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Selective angiotensin II AT_2 receptor agonists: Benzamide structure–activity relationships

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ABSTRACT

In the investigation of the structure–activity relationship of nonpeptide AT_2 receptor agonists, a series of substituted benzamide analogues of the selective nonpeptide AT_2 receptor agonist M024 have been synthesised. In a second series, the biphenyl scaffold was compared to the thienylphenyl scaffold and the impact of the isobutyl substituent and its position on AT_1/AT_2 receptor selectivity was also investigated. Both series included several compounds with high affinity and selectivity for the AT_2 receptor. Three of the compounds were also proven to function as agonists at the AT_2 receptor, as deduced from a neurite outgrowth assay, conducted in NG108-15 cells.

1. Introduction

Angiotensin II (Ang II) is the major component of the renin–angiotensin system (RAS), which plays an important role in the regulation of blood pressure and fluid and electrolyte homeostasis. This octapeptide mediates its effects through stimulation of two receptor subtypes, the AT_1 receptor and the AT_2 receptor. Both receptors are G-protein coupled receptors, but exhibit only 32–34% homology in their amino acid sequences.[1,2] The AT_1 receptor is associated primarily with classical Ang II effects, such as vasoconstriction, aldosterone and vasopressin release, retention of sodium and water, and cellular growth and proliferation. Activation of the AT_2 receptor on the other hand frequently renders opposing effects and stimulation will, for example, induce vasodilatation, antiproliferation and apoptosis.[1–3] Furthermore, stimulation of neuronal cell AT_2 receptors leads to neurite outgrowth, which is characteristic for neuronal differentiation.[1,4,5] Another feature of the AT_2 receptor, which is connected to its involvement in cell differentiation, is the suggested importance in fetal development. The AT_2 receptor is widely expressed in the fetal tissues, but its expression drops dramatically after birth.[6] Notably in the adults the AT_2 receptor is up-regulated during certain pathological conditions such as myocardial infarction, vascular injury, brain ischemia, and renal failure as well as in cutaneous wounds.[2,7] The RAS is an established target in the treatment of hypertension and it was recently postulated that some of the positive effects of AT_1 receptor antagonists could be ascribed to a stimulation of the AT_2 receptor.[8,9] We have previously reported the first selective nonpeptide AT_2 receptor agonist, M024,[10] and we are now further exploring the structure–activity relationship for selective nonpeptide AT_2 receptor agonists. M024 was derived from the nonselective agonist L-162,313 by stepwise simplification of the nitrogen containing heterocyclic ring system (Fig. 1).[10,11]

The *N*-benzylimidazole fragment in M024 was initially believed to be a strong determinant for AT_2 receptor selectivity. Recently, however, we disclosed two series of compounds, including highly selective AT_2 receptor agonists, where the imidazole has been replaced by amides. We investigated analogues with both the nitrogen in the same position as the alkylated nitrogen in the imidazole ring (**48**, Fig. 2), as well as inverted amide analogues (**49**).[12] In both series amides with small substituents showed the best affinity to the AT_2 receptor.

Herein, we report a series of benzamides (series A, Fig. 2) with good AT_2 receptor affinity and selectivity including ligands proven to serve as agonists. Furthermore, we have compared the biphenyl and thienylphenyl scaffolds and examined the impact of the isobutyl substituent and its position on the AT_1/AT_2 receptor selectivity (series B, Fig. 2).

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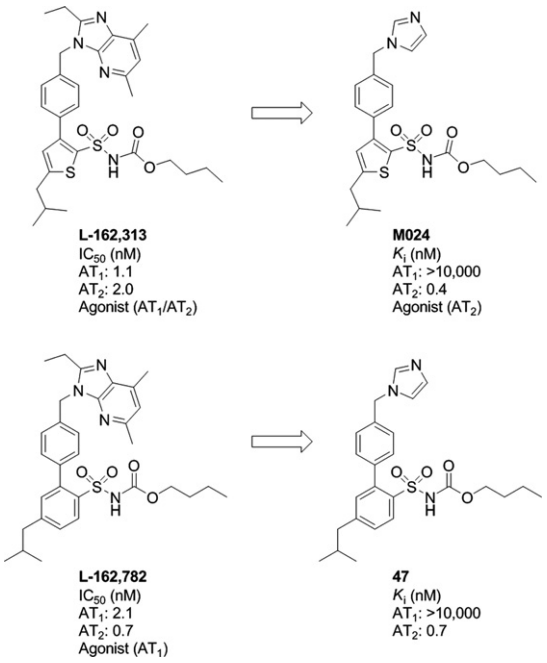


Figure 1.

2. Results

2.1. Chemistry

The synthesis of series A, outlined in Scheme 1, started with the thiopheneboronic acid **1** which was prepared essentially as described by Kevin et al.[13,14] The thiopheneboronic acid **1** was coupled with 4-iodobenzoic acid in a Suzuki reaction with $Pd(PPh_3)_4$ as catalyst and Na_2CO_3 as base under microwave irradiation (110 °C, 5 min), rendering compound **2** in 65% yield. The carboxylic acid **2** was transformed into the corresponding acyl chloride **3** by treatment with oxalyl chloride and a catalytic amount of DMF. The acyl chloride **3** served as a good starting material for preparation of the first series of compounds (**4**–**15**) in a parallel manner. Compound **3** was dissolved in dry DCM and added to sample vials loaded with a diverse set of amines. The resulting amides were then treated with borontrichloride or TFA to remove the *tert*-butyl protecting group.[15] Finally, the primary sulfonamides were acetylated with *n*-butyl chloroformate in DCM and TEA to yield the final compounds **4**–**15**, in 15–72% yield after purification by chromatotron or preparative LC–MS.

