Supporting Information

for

Design, Synthesis and Biological Evaluation of Novel Biphenylsulfonamide Derivatives as Selective AT2 Receptor Antagonists

Danhui Wang a, Wenjie Zhao a, Zuzhi Zhang a, Yanchun Zhang * a,b, Jiaming Li a,b, Weijun Huang a

a College of Pharmacy, Anhui University of Chinese Medicine, Hefei, 230012, China
b Anhui Province Key Laboratory of Chinese Medicinal Formula, Hefei, Anhui, 230012, China

* Corresponding author: Yanchun Zhang, E-mail address: yczhang2017@163.com
† These authors contributed equally to this work.

Table of contents:
1. Synthesis of intermediate 4a-4c
2. NMR and HRMS spectra of compounds 8a-8l, 9a-9h
3. Radioligand binding assay steps
4. NG108-15 cell experiment steps
1. **Synthesis of intermediate 4a-4c**

1.1 **General procedure A: Synthesis of intermediates (2a-2c)**

Isobutylbenzene, butylbenzene or butoxybenzene (74.50 mmol) was dissolved in the CH₂Cl₂ (200 mL). Subsequently, chlorosulfonic acid (298.01 mmol) was added slowly to the solution in an ice bath and the mixture was stirred at rt for 1 h. The reaction was quenched by dropped ice-cold water (50 mL) into the mixture. The reaction mixture was extracted twice with CH₂Cl₂ (20 mL) and washed with water (30 mL×2) and brine (30 mL×2). The organic layer was dried with anhydrous Na₂SO₄, filtered, and evaporated to achieve 2a-2c.

1.2 **General procedure B: Synthesis of intermediates (3a-3c)**

To a 250 mL closed pressure vessel was added 2a, 2b, or 2c (70.90 mmol) dissolved in DCM (200 mL). Tert-butylamine (11.18 mL, 106.35 mmol) was added to the mixture in an ice bath. The reaction was stirred at r.t for 8 h. The reaction was extracted with DCM (50 mL), the organic layers were combined and washed with NaCl, dried over Na₂SO₄, and concentrated to give 3a-3c.

1.3 **General procedure C: Synthesis of intermediates (4a-4c)**

To a 100 mL round-bottom flask were added 3a, 3b, 3c (7.42 mmol) followed by dry THF (20 mL). N-BuLi (1.6 M in hexane, 9.28 mL, 14.85 mmol) was added to the mixture at -78 °C. The reagent was added under nitrogen and the reaction was stirred for 1 h. After the flask was warmed to -20 °C, kept for 3 h and subsequently decreased to -78 °C. Triisopropyl borate (2.57 mL, 11.14 mmol) was then added. The reaction mixture was stirred overnight at room temperature. The reaction mixture was treated with an excess of 2 M HCl solution in an ice bath. The mixture was extracted with ethyl acetate (50 mL x 2). The combined organic phase was washed with water and brine, dried with Na₂SO₄, filtered and evaporated. Using silica gel column chromatography (PE/EA as eluent), the residue was purified to obtain 4a-4c.
2. NMR and HRMS spectra of compounds 8a-8l, 9a-9h.

$^1$H and $^{13}$C NMR spectra were recorded at room temperature at 400 MHz and 100 MHz respectively using a QNP probe. NMR spectra were recorded in deuterated dimethyl sulfoxide (DMSO-$d_6$) at room temperature unless otherwise stated. Chemical shifts (δ values) are reported in parts per million, and are referenced to the deuterated residual Solvent peak. NMR data was reported as: δ value (chemical shift, J-value(Hz), integration, where s = singlet, d = doublet, t = triplet, q = quartet, brs = broad singlet). High-resolution mass spectra (HRMS) were recorded with a Bruker microTOF ESI-TOF mass spectrometer in positive ion mode unless otherwise specified.

