Pharmacological evaluation on antihypertensive activity of a novel AT1 angiotensin II receptor antagonist

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Hypertension is a major risk factor for human cardiovascular health, which can damage heart, brain, kidneys, etc. In this study we aimed to develop novel angiotensin II receptor blockers (ARBs) that prevent the increase of blood pressure for treatment of hypertension. (2-(4-((2-Amyl-5-nitro-1H-benzo[d]-imidazol-1-yl) methyl)-1H-indol-1-yl) tetrazole; compound 1a) was one of the ARBs designed and synthesized. It was prepared and orally administered to spontaneous hypertensive rats to study the antihypertensive effects. The maximum reduction in blood pressure reached 50 mmHg after dosing compound 1a for 5 h. Acute toxicity test was carried out on healthy 4 week old 30 male and 30 female ICR mice and LD_{50} for 1a was found to be 2864.03 mg/kg. High performance liquid chromatography was employed to determine the level of 1a plasma concentration at various time points after administration. The plasma concentration of 1a increased after 2 h, declined gradually and was still detectable in the plasma after 72 h. The drug distribution analysis of 1a was performed on healthy Wistar rats. It was present in the liver with the highest concentration, in kidney with a lower concentration, and in the spleen, lung, heart and brain with the lowest concentration. It displayed high affinity to AT1 receptor, and had an efficient and long-lasting effect in reducing blood pressure, which lasted for more than 12 h. Due to its biological safety, 1a could be absorbed quickly, metabolized smoothly, and can be distributed in important organs. Therefore, 1a could be considered as a suitable ARB candidate for further studies.

Keywords: Angiotensin II receptor blockers, antagonistic activity, antihypertension, pharmacological evaluation.

CARDIOVASCULAR and cerebrovascular diseases are regarded as the leading cause of death worldwide and hypertension is becoming one of the major risk factors among patients due to poor lifestyle. According to the diagnostic criteria of hypertension, 2002 National Nutrition and Health Survey data, that is, systolic blood pressure (SBP) is greater than 140 mmHg and/or diastolic blood pressure (DBP) is less than 90 mmHg, showed that the prevalence crude rate of hypertension accounted for 18.8% in China and was found to increase with each year[1]. Although there are several drugs for treating high BP, still one-third of the patients lack complete cure[2,3]. It is recommended that hypertension awareness and control can significantly reduce the mortality of cardiovascular and cerebrovascular diseases, and help improve the quality of life.

Angiotensin II receptor blockers (ARBs), which are novel hypertension drugs are gaining attention in patients with hypertension. ARBs play a significant role of AT1 receptor blocker, blocking the combination of Angiotensin II (Ang II) and AT1 receptor, and preventing increase in BP. In addition, ARBs have no effect on blocking AT2. Therefore, Ang II can bind AT2 receptor and lower BP[4,5]. 'The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure' provides a new way for managing and preventing hypertension[6–14]. At the same time, adverse reactions arise from ARBs, such as cough and angioedema, through slightly lower than those in angiotensin-converting-enzyme inhibitor (ACEI) classes. If patients are unable to tolerate ACEI class of antihypertensive drugs, ARBs are recommended for use. On the other hand, it has been reported that ARBs can present hypotension in overweight patients[15,16].

Sartans, a class of non-peptide drugs, are commonly used to treat hypertension after the application of ACEI and calcium antagonists[17–19]. Among these, losartan is now the most widely used antihypertensive agent due to its long-lasting effect, mild toxicity, less side effects and easy administration[20,21]. It can be used as an ideal drug in the treatment of hypertension and congestive heart failure. However, losartan has side effects such as headache, asthma, nausea, occasional high potassium and may also cause renal dysfunction in sensitive individuals and abnormal renal artery stenosis patients[22]. Losartan has also been reported to influence to a certain extent[23,24].

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The aim of the present study was to explore the synthesis of a novel type of ARBs, which has the advantages of losartan and also does not cause or reduce side effects of antihypertensive drugs. We synthesized compound **1a** (2-(4-((2-amyl-5-nitro-1H-benzo[d]imidazol-1-yl) methyl)-1H-indol-1-yl) tetrazole), and explored its antihypertensive effects through various experiments. In the AngII receptor binding experiments, **1a** exhibited high affinity. In antihypertensive activity measurements, it had a better antihypertensive effect than losartan. It could reduce BP for more than 12 h, and did not affect heart rate. Pharmacokinetic experiments showed that **1a** can be absorbed quickly and metabolized smoothly. Therefore, it has good hypotensive effect. It is safe, long-lasting, and can be used as a antihypertensive medication.