The series B compounds were prepared from a set of different benzenesulfonamides (Schemes 2 and 3). The synthesis of 3-, 4-isobutyl- and 3-, 4-benzylbenzenesulfonamide (**18**–**21**, Scheme 2) started with the conversion of 3- and 4-bromobenzenesulfonyl chloride to the corresponding *N*-*tert*-butylsulfonamides, compounds **16** and **17**, in excellent yields (95–96%). The compounds **16** and **17** were then alkylated under Negishi coupling conditions with freshly prepared isobutylzinc bromide or benzylzinc chloride, LiBr and with (1,3-diisopropylimidazol-2-ylidene)(3-chloropyridyl)palladium(II) dichloride (PEPPSI)[16] as the catalyst to yield the desired compounds **18**–**21** in good to excellent yields (77–95%). Benzenesulfonamide, 4-phenylbenzenesulfonamide, and phenoxybenzenesulfonamide (**22**–**24**) were synthesized from their corresponding benzenesulfonyl chlorides and isolated in good to excellent yields (73–99%).

The benzenesulfonamides (compounds **18**–**24**) were transformed into the corresponding boronic acids by selective ortho lithiation and subsequent boronation. In the boronation of compounds **19** and **21** (see Scheme 3) only one product was formed (compounds **27** and **29**, respectively), the formation of the di-*ortho*-substituted boronic acids was not observed, presumably due to steric hindrance. Compound **25** was prepared from *para*-iodobenzoic acid, through formation of the corresponding acyl chloride by reaction with oxalyl chloride, and subsequent amidation with diethylamine, which after isolation yielded the desired compound in 83% yield. The boronic acids (**26**–**32**) were coupled with compound **25** in a Suzuki coupling reaction with $Pd(PPh_3)_4$ as the catalyst and Na_2CO_3 as base under microwave irradiation (110 °C, 5 min) to form compounds **33**–**39** in moderate to good yields (46–92%). Compounds **40**–**46** were formed by deprotection of the sulfonamide with borontrichloride and subsequent acylation with *n*-butyl chloroformate in dichloromethane and TEA to yield the final products (**40**–**46**) in 28–88% yield after purification by column chromatography or preparative LC–MS (Scheme 3).

2.1.1. Binding assays

Compounds **4**–**15**, **40**–**46** were evaluated in radioligand-binding assays by displacement of [^{125}I]Ang II from AT_1 receptors in rat liver membranes and from AT_2 receptors in pig uterus membranes as described previously.[17,18] The natural substrate Ang II and the selective AT_1 receptor antagonist losartan were used as reference substances.[19] The affinity results are presented in Tables 1 and 2.

In series A all compounds exhibited moderate to high affinity for the AT_2 receptor and none of the compounds possessed any affinity for the AT_1 receptor (Table 1). Compound **4** with a primary amide moiety had a much lower affinity compared to the secondary and tertiary amides. Introducing methyl groups to the amide (**5**, 36.9 nM) increased the affinity 2.5 times. The affinity was further improved 10 times when ethyl groups were applied (**6**, 3.0 nM). The isopropyl amide **7** showed a better affinity than the cyclopropyl amide **8**. The cyclopropyl amide seems to be equipotent to the thiazolyl amide, compound **12**, while the cyclohexyl amide, **9**, resulted in two times higher affinity. Among the secondary amides the benzyl substituent, compound **10**, exhibited the highest affinity, 1.0 nM. When exchanging the amide hydrogen in compound **10** for a methyl group, rendering compound **11**, the affinity was reduced 30 times. The cyclic substituents in compounds **13** and **14** resulted in good affinity, very similar to the open *N*,*N*-diethylamide, but changing the piperidino moiety of compound **14** to the morpholino of compound **15** dramatically lowered the affinity.

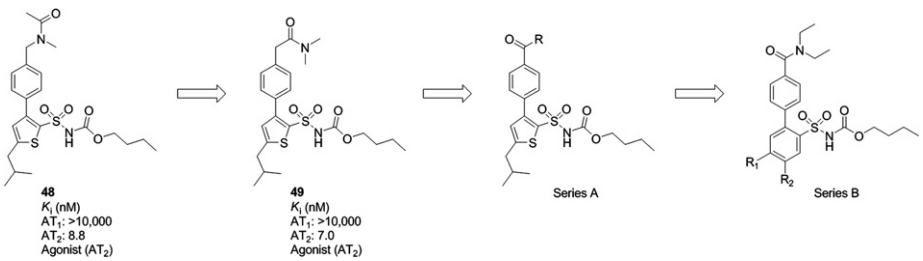
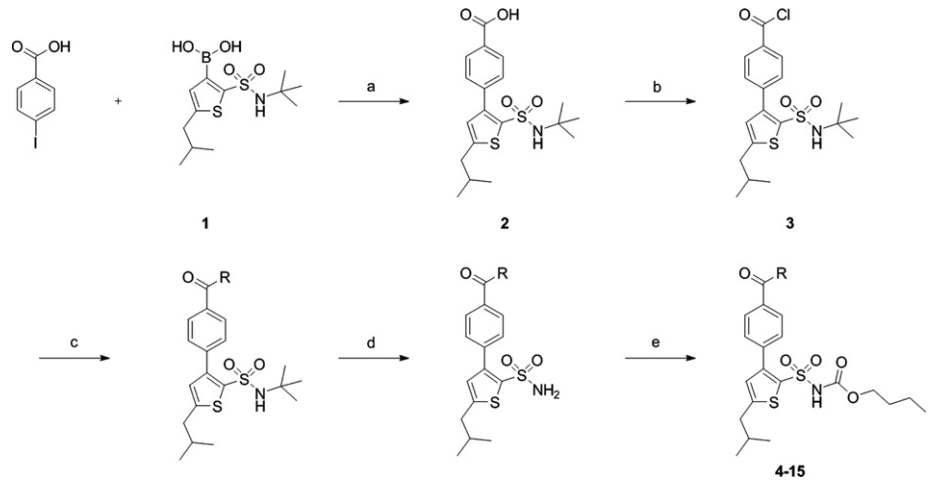
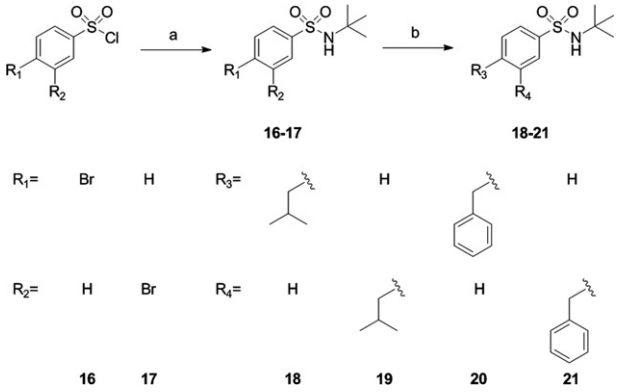


Figure 2.



