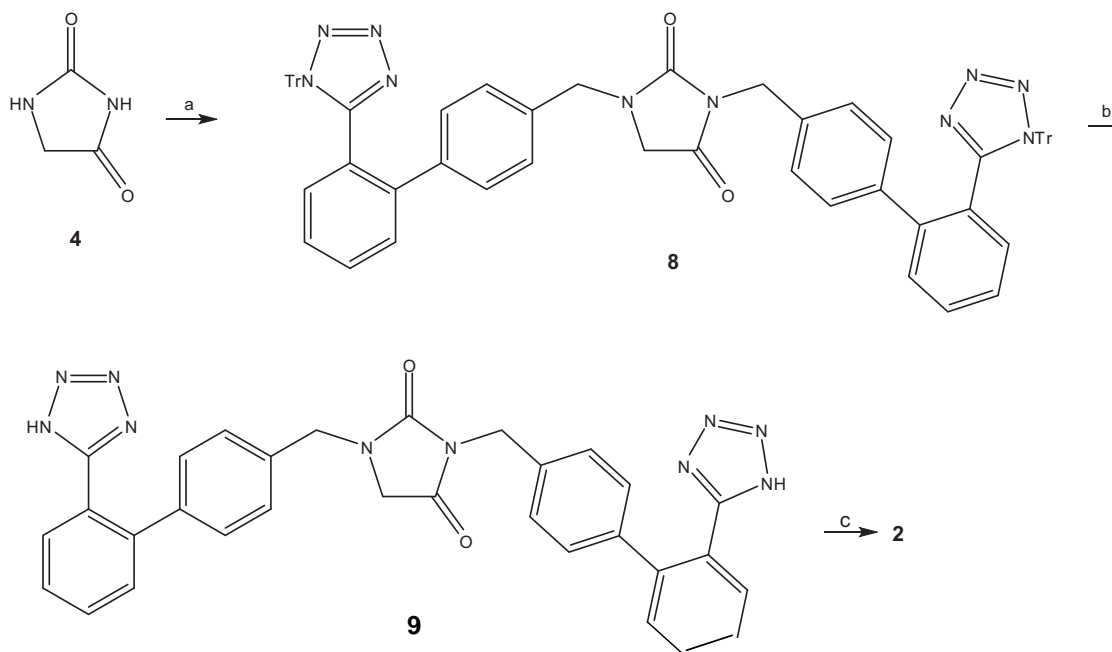
The same synthetic strategy described above to compound **1** was employed to obtain the dimeric compound **2**. The *N-N'*-dialkylated product **8** was obtained from 2.0 equivalents of bromide **4** in presence of base in excess. Deprotection reaction of triphenylmethyl group in presence of methanol under reflux resulted in compound **9** in a good yield.

Thus, treatment of compound **9** with 2.0 equivalents of potassium bicarbonate gave compound **2** with 58% yields (3 steps from **4**) (Scheme 1).