8a:
8b:
8c:
Spectrum from MSSI20201214.wav (sample 8) - 1G, +TOF... - 1G, +TOF MS (50 - 1000) from 0.449 to 0.493 min

8d:
Spectrum from MASS20201214.wiffZ (sample 9) - 1H, *TOF MS (50 - 1000) from 0.783 to 0.818 min

8e:

[Structural diagram]
8f: 

Chemical structure diagram
8h:
8i:
Spectrum from MASS20201214.wff2 (sample 4) - 3A. + TOF MS (50 - 1000) from 0.088 to 0.123 min
Spectrum from MASS20201214.wiff2 (sample 7) - 3D, + TOF MS (50 - 10000) from 0.343 to 0.378 min

9a:
Spectrum from 07062.wff (sample 10) - 1C, Experiment 1, +TOF MS (50 - 1000) from 0.146 to 0.177 min
Spectrum from 07062.wiff (sample 12) - 1E, Experiment 1, +TOF MS (50 - 1000) from 0.145 to 0.177 min

9d:
9e:
Spectrum from 07062.wiff (sample 16) - 2C, Experiment 1, +TOF MS (50 - 1000) from 0.146 to 0.177 min

9f:
9g:
Spectrum from 07062.wiff (sample 14) - 2E, Experiment 1. +TOF MS (50 - 1000) from 0.145 to 0.177 min

9h:
3. Radioligand binding assay steps

3.1 Rat Liver Membrane AT1 Receptor Binding Assay.

Rat liver membranes were prepared according to the method of Dudley et al. Binding of \(^{125}\text{I}\)-Ang II to membranes was conducted in a final volume of 0.5 mL containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl\(_2\), 1 mM EDTA, 0.025% bacitracin, 0.2% BSA (bovine serum albumin), liver homogenate corresponding to 5 mg of the original tissue weight, \(^{125}\text{I}\)-Ang II (80000-85 000 cpm, 0.03 nM), and variable concentrations of test substance. Samples were incubated at 25 °C for 2 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets, which had been presoaked overnight with 0.3 % polyethylamine, using a Brandel cell harvester. The filters were washed with 3×3 mL of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a \(\gamma\)-counter. The characteristics of the Ang II binding AT1 receptor was determined by using six different concentrations (0.03-5 nmol/L) of the labeled \(^{125}\text{I}\)-Ang II. Nonspecific binding was determined in the presence of 1 \(\mu\)M Ang II. The specific binding was determined by subtracting the nonspecific binding from the total bound \(^{125}\text{I}\)-Ang II. The apparent dissociation constant Ki values were calculated from IC\(_{50}\) values using the Cheng-Prusoff equation. The binding data were best fitted with a one-site fit. All determinations were performed in triplicate.

3.2 HEK-293 cell AT2 Receptor Binding Assay.
After HEK-293 cells were transfected with AT\(_2\) receptor, lysis buffer (150 mM NaCl, 0.1% Triton X-100, 50 mM Tris-HCl, 1 mM EDTA, protease inhibitor) was used to separate the cell membrane of HEK-293 cells\(^2\), using 27-G Resuspend and mix the lysis solution, and centrifuge at 12,000 g for 10 minutes at 4 °C. The supernatant was collected and centrifuged for a further 60 minutes. The precipitated membrane extracts were buffered (150 mM NaCl, 10 mM MgCl\(_2\), 0.5% SDS, 1% Triton X-100, 50 mM Tris-Cl, 1 mM EDTA, protease inhibitor) for resuspension. Binding of \([^{125}\text{I}]\)-CGP42112A to membranes was conducted in a final volume of 0.5 mL containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl\(_2\), 1 mM EDTA, 0.025% bacitracin, 0.2% BSA (bovine serum albumin), cell homogenate corresponding to 5 mg of the original tissue weight, \([^{125}\text{I}]\)-CGP42112A (80000-85 000 cpm, 0.03 nM), and variable concentrations of test substance. Samples were incubated at 25 °C for 2 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets, which had been presoaked overnight with 0.3% polyethylamine, using a Brandel cell harvester. The filters were washed with 3×3 mL of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a γ-counter. The characteristics of the Ang II binding AT\(_1\) receptor was determined by using six different concentrations (0.03-5 nmol/L) of the labeled \([^{125}\text{I}]\)-CGP42112A. Nonspecific binding was determined in the presence of 1 µM Ang II. The specific binding was determined by subtracting the nonspecific binding from the total bound \([^{125}\text{I}]\)-CGP42112A. The apparent dissociation constant \(K_i\) values were calculated from IC\(_{50}\) values using the Cheng-Prusoff equation. The binding data were best fitted with a one-site fit. All determinations were performed in triplicate.