Scheme 1. Reageants: (a) $Pd(PPh_3)_4$, Na_2CO_3, DME, EtOH, H_2O; (b) oxalyl chloride, 1,2-dichloroethane, cat. DMF; (c) amine, DCM; (d) TFA or BCl_3, DCM; (e) *n*-butyl chloroformate, TEA, DCM.



Scheme 2. Reageants: (a) tertbutylamine, DCM (b) isobutylmagnesium bromide or benzylmagnesium chloride, $ZnBr_2$, LiBr, PEPPSI, THF, NMP.

Considering series B, the thienylphenyl scaffold is far superior to the biphenyl scaffold (Table 2). Biphenyl compounds **40** and **41** are 50 times less potent than the corresponding thienylphenyl compound **6**. Compound **44**, lacking substituent on the phenyl ring, exhibited a K_i of only 402 nM, indicating the importance of the lipophilic side chain for good binding. The isobutyl substituent results in better affinities in both positions 4 and 5 as compared to the benzyl substituent (**40**, **41** vs **42**, **43**). With regards to the benzyl substituted compounds (**42** and **43**), a more pronounced difference was observed as result of the position of the substituent. Compound **42**, with the benzyl in the 4-position, exhibited twice as high affinity as compound **43**, which contained the benzyl group in the 5-position. Exchanging the benzyl substituent of **42** into a phenoxy (**46**) increased the affinity to 91.7 nM and exchanging it to a phenyl (**45**) rendered an affinity of 52.0 nM, which was the most potent compound in series B.

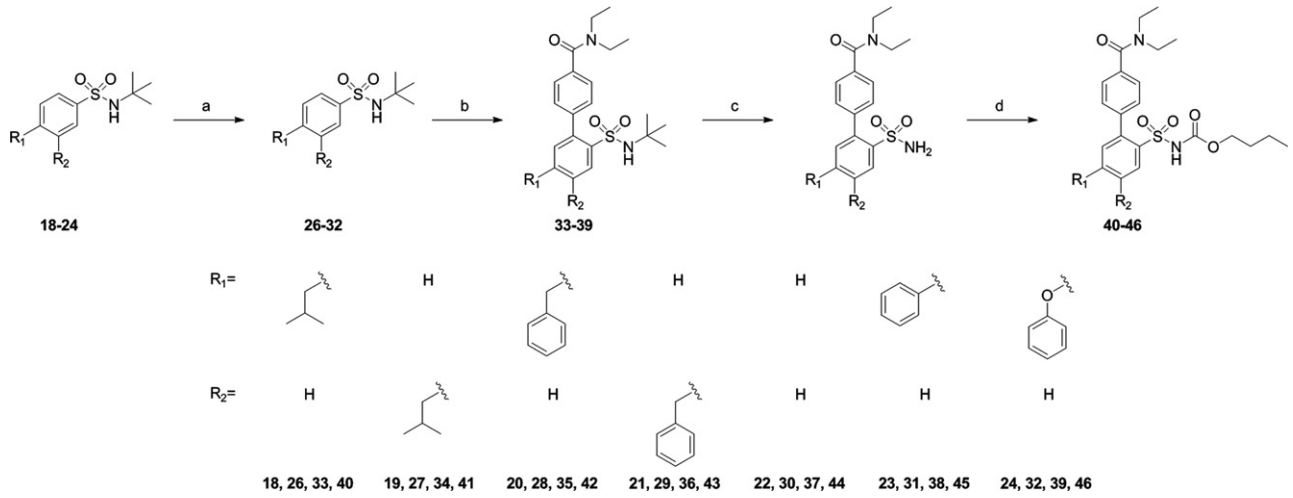
2.1.2. In vitro morphological effects induced by compounds 6, 10, and 45 in NG108-15 cells

We have shown previously that these cells express only the AT_2 receptor and that a 3-day treatment with Ang II or the selective peptidic AT_2 receptor agonist CGP-42112 induces neurite outgrowth.[20,4] The signaling pathways involve a sustained increase in Rap1/B-Raf/p42/p44^{mapk} activity and activation of the nitric oxide/guanylyl cyclase/cGMP pathway.[21–23] The cells were plated as described in Section 5 and adequate test concentrations for the three compounds **6**, **10**, and **45** were determine by testing various concentrations ranging from 1 pM to 1 μM. For all the compounds, it was only at the highest concentration that any evidence of cell death was observed. Cells were then treated in the absence or in the presence of Ang II (100 nM), compounds **6** (1 and 10 nM), **10** (1 and 10 nM), and **45** (100 nM) in the absence or in the presence of PD-123,319 (10 μM), an AT_2 receptor antagonist introduced daily 30 min prior to Ang II, compounds **6**, **10**, or **45**. After 3 days of treatment, cells were examined under a phase contrast microscope and micrographs were taken. As can be seen in Figure 3 compounds **6**, **10**, and **45** all induced neurite outgrowth in an extent comparable to the endogenous agonist Ang II, treatment with the higher concentration of compounds **6** and **10** (10 nM) gave a slightly higher induction of neurite outgrowth (data not shown). Coincubation with the selective AT_2 receptor antagonist PD 123,319 (10 μM) reduced neurite outgrowth to control levels, 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 (data not shown).

3. Discussion

The benzamides presented herein exhibit generally a higher affinity to the AT_2 receptor as compared to the previously disclosed, somewhat larger amides.[12] This observation is consistent with the trends seen in the previous series where smaller substituents improved the AT_2 receptor affinity.[12] However, the primary benzamide **4**, with a K_i value of 105.6 nM was found to be a relatively poor binder while the corresponding primary phenylacetamide exhibited a K_i of 51 nM.[12] Conversion of the benzamide to the *N*,*N*-dimethylamide **5** afforded an improved affinity (36.9 nM) but still lower than the *N*,*N*-dimethylphenylacetamide (**49**, 7.0 nM), the later one being the most potent in the phenylacetamide series.[12] Extending the size of the benzamide substituent further results in ligands more potent than **49**, for example, compound **6**, **7**, **10**, and **14** exhibiting K_i values ranging from 1.0 to 3.2 nM.