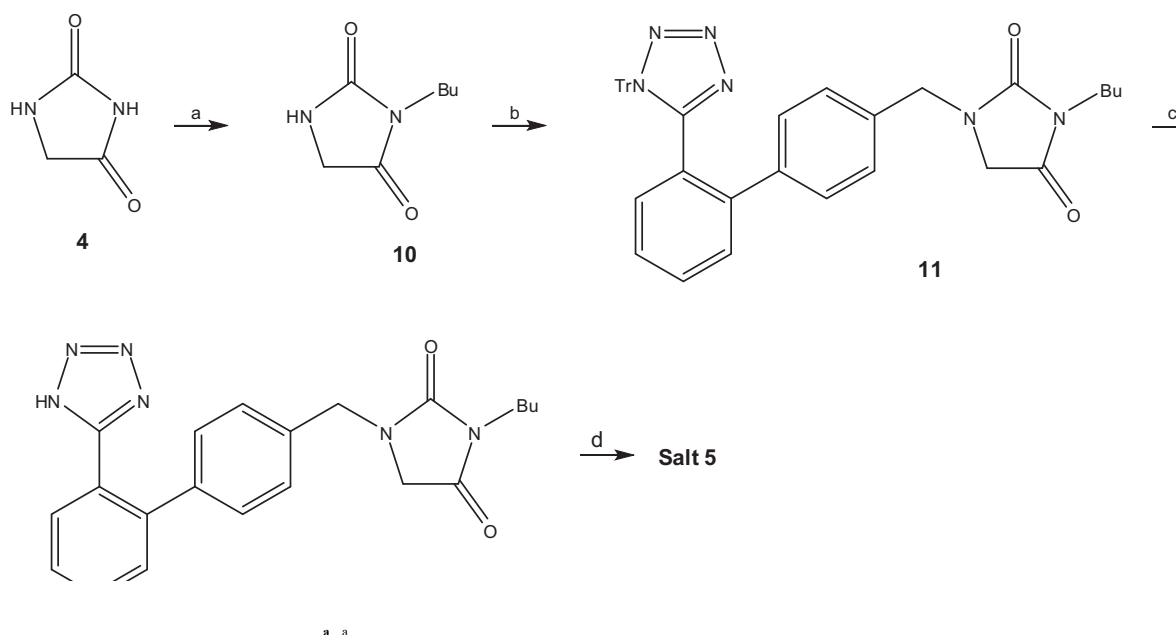
Compound **3** was prepared as shown in Scheme 3. The first step involved the reaction of **4** with 1-bromobutane in presence of base and catalytic amount of potassium iodide to afford compound **10** with 63% yield. Alkylation of compound **10** with bromide **5** and deprotection of compound **11** gave the compound **12** with 44% yield (2 steps). Finally, compound **3** was obtained with 78% yield by the treatment of compound **12** with potassium bicarbonate in methanol under reflux.



Scheme 1. Synthesis of compound 1^a. ^aReagents and conditions: (a) 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1H-tetrazole (**5**) (1.0 equiv.), K₂CO₃ (1.2 equiv.), DMF, rt, 18h, 75%; (b) Method A: 37% HCl, THF/H₂O (4:1), rt, 24h, 82%; or Method B: MeOH, reflux, 16h, 95%; (c) KHCO₃ (1.0 equiv.), MeOH, reflux, 18h, 74%.



Scheme 2. Synthesis of compound 2^a. ^aReagents and conditions: (a) 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1H-tetrazole (**5**) (2.0 equiv.), K₂CO₃ (3.0 equiv.), DMF, rt, 72h, 82%; (b) MeOH, reflux, 72h, 82%; (c) KHCO₃ (1.0 equiv.), MeOH, reflux, 18h, 74%.



Scheme 3. Synthesis of compound 3^a. ^aReagents and conditions: (a) 1-bromobutane (1.5 equiv.), K₂CO₃ (1.5 equiv.), KI (10 mol%), DMF, rt, 48h, 63%; (b) 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1H-tetrazole (5) (1.1 equiv.), K₂CO₃ (1.5 equiv.), KI (5 mol%), DMF, rt 72h, 92%; (c) MeOH, reflux, 28h, 48%; (d) KHCO₃ (1.0 equiv.), MeOH, reflux, 18h, 78%.

In Vitro and *In Vivo* Biological Evaluations

In Vitro Binding Studies

The affinities of the compounds for the AT₁ receptor were performed using competition binding assays in CHO cells stably expressing the AT₁ receptor. We used Losartan as positive control and ³H-AngII as the radioligand compound (Fig. 2).

Our results showed that only the compound 2 was able to bind to the AT₁ receptor, however its affinity was very low (0.3μM) when compared with the Losartan (2.7 nM).