4. NG108-15 cell experiment steps

We have previously shown that NG108-15 cells in their undifferentiated state express only the AT\(_2\) receptor and that a 3-day treatment with Ang II or the selective peptidic AT\(_2\) receptor agonist CGP-42112A induces neurite outgrowth\(^3,4\). The signaling pathways involve a sustained increase in Rap1/BRaf/p42/p44\(^{\text{mapk}}\) activity and activation of the nitric oxide/guanylyl cyclase/cGMP pathway\(^5-7\). Cells were plated as described in the Experimental Section, and adequate test concentrations for
each compound were determined by testing a dilution series of each compound ranging from 1 pM to 1 uM. For all the compounds it was only at the highest concentration that any evidence of cell death was observed. Antagonistic effect was verified through co-incubation with Ang II resulting in reduced Ang II-induced neurite outgrowth, verifying blockage of the AT$_2$ receptor. Agonistic effect was verified through co-incubation with the selective AT$_2$ receptor antagonist PD-123,319, which reduced neurite outgrowth, verifying that the effect was mediated through the AT$_2$ receptor. Treatment with PD-123,319 alone did not alter the morphology compared to untreated cells.

The chemicals used in the present study were obtained from the following sources: Dulbecco’s modified Eagle’s medium (DMEM), heat-inactivated fetal bovine serum (FBS), HAT supplement (hypoxanthine, aminopterin, thymidine), gentamycin from Gibco BRL (Burlington, Ontario, Canada), and Ang II from MedChemExpress (Monmouth Junction, NJ, USA). PD-123,319 was obtained from MedChemExpress (Monmouth Junction, NJ, USA). All other chemicals were of grade A purity.

For all experiments, cells were plated at the same initial density of $3.6 \times 10^4$ cells/35 mm Petri dish. To determine a good test concentration, all compounds were tested at various concentrations ranging from 1 pM to 1 uM. It was only at the highest concentration of compounds 8d and 8h that any evidence of cell death was observed, and that was most probably due to a higher concentration of DMSO (due to low solubility). Cells were treated without (control cells), or with Angiotensin II (100 nM) or with compound 8d (100 nM), 8h (100 nM), 9h (10 and 100 nM), 8i (10 and 100 nM), 8j (100 nM), 8k (100 nM), or 8l (10 and 100 nM) in the absence or in the presence of PD-123,319 (10 uM), an AT$_2$ receptor antagonist. The antagonist was introduced daily 30 min prior to Ang II, compound 8d, 8i, 8k, 8l, 8h or 9h, to evaluate antagonistic properties.

Cells were examined under a phase contrast microscope, and micrographs were taken after 3 days under the various experimental conditions. Cells with at least one neurite longer than a cell body were counted as positive for neurite outgrowth. The number of cells with neurites represents the percentage of the total amount of cells in
the micrographs. At least three different experiments were conducted for each condition, each in duplicate\(^{(3)}\). At least five images were taken per petri dish. Hence, a total of 250–400 cells from each of the duplicate dishes were examined.

References

[1] Dudley DT, Panek RL, Major TC, et al. Subclasses of angiotensin II binding sites and their functional significance [J]. Mol Pharmacol, 1990, 38 (3): 370-377.

[2] Grieger JC, Soltys SM, Samulski RJ. Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector [J]. Molecular Therapy, 2016, 24 (2): 287-296.

[3] Buisson B, Bottari SP, Gasparo MD, et al. The angiotensin AT\(_2\) receptor modulates T-type calcium current in non-differentiated NG108-15 cells [J]. Febs Letters, 1992, 309 (2): 1-9.

[4] Gasparo, DM. Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells. Effect counteracted by the AT1 receptors [J]. J Biol Chem, 1996, 271 (37): 22729-22735.

[5] Gendron, L. Cyclic AMP-independent involvement of Rap1/B-Raf in the angiotensin II AT2 receptor signaling pathway in NG108-15 cells [J]. J Biol Chem, 2003, 278 (6): 3606-3614.

[6] Gendro Louis, Laflamme Liette, Nathalie R, et al. Signals from the AT\(_2\) (angiotensin type 2) receptor of angiotensin II inhibit p21ras and activate MAPK (mitogen-activated protein kinase) to induce morphological neuronal differentiation in NG108-15 cells [J]. Mol. Endocrinol. 1999, 13, 1615-1626.

[7] Gendron, Louis, Cote, et al. Nitric Oxide and Cyclic GMP Are Involved in Angiotensin II AT[sub 2] Receptor Effects on Neurite Outgrowth in NG108-15 Cells [J]. Neuroendocrinology, 2002, 75, 70-81.