It has previously been shown that the biphenyl scaffold is exchangeable to the thienylphenyl scaffold in this type of ligands, see Figure 1.[24,25] However, the *N*,*N*-diethylbenzamide thienylphenyl containing compound, **6** (3.0 nM), was 50 times more potent than the corresponding biphenyl compounds (**40** and **41**). The position of the substituent had little effect on compound potency. Compound **41**, with the isobutyl in the 5-position, showed slightly better affinity compared to compound **40**. It appears as though the thienylphenyl scaffold positions the isobutyl substituent in a



Scheme 3. Reageants: (a) *n*-BuLi, THF, triisopropyl borate, HCl (aq); (b) $Pd(PPh_3)_4$, Na_2CO_3, DME, EtOH, H_2O; (c) BCl_3, DCM; (d) *n*-butyl chloroformate, TEA, DCM.

more favorable position than the 4- or the 5-position on the biphenyl scaffold in the *N*,*N*-diethylamide series. Accordingly, we believe that the *N*,*N*-diethylbenzamide and the *N*-benzylimidazole group, as in M024, are probably binding to the receptor in slightly different ways. It is, however, interesting to see that both position 4 and 5 render almost equipotent ligands and that the isobutyl substituent is necessary for good AT_2 affinity since compound **44** (402.2 nM), which lacks the isobutyl substituent, possess significantly lower affinity than compounds **6** (3.0 nM) and **40** (144.5 nM).

Both the benzyl substituted compounds, **42** and **43**, had a lower affinity toward the AT_2 receptor as compared to the isobutyl substituted analogues and a larger difference between the 4- and 5-positions was observed. Contrary to the previously reported *m*-metoxybenzyl analogue of M024, which lacks affinity for the AT_2 receptor,[25] compound **42** exhibited affinity, although not in the lower nanomolar range. Compound **46**, with a phenoxy substituent instead of the isobutyl, showed almost twice as high affinity as compared to compound **42**. Ethers in the isobutyl position have previously been demonstrated to either increase or reduce the affinity to the AT_2 receptor depending on the molecular framework studied.[25] The big difference seen in this particular case is difficult to explain, but might be accounted for by the higher hydrophilicity or higher electron density.

The good affinity exhibited by the triphenyl, compound **45**, encouraged us to assess if the compound acts as an AT_2 receptor agonist or antagonist. Although, higher concentrations were needed to induce the same agonism as with **6** and **10** this very rigid compound may be a valuable tool in future studies of bioactive conformations.

Neither of the compounds in series A or B possessed any affinity toward the AT_1 receptor. According to the literature any change in the size of the substituent in the isobutyl position will diminish the affinity for the AT_2 receptor and enhance the affinity for the AT_1 receptor.[13,26] However, in series B, where these kinds of alterations were performed (**44**, 402.2 nM, **42**, 162.5 nM) we observed a decrease in AT_2 affinity but no AT_1 affinity was encountered. The substitution pattern in this part of the molecules does not seem to influence the AT_1/AT_2 selectivity in these smaller compounds. So far we have not seen any affinity for the AT_1 receptor from compounds lacking the bicyclic heteroaromatic substituents in the benzylic position.[10–12] Thus, the smaller heterocyclic and linear substituents strongly discriminate between the two receptors, favouring an interaction with the AT_2 receptor.

4. Conclusion

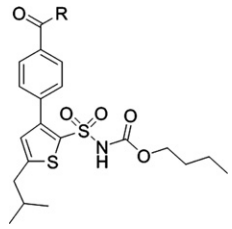
In conclusion, we have demonstrated that substituted benzamides function well in producing high affinity nonpeptide AT_2 receptor selective agonists, for example, **6** and **10**. We have also shown that the thienylphenyl scaffold is not generally exchangeable to the biphenyl scaffold. Interestingly, substituents in both positions 4 or 5 of this scaffold result in active and selective AT_2 ligands. Furthermore, the triphenyl analogue **45** possesses a higher affinity compared to both the corresponding isobutyl and benzyl compounds and it acts as an agonist at the AT_2 receptor. This rigid compound will serve as a very valuable tool in future modeling of the bioactive conformation of selective nonpeptide AT_2 receptor agonists. Furthermore, these small ligands strongly discriminate between the AT_1 and the AT_2 receptors, favouring an interaction with the AT_2 receptor.

5. Experimental

5.1. Chemistry: general considerations

^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Varian Mercury 400 at ambient temperature. Chemical shifts are given as δ values (ppm) downfield from tetramethylsilane and referenced to δ 7.26 and δ 77.16 for $CDCl_3$ and δ 3.31 and δ 49.00 for CD_3OD. Analytical GC–MS with EI ionization was performed on a Varian 3800 or 3900 equipped with a CP-SIL 5 CB Low Bleed (30 m × 0.25 mm) or CP-SIL 8 CB Low Bleed (30 m × 0.25 mm) and using He as carrier gas. Analytical RP-LC–MS was performed on a Gilson HPLC system equipped with a Zorbax SB C8, 5 μm (4.6 × 50 mm) column, and a Finnigan AQA quadrupole mass spectrometer at a flow rate of 4 mL/min. The mobile phase consisted of H_2O/CH_3CN/0.05% HCOOH. Preparative RP-LC–MS was performed on a Gilson-Finnigan Thermo Quest AQA system equipped with a Zorbax SB-C8, 5 μm 21.2 × 150 mm (Agilent technologies) column at a flow rate of 15 mL/min. The mobile phase consisted of H_2O/CH_3CN/0.05% HCOOH. Elemental analyses were performed by Analytische Laboratorien, Lindlar, Germany. Exact molecular masses (HRMS) were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source. Dry CH_2Cl_2 was distilled over calcium hydride and dry THF over sodium. Other chemicals and solvents used were of analytical grade and pur-