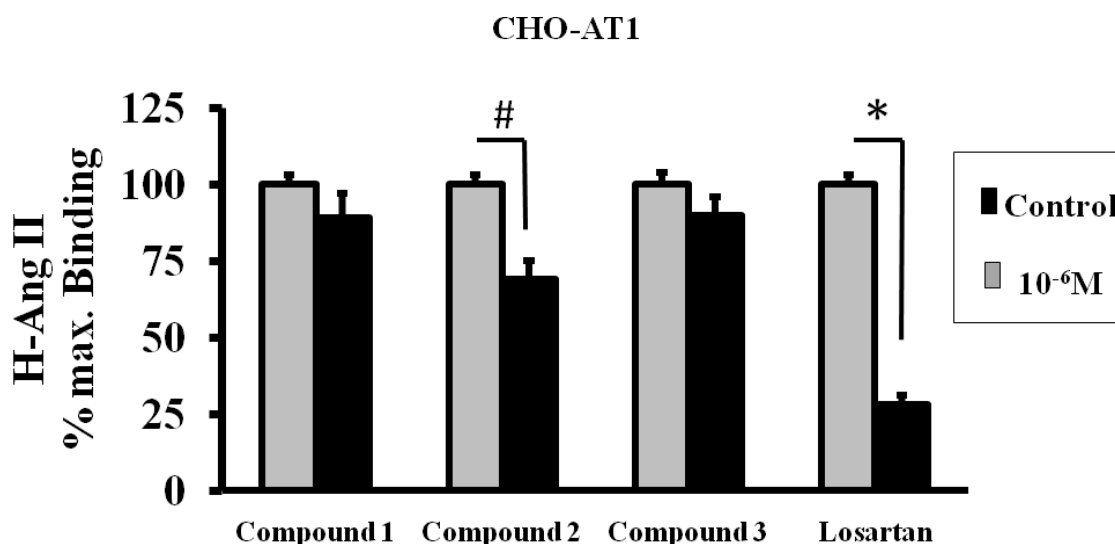


Fig. (2). Competition Binding Assays. Maximum binding obtained in the presence of the AT₁ receptor antagonist Losartan or compounds 1-3 (10⁻⁶M) in CHO cells stably expressing the AT₁ receptor. Values are means ± SE of three independent experiments done in duplicate. (#, *p* < 0.05 and *, *p* < 0.001).

Studies In Vivo

Body Weight

The animals body weight had a small variation between the groups which was expected (250 – 300g). Importantly, the intravenous doses (i.v.) of the compounds were administrated based on body weight of each animal (50 mg/Kg) (Table 2 and Fig. 3).

Table 2. Body weight of the all groups studied.

	Groups	Body Weight
SHR	Control (n=12)	280.3 ± 13.8
	Losartan (n=11)	288.7 ± 16.3
	Captopril (n=12)	268.5 ± 19.4
	Compound 1 (n=12)	285.0 ± 11.3
	Compound 2 (n=12)	273.1 ± 11.6
	Compound 3 (n=12)	255.8 ± 18.2

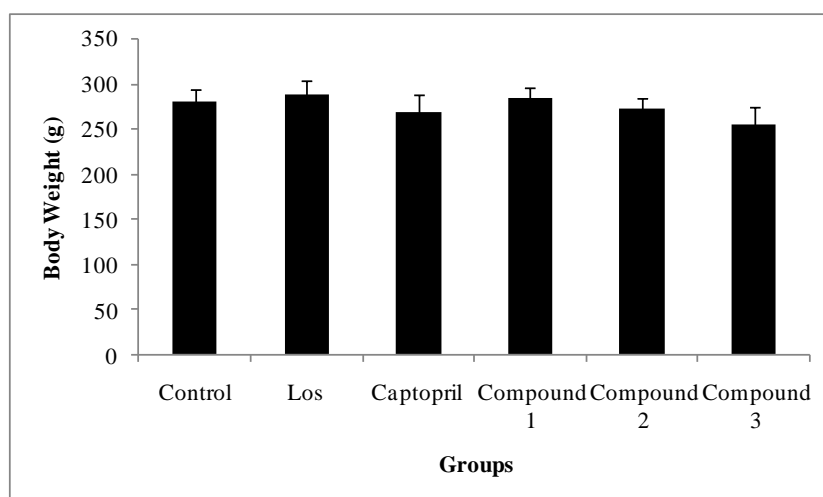


Fig. (3). Body weight of the experimental groups. Data are expressed as mean ± standard deviation.

Blood Pressure Measurements

Values of arterial BP in SHR after a single dose of compounds 1-3, Losartan or Captopril are described in Table 3 and Fig. (4). The arterial BP after Losartan or Captopril administration (i.v.) reduced significantly in all studied time when compared to respective zero time.

Table 3. Different times of Systolic Blood Pressure the experimental groups.

Groups SHR	Systolic Blood Pressure (mmHg)					
	Zero minute	15 minutes	30 minutes	60 minutes	120 minutes	240 minutes
Control (n=12)	185.2 ± 2.6	185.0 ± 1.8	185.5 ± 2.5	185.9 ± 1.8	186.1 ± 3.4	183.4 ± 2.8
Losartan (n=11)	178.2 ± 10.3	165.1 ± 6.9*	164.2 ± 10.2*	159.9 ± 11.6*	144.2 ± 12.6*	144.4 ± 11.1*
Captopril (n=12)	183.6 ± 17.0	169.4 ± 15.7*	167.7 ± 12.0*	167.4 ± 11.8*	166.8 ± 17.8*	161.1 ± 17.4*
Compound 1 (n=12)	195.1 ± 6.5	191.2 ± 5.0	191.3 ± 5.4	191.3 ± 6.1	191.1 ± 5.4	186.3 ± 5.7*
Compound 2 (n=12)	177.3 ± 12.2	174.8 ± 16.2	182.6 ± 9.2	179.1 ± 9.9	178.7 ± 7.1	179.4 ± 8.5
Compound 3 (n=12)	179.1 ± 12.6	180.9 ± 13.6	179.0 ± 12.9	173.3 ± 17.4	173.4 ± 9.3	171.2 ± 6.2

Values are means ± SD.

* $P < 0,05$. The groups were compared with their respective control.

Compound 1 showed a significant reduction in BP (8.8 mmHg) 240 min after the administration when compared

with zero time. For compound **2** no changes in BP were observed after its administration, although this salt had demonstrated some affinity for the AT₁ receptor according to the binding studies. Compound **5** induced a decrease not statistically significant in BP 240 min after its administration.

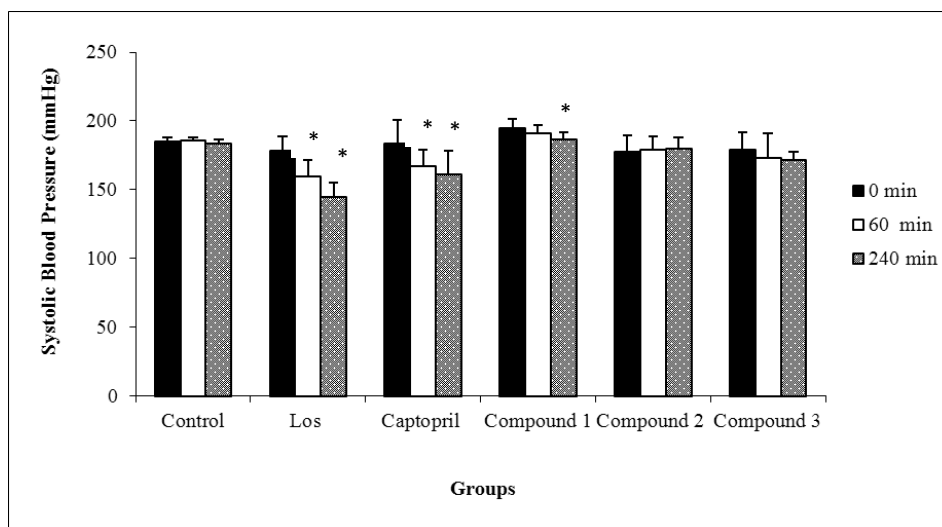


Fig. (4). SBP of different groups. Data are expressed as mean \pm SD. All groups were compared with their respective Control (Tukey's test, * $P < 0,05$).

ACE Activity

As we observed a decrease in BP on animals treated with compound **1** and in the binding assays this compound showed no affinity for the AT₁ receptor, we decided to investigate if this compound could act as ACE inhibitor.

Our results showed that serum ACE activity using ZPhe-HL as substrate decreased significantly in the Captopril and compound **1** groups when compared with Control (saline group). In Captopril and compound **1** groups ACE activity was decreased 30.6 and 32.4%, respectively (Fig. 5). No significant changes in ACE activity were detected for the other groups when compared to Control (saline group).