Table 1
Series A



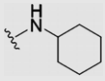
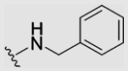
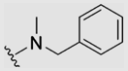
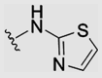
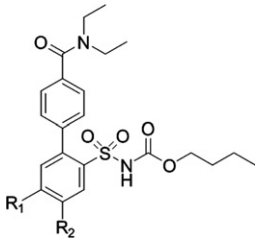
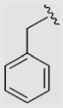
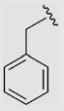
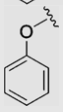
Compound	R	K_i (nM)	
		AT_2	AT_1
4		105.6 ± 0.7	>10,000
5		36.9 ± 1.8	>10,000
6		3.0 ± 0.3	>10,000
7		2.6 ± 0.2	>10,000
8		10.5 ± 0.5	>10,000
9		6.4 ± 0.5	>10,000
10		1.0 ± 0.08	>10,000
11		31.5 ± 0.7	>10,000
12		10.9 ± 0.4	>10,000
13		6.4 ± 0.3	>10,000
14		3.2 ± 0.2	>10,000
15		348 ± 5.4	>10,000

Table 2
Series B



Compound	R_1	R_2	K_i (nM)	
			AT_2	AT_1
40		H	144.5 ± 3.4	>10,000
41	H		121.8 ± 1.8	>10,000
42		H	162.5 ± 1.0	>10,000
43	H		299.8 ± 2.9	>10,000
44	H	H	402.2 ± 10.1	>10,000
45		H	52.0 ± 4.3	>10,000
46		H	91.7 ± 2.3	>10,000

chased from commercial suppliers, and used without further purification unless stated. Thin-layer chromatography was performed on aluminium plates precoated with silica gel 60 F_{254} (Merck) and visualized in UV light. Flash chromatography was performed on silica gel 60 (0.040–0.063 mm, Merck).

5.1.1. *N-tert*-Butyl-3-(4-carboxyphenyl)-5-isobutylthiophene-2-sulfonamide (2)

Eight Smith Process Vials™ (2–5 mL) were charged with compound **1** (total amount 1.82 g, 5.7 mmol) and *para*-iodobenzoic acid (total amount 1.28 g, 5.2 mmol). 2.8 mL of DME, 0.7 mL of ethanol, 0.8 mL of water, Na_2CO_3 (2 M, 0.6 mL, 1.2 mmol), and $Pd(PPh_3)_4$ (20 mg, 0.017 mmol) were added to each tube. The reaction mixtures were flushed with nitrogen, sealed, and heated by microwave irradiation to 110 °C for 5 min. The reaction mixtures were combined and the organic solvents were removed under vacuum. The residue was dissolved in slightly basic water and washed with EtOAc. The aqueous phase was acidified and extracted with diethylether. The combined organic phase was washed with water and brine, dried with $MgSO_4$, filtered, and concentrated in vacuo. The same extraction procedure was repeated three times to isolate compound **2**, without further purification, as white crystals in 65% yield (672 mg, 1.7 mmol). ^{1}H NMR ($CDCl_3$ + CD_3OD), δ, ppm: 8.10–8.06 (m, 2H), 7.67–7.64 (m, 2H), 6.76 (t, J = 0.8 Hz, 1H), 2.62 (dd, J = 7.2, 0.8 Hz, 2H), 1.96–1.86 (m, 1H), 0.99 (s, 9H), 0.95 (d, J = 6.6 Hz, 6H). ^{13}C NMR (CD_3OD), δ, ppm: 168.4, 148.8, 142.4, 139.4, 137.1, 130.2, 129.8, 129.2, 129.0, 54.5, 39.2, 30.6, 29.4, 22.1. Anal. ($C_{19}H_{25}NO_4S_2$) C, H, N.

5.1.2. *N-tert*-Butyl-3-(4-chloroformylphenyl)-5-isobutylthiophene-2-sulfonamide (3)

Compound **2** (740 mg, 1.9 mmol), 60 mL 1,2-dichloroethane, three drops DMF and oxalylchloride (10 mL, 120 mmol) were mixed together under a nitrogen atmosphere. The reaction mixture was heated to 40 °C over night. The reaction mixture was then cooled and the solvents were removed under vacuum and the residue was co-evaporated with dry CH_2Cl_2four times and left under vacuum. Compound **3** was used without further purification.

5.2. General procedure of compounds 4–15

Compound **3** (65 mg, 016 mmol) was dissolved in 2 mL dry CH_2Cl_2. An excess of the respective amine or in combination with

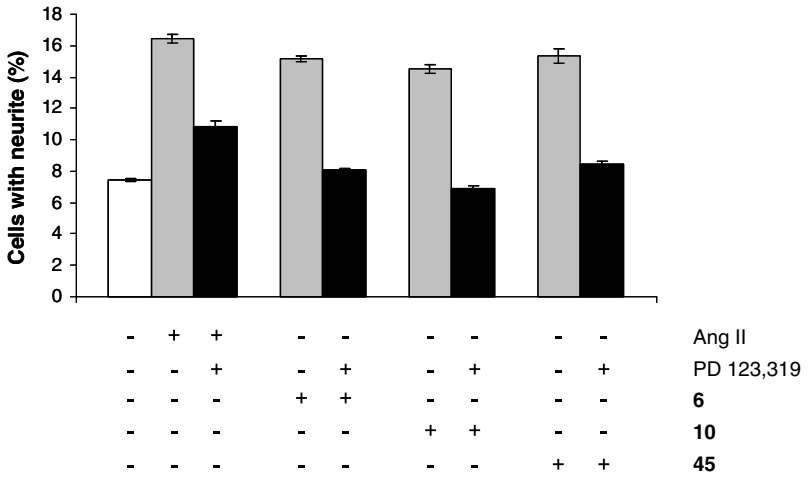


Figure 3. Effect of compounds **6**, **10**, and **45** on neurite outgrowth in NG108-15 cells. The cells were plated at a cell density of 3.6×10^4 cells/35 mm Petri dishes and were cultured for 3 days in the absence or presence of 100 nM Ang II, 1 nM compound **6**, 1 nM compound **10** or 100 nM compound **45** alone or in combination with 1 μM PD 123,319. Cells with at least one neurite longer than a cell body were counted as positive for neurite outgrowth. The number of cells with neurites was expressed as the percentage of the total number of cells in the micrographs (at least 290 cells according to the experiment).

triethylamine was added under nitrogen. The reaction mixture was stirred at ambient temperature over night and diluted with CH_2Cl_2 and washed with 10% citric acid, water and brine. The organic layer was dried with $MgSO_4$, filtered, and evaporated. The residue was dissolved in CH_2Cl_2 and reacted with an excess of 1.0 M BCl_3 (500 μL) in hexane fractions under nitrogen for 1 h. More BCl_3 (500 μL) was added if the reaction had not reached full conversion after this time. The reaction mixture was diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried with $MgSO_4$, filtered, and evaporated. Compounds **1** and **12** was deprotected using trifluoroacetic acid and anisole instead of BCl_3. The residue was dissolved in dry CH_2Cl_2 (5 mL) and triethylamine (100 μL, 0.72 mmol, if not otherwise stated) and *n*-butyl chloroformate (30 μL, 0.24 mmol, if not otherwise stated) were added under nitrogen and the reaction was left for 2 h. The reaction mixture was diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried with $MgSO_4$, filtered, evaporated and purified by chromatotron or preparative HPLC to give the pure compounds **4**–**15**.

5.2.1. *N*-Butoxycarbonyl-3-[4-(*N*,*N*-diethylcarbamoyl)phenyl]-5-isobutylthiophene-2-sulfonamide (6)

Compound **3** was used according to the general procedure and reacted with triethylamine (42 μL, 0.30 mmol) and diethylamine (24 μL, 0.23 mmol). Compound **6** was purified by chromatotron (100% CH_2Cl_2 to 3% MeOH in CH_2Cl_2) to give the pure product in 45% yield (36 mg, 0.072 mmol). ^{1}H NMR ($CDCl_3$), δ, ppm: 7.53–7.50 (m, 2H), 7.43–7.40 (m, 2H), 6.77 (t, J = 0.8 Hz, 1H), 4.04 (t, J = 6.6 Hz, 2H), 3.62–3.48 (m, 2H), 3.36–3.22 (m, 2H), 2.71 (dd, J = 7.1, 0.8 Hz, 2H), 2.00–1.90 (m, 1H), 1.54–1.47 (m, 2H), 1.32–1.08 (m, 8H), 1.00 (d, J = 6.6 Hz, 6H), 0.88 (t, J = 7.3 Hz, 3H). ^{13}C NMR ($CDCl_3$), δ, ppm: 171.0, 151.5, 150.5, 145.5, 137.1, 135.1, 131.6, 129.4, 129.2, 126.5, 66.8, 43.5, 39.5, 39.4, 30.7, 30.5, 22.4, 18.9, 14.4, 13.7, 13.0. HRMS (M+H$^+$): 495.1964, $C_{24}H_{36}N_2O_5S_2$ requires 495.1987. Anal. ($C_{24}H_{35}N_2O_5S_2$) C, H, N.

5.2.2. *N*-Butoxycarbonyl-3-[4-(*N*-benzylcarbamoyl)phenyl]-5-isobutylthiophene-2-sulfonamide (10)

Compound **3** was used according to the general procedure and reacted with benzylamine (36 μL, 0.33 mmol). Compound **10** was purified by chromatotron (100% CH_2Cl_2 to 4% MeOH in CH_2Cl_2) to give the pure product in 46% yield (39 mg, 0.074 mmol). ^{1}H NMR (CD_3OD + $CDCl_3$), δ, ppm: 7.90–7.86 (m, 2H), 7.59–7.56 (m, 2H), 7.36–7.29 (m, 4H), 7.26–7.21 (m, 1H), 6.84 (t, J = 0.8 Hz, 1H), 4.60 (s, 2H), 3.99 (t, 2H, J = 6.5 Hz), 2.74 (dd, J = 7.1, 0.8 Hz, 2H), 2.00–1.90 (m, 1H), 1.53–1.46 (m, 2H), 1.29–1.14 (m, 2H), 0.99 (d, J = 6.6 Hz, 6H), 0.87 (t, J = 7.3 Hz, 3H). ^{13}C NMR ($CDCl_3$), δ, ppm: 167.2, 152.0 150.6, 145.6 138.3, 137.4, 134.3, 131.4, 129.3, 129.2, 128.8, 128.3, 127.7, 127.3, 67.0, 44.3, 39.4, 30.7, 30.6, 22.4, 18.9, 13.7. HRMS (M−H$^+$): 527.1700, $C_{27}H_{31}N_2O_5S_2$ requires 527.1674. Anal. ($C_{27}H_{32}N_2O_5S_2$) C, H, N.

5.3. General procedure for compound 16–17 and 22–24

The respective sulfonylchloride was dissolved in dry CH_2Cl_2. The mixture was cooled on an ice bath and *tert*-butylamine was added dropwise. After completion of addition, the reaction mixture was left stirring at ambient temperature for 1 h to over night. The reaction mixture was diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried with $MgSO_4$, filtered, and evaporated to achieve the pure compounds **16**–**17** and **22**–**20** without further purification.

5.3.1. *N-tert*-Butyl-4-phenylbenzenesulfonamide (23)

4-Phenylbenzenesulfonylchloride (1.00 g, 4.6 mmol) dissolved in 50 mL dry CH_2Cl_2 was used according to the general procedure and reacted with *tert*-butylamine (2.40 mL, 23 mmol) for 3 h. Compound **23** was achieved as white crystals in 77% yield (1.02 g, 3.5 mmol). ^{1}H NMR ($CDCl_3$), δ, ppm: 7.96–7.93 (m, 2H), 7.71–7.68 (m, 2H), 7.63–7.60 (m, 2H), 7.50–7.40 (m, 3H), 4.44 (br s, 1H), 1.27 (s, 9H). ^{13}C NMR ($CDCl_3$), δ, ppm: 145.2, 142.2, 139.6, 129.2, 128.5, 127.64, 127.62, 127.4, 54.9, 30.4. Anal. ($C_{16}H_{19}NO_2S$) C, H, N.