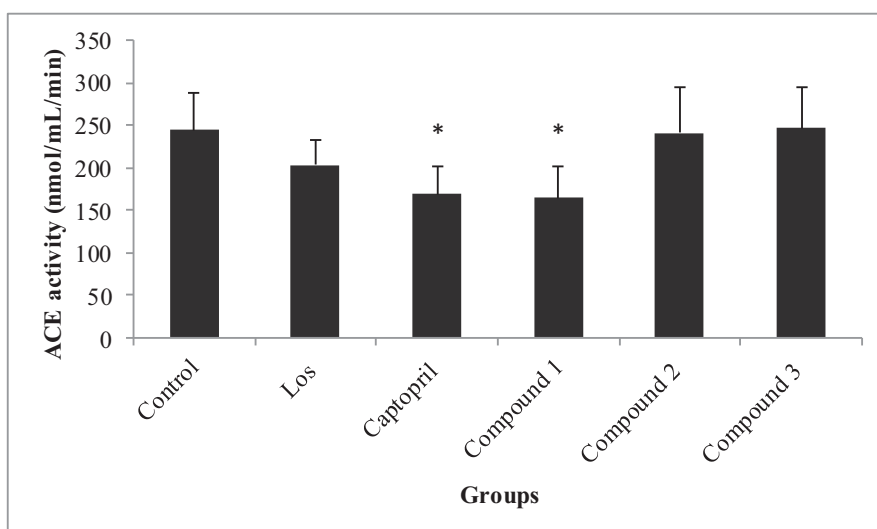


Fig. (5). ACE activity (nmol/mL/min) in serum of studied animals after 60 minutes intravenous administration of the compounds. Results are mean \pm SD. All groups were compared with their respective control (Tukey's test, * $P < 0,05$).

DISCUSSION AND CONCLUSIONS

Novel compounds **1-3** designed as antagonist of the AT₁ receptor having hydantoin group moiety linked to biphenyl-1*H*-tetrazole scaffold were prepared according to reported methods described in outlined Schemes **1 - 3**. To afford these compounds, the advanced intermediate 5-(4'-bromomethyl-biphenyl-2-yl)-1-trityl-1*H*-tetrazole (**5**) was employed. Compounds **1-3** were obtained in good to moderate overall yields (53%, 58% and 22%, respectively). The process employed was able to furnish from 500 mg to 1g of compounds **1-3** to conduct all biological assays.

Biological evaluations *in vitro* and *in vivo* were performed to verify the pharmacological activities of compounds **1-3** as AT₁ receptor antagonists with antihypertensive effect. *In vitro* binding assays performed in cells expressing the AT₁ receptor showed that only the compound **2** was able to compete with the radioligand ³H-Ang II although with a very low affinity when compared with the AT₁ receptor antagonist Losartan.

Aiming to evaluate the antihypertensive effect of these compounds it was carried out an *in vivo* study. These experiments demonstrated that compounds **1-3** showed no antihypertensive effect when compared with Losartan and Captopril. On the other hand, the compound **1** had no anti-hypertensive effect in the early minutes, but after 240 min it was able to significantly reduce BP by 8.8 mmHg. This reduction in BP after 240 min may be due to the formation of secondary metabolites which would have anti-hypertensive activity or this compound could act as ACE inhibitor.

Based on decrease in BP observed in animals treated with compound **1**, we decided to measure serum ACE activity in all groups. In compounds **2** and **3** groups serum ACE activity had no difference when compared to Control group. Compound **1** and Captopril showed a similar decrease in the ACE activity and this reduction was statistically significant when compared to Control (saline group).

As presented in the Introduction, hydantoin groups after insertion into a molecule could add to it different pharmacological properties. We believe that compound **1** (or its secondary metabolites) can play a role as an ACE inhibitor, and further studies should be conducted concerning this regard to understand the involved mechanisms as well as signaling pathways.

ABBREVIATIONS

ACE	=	Angiotensin converting enzyme;
Ang II	=	Angiotensin II;
BP	=	Blood pressure;
BSA	=	Bovine serum albumine;
CHO	=	Chinese hamster ovary cells;
DMF	=	<i>N,N</i> -dimethylformamide;
HPLC	=	High-performance liquid chromatography;
HRMS	=	High-resolution mass spectra;
IR	=	Infrared; i.v., intravenous;
MeOH	=	Methanol ;
min	=	Minutes;
MS	=	Mass spectra;
NCE	=	New chemical entity;
RAAS	=	Renin–angiotensin–aldosterone system;
RAS	=	Renin-angiotensin system;
SAR	=	Structure activity relationship;
SHR	=	Spontaneously hyperensive rats;
THF	=	Tetrahydrofuran;
TMS	=	Tetramethylsilane.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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