5.4. General procedure for compound 18–21

A solution of $ZnBr_2$ in dry THF was prepared under inert conditions and cooled on an ice bath. The respective Grignard reagent was added dropwise and a white precipitate was formed. The reaction was left under nitrogen at ambient temperature for 1 h. A dry vial was charged with LiBr and the respective bromide and thoroughly flushed with nitrogen. Dry NMP was added to the solids which dissolved upon exposure to an ultrasonic bath. A dry vial was charged with (1,3-diisopropylimidazol-2-ylidene)(3-chloropyridyl)palladium(II) dichloride and thoroughly flushed with nitro-

gen. The catalyst was dissolved in dry THF and transferred to the NMP solution. The three component solution was added dropwise under nitrogen to the zinc reagent. The reaction mixture was left under nitrogen at ambient temperature over night. The next day the reaction was cooled on an ice bath and 1 M HCl was added until a clear dark solution with acidic pH was achieved. The mixture was extracted with diethylether and the combined organic phase was washed water and brine. The organic layer was dried with $MgSO_4$, filtered, and evaporated. The crude product was purified by column chromatography to achieve the pure compounds **18**–**21**.

5.4.1. 4-Iodo-(*N*,*N*-diethyl)benzenamide (25)

Para-iodobenzoic acid (3.46 g, 14 mmol) was dissolved in 200 mL dry CH_2Cl_2 under a nitrogen atmosphere and 15 drops DMF and oxalylchloride (22 mL, 28 mmol) were subsequently added. The reaction mixture was heated to 40 °C and stirred over night. The reaction mixture was then cooled and the solvents were removed under vacuum and the residue was co-evaporated with $CHCl_3$ three times and left under vacuum. The crude product was dissolved in 250 mL dry CH_2Cl_2 under a nitrogen atmosphere and triethylamine (15.6 mL, 113 mmol) and diethylamine (7.30 mL, 71 mmol) were added and the reaction was left under nitrogen at ambient temperature over night. The reaction was washed with 1 M HCl, water, and brine. The organic layer was dried with $MgSO_4$, filtered, and evaporated. The crude product was purified by column chromatography (isohexane/EtOAc 20:1 first then 3:1) to isolate compound **25** as white crystals in 83% yield (3.52 g, 11.62 mmol). ^{1}H NMR ($CDCl_3$), δ, ppm: 7.76–7.73 (m, 2H), 7.13–7.10 (m, 2H), 3.64–3.41 (m, 2H), 3.33–3.15 (m, 2H), 1.32–1.04 (m, 6H). ^{13}C NMR ($CDCl_3$), δ, ppm: 170.4, 137.7, 136.8, 128.2, 95.2, 43.4, 39.5, 14.4, 13.0. Anal. ($C_{11}H_{14}INO$) C, H, N.

5.4.2. General procedure for compound 33–39

To a cooled (−78 °C) solution of compounds **18**–**24** in dry THF was added *n*-BuLi (1.6 M in hexane) under nitrogen and the reaction was stirred for 1 h. The temperature was raised to −30 °C and kept for 3 h and subsequently decreased to −40 °C. Triisopropyl borate was then added. The reaction mixure was stirred over night at room temperature. The reaction mixture was cooled (0 °C) and treated with an excess of 2 M HCl solution. The mixture was extracted with EtOAc and the combined organic phase was washed with water and brine. The organic layer was dried with $MgSO_4$, filtered and evaporated. The crude product (**26**–**32**) was then used in the next step without further purification. A Smith Process Vial™ (2–5 mL) was charged with the crude boronic acid (**26**–**32**), compound **25**, DME, ethanol, water, 2 M Na_2CO_3, and $Pd(PPh_3)_4$. The reaction mixture was flushed with nitrogen, sealed, and heated by microwave irradiation to 110 °C for 5 min. The reaction mixture was diluted with EtOAc, washed with water and brine, dried with $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by column chromatography or preparative HPLC to isolate the pure compounds **33**–**39**.

5.4.3. *N*-*tert*-Butyl-2-[4-(*N*,*N*-diethylcarbamoyl)phenyl]-4-phenylbenzenesulfonamide (38)

According to the general procedure compound **23** (0.997 g, 3.45 mmol) was dissolved in dry THF (20 mL) and reacted with *n*-BuLi (5.4 mL, 1.6 M in hexane, 8.62 mmol) and triisopropyl borate (1.2 mL, 5.18 mmol). Two Smith Process Vials™ (2–5 mL) were charged with the crude boronic acid **31** (total amount 240 mg, <0.72 mmol) and compound **25** (total amount 133 mg, 0.43 mmol). 2.0 mL of DME, 0.5 mL of ethanol, 0.5 mL of water, Na_2CO_3 (2 M, 0.4 mL, 0.88 mmol) and $Pd(PPh_3)_4$ (15 mg, 0.013 mmol) were added to each tube. The crude product was purified by preparative HPLC to isolate compound **38** as white crystals in 46% yield (92 mg, 0.20 mmol). ^{1}H NMR ($CDCl_3$), δ, ppm: 8.24 (d, J = 8.3 Hz, 1H), 7.72 (dd, J = 8.3 Hz, J = 2.0 Hz, 1H), 7.65–7.61 (m, 4H), 7.52 (d, J = 2.0 Hz, 1H), 7.50–7.39 (m, 5H), 3.65–3.48 (m, 3H), 3.40–3.26 (m, 2H), 1.33–1.09 (m, 6H), 1.05 (s, 9H). ^{13}C NMR ($CDCl_3$), δ, ppm: 170.9, 144.9, 141.0, 140.8, 139.9, 139.2, 137.4, 131.1, 130.2, 129.3, 129.3, 128.7, 127.5, 126.6, 126.4, 54.7, 43.6, 39.7, 30.1, 14.5, 13.1. Anal. ($C_{27}H_{32}N_2O_3S$) C, H, N.

5.5. General procedure for compound 40–46

Compound **33**–**39** were dissolved in dry CH_2Cl_2 (5–10 mL) and cooled to 0 °C and reacted with an excess of 1.0 M BCl_3 in hexane fractions under nitrogen. The reaction was left at room temperature for 1 h. More BCl_3 was added if the reaction had not reached full conversion after this time. The reaction mixture was then evaporated and co-evaporated several times with $CHCl_3$. The residue was dissolved in dry CH_2Cl_2 (5 mL) and triethylamine and *n*-butyl chloroformate were added under nitrogen and the reaction was left for 2 h at ambient temperature. The reaction mixture was diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried with $MgSO_4$, filtered, evaporated, and purified by chromatotrone or preparative HPLC to give the pure products **40**–**46**.

5.5.1. *N*-Butoxycarbonyl-2-[4-(*N*,*N*-diethylcarbamoyl)phenyl]-4- phenylbenzenesulfonamide (45)

According to the general procedure compound **38** (92 mg, 0.20 mmol) was reacted with BCl_3 (1.0 mL + 0.50 mL, 1.0 M in hexane) and afterwards with triethylamine (110 μL, 0.80 mmol) and *n*-butyl chloroformate (31 μL, 0.24 mmol). The crude product was purified by preparative HPLC to isolate compound **45** in 28% yield (28 mg, 0.056 mmol). ^{1}H NMR ($CDCl_3$), δ, ppm: 8.34 (d, J = 8.3 Hz, 1H), 7.78 (dd, J = 8.3 Hz, J = 1.9 Hz, 1H), 7.64–7.61 (m, 2H), 7.54 (d, J = 1.9 Hz, 1H), 7.52–7.41 (m, 7H), 7.10–6.94 (br s, 1H), 4.03 (t, J = 6.5 Hz, 2H), 3.66–3.49 (m, 2H), 3.42–3.24 (m, 2H), 1.53–1.46 (m, 2H), 1.35–1.10 (m, 8H), 0.86 (t, J = 7.3 Hz, 3H). ^{13}C NMR ($CDCl_3$), δ, ppm: 171.0, 150.4, 146.3, 141.0, 139.6, 138.8, 137.3, 136.0, 131.4, 130.9, 129.5, 129.2, 129.0, 127.6, 126.6, 126.2, 66.9, 43.6, 39.6, 30.6, 18.9, 14.5, 13.7, 13.1. Anal. ($C_{28}H_{32}N_2O_5S$) C, H, N.

5.6. Rat liver membrane AT_1 receptor-binding assay

Rat liver membranes were prepared according to the method of Dudley et al.[17] Binding of [^{125}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}I]Ang II (80,000–85,000 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 pre-soaked 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 were determined by using six different concentrations (0.03–5 nmol/L) of the labeled [^{125}I]Ang II. 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}I]Ang II. The apparent dissociation constant K_i values were calculated from IC_{50} values using the Cheng–Prusoff equation (K_d = 1.7 ± 0.1 nM, [L] = 0.057 nM). The binding data were best fitted with a one-site fit. All determinations were performed in triplicate.

5.7. Porcine (pig) myometrial membrane AT_2 receptor-binding assay

Myometrial membranes were prepared from porcine uteri according to the method by Nielsen et al.[18] A presumable interference by binding to AT_1 receptors was blocked by addition of 1 μM losartan. Binding of [^{125}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, homogenate corresponding to 10 mg of the original tissue weight, [^{125}I]Ang II (80,000–85,000 cpm, 0.03 nM) and variable concentrations of test substance. Samples were incubated at 25 °C for 1.5 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_2 receptor were determined by using six different concentrations (0.03–5 nmol/L) of the labeled [^{125}I]Ang II. 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}I]Ang II. The apparent dissociation constant K_i values were calculated from IC_{50} values using the Cheng–Prusoff equation (K_d = 0.73 ± 0.06 nM, [L] = 0.057 nM). The binding data were best fitted with a one-site fit. All determinations were performed in triplicate.

5.8. In vitro morphological effects: general

The chemicals used in the present study were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), HAT supplement (Hypoxanthine, Aminopterin, Thymidine), gentamycin from Gibco–BRL (Burlington, Ont, Canada); [Val^5]angiotensin II from Bachem (Marina Delphen, CA, USA). PD-123,319 was obtained from RBI (Natick, MA, USA). All other chemicals were of grade A purity.

5.9. Cell culture

To study the in vitro morphological effects NG108-15 cells (provided by Drs. M. Emerit and M. Hamon; INSERM, U. 238, Paris, France) were used as well as transfected NG108-15/pcDNA3 cells. The transfected cell line has previously been shown to have the same behavior as the native cell line.[21] In their undifferentiated state, neuroblastoma × glioma hybrid NG108-15 cells have a rounded shape and divide actively. Both cell lines were cultured (NG108-15 passage 18–28, NG108-15/pcDNA3 passage 12–18) in Dulbecco's modified Eagle's medium (DMEM, Gibco–BRL, Burlington, Ont., Canada) with 10% fetal bovine serum (FBS, Gibco), HAT supplement and 50 mg/L gentamycin at 37 °C in 75 cm^2 Nunclon Delta flasks in a humidified atmosphere of 93% air and 7% CO_2, as previously described.[4,20] The transfected cell line was kept stable by addition of Geneticin (G-418, 200 μg/mL) to the media.[21] Subcultures were performed at subconfluency. Under these conditions, cells express only the AT_2 receptor subtype.[4,20] Cells were treated during three days, once a day (first treatment 24 h after plating), and micrographs were taken the fourth day. For all experiments, cells were plated at the same initial density of 3.6×10^4 cells/35 mm Petri dish. To determine a good test concentration compounds **6**, **10**, and **45** were tested at various concentrations ranging from 1 pM to 1 μM. For all the compounds it was only in the highest concentration that a tendency of cell death was observed. Cells were treated without (control cells), or with [Val^5]angiotensin II (100 nM) or compound **6** (1 and 10 nM), compound **10** (1 and 10 nM) and compound **45** (100 nM) in the absence or in the presence of PD 123,319 (RBI Natick, MA, USA) (10 μM), an AT_2 receptor antagonist introduced daily 30 min prior to Ang II, compounds **6**, **10** or **45**. During the three days treatment the transfected cell line was cultured without Geneticin.

5.10. Determination of cells with neurites

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 was reported as the percentage of the total amount of cells in the micrographs and at least 290 cells were counted in three independent experiments and each condition was performed in duplicate.[23] The data are represented as means ± SEM of the average number of cells on a micrograph.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.05.066.

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