Materials and method

Rotary evaporator (RE-3000A, Shanghai Yarong Instrument Company, Shanghai, China), gamma counter (SN-682, Shanghai Hesuo Rihuan Photo Electronic Instrument Corporation, Shanghai, China), high performance liquid chromatography (HPLC; L-3000, Beijing Jingke Rida Technology Instrument Corporation, Beijing, China), tissue homogenate machine (PRO200, Shanghai Sustained High Biotechnology Limited Company, Shanghai, China) were employed in this study.

The chemical reagents used were 2-pentyl-5-nitro-1-benzimidazole (ITC Chemical Industry Development Co. Ltd). K_2CO_3, $MgSO_4$, MeOH, NaOH (Sinopharm Chemical Reagent Co. Ltd), saturated salt water, ethyl acetate, dichloromethane (DCM), dimethylformamide (DMF; Shanghai Lingfeng Chemical Reagent Co Ltd). All the chemical reagents were of analytical grade and did not need to be purified. Losartan (Shanghai Zhongkang Weiye Biological Science and Technology Co Ltd)^{125}I-AngII radioimmunoassay kit (Beijing North Institute of Biological Technology) were also used in the study.

*Synthesisof compound **1a***

A mixture of 2-butyl-5-nitro-1h-benzo[d]imidazole (500 mg, 2.28 mmol) and ((4-bromomethyl)-1H-indol-1-yl)(phenyl) methanone（765 mg, 2.44 mmol) in 50 ml of acetone was added to K_2CO_3 solution (435 mg, 3.15 mmol). The mixture was stirred for 6 h at 60°C. The remaining solution was diluted with water and extracted three times using 50 ml of ethyl acetate. The organic layer was washed with 75 ml of saturated brine and dried using $MgSO_4$. After filtration and reduced pressure distillation, the solvent was removed to obtain a product in the first step. Product a (158 mg, 0.45 mmol) and K_2CO_3 were dissolved in 10 ml of dimethyl formamide. Next, 2-Fluorobenzene nitrile (0.08 ml, 0.08 mmol) was added to the mixture, followed by heating and refluxing under nitrogen atmosphere for 5 h. The remaining liquid was diluted with water and extracted four times with 30 ml of ethanol. The organic phase was washed three times with saturated sodium chloride solution and dried using $MgSO_4$ to get product b in the second step. Product b (107 mg, 0.24 mmol) was dissolved in 10 ml of 5 M NaOH and 10 ml of methanol. The reaction mixture was heated with continuous stirring for 8 h in a condenser. The pH value was carefully adjusted between 5 and 6 with hydrochloric acid aqueous solution (6 M). The remaining liquid was extracted five times with 20 ml of methylene chloride. The organic layer was washed two times with saturated brine and dried with $MgSO_4$. The organic solvent was removed under reduced pressure. Compound **1a** was prepared.

Receptor binding experiment: cell culture

The primary vascular smooth muscle cells (VSMCs) were obtained from thoracic aorta of SPF SD rats (Shanghai SLAC Laboratory Animal Co. Ltd). Cell growth was observed using an inverted microscope and the cells were identified by immune histochemical methods. In order to measure cell viability, the Trypan blue counting method was employed[25]. A 1 mm × 1 mm tissue block was cut from the thoracic aorta and then transferred to a cell culture bottle. The cell culture medium containing 10% foetal bovine serum and 1% streptomycin and penicillin was added when the tissue adhered uniformly to a tissue culture plastic ware. Then the culture bottle was incubated at 37°C and 5% of CO_2 environment for 3–5 h. Cells grew and gradually covered the entire wall. All the cells were incubated at 37°C, and 5% of CO_2 environment for continuous culture.

^{125}I-Ang II receptor binding assay

Three to six generations of VSMCs were used for binding experiments. Compound **1a** and losartan were dissolved in DMSO and diluted to different concentrations with PBS before the experiments.^{125}I-Ang II (Beijing North Institute of Biological Technology, Beijing, China) was dissolved with PBS at 37°C. VSMCs (10^6 cells/well, 500 ml) were seeded into 24-well plates and with 5% of CO_2. After the cells adhered to the wall, they were washed and incubated in PBS containing 0.1 nM ^{125}I-Ang II and compounds of different concentrations and then cultivated in 4°C, for 150 min. The final concentrations of the compound **1a** was in the range 10^{-12}–10^{-6} M and non-specific binding represented 5%–10% of total binding, which was measured in the presence of 1 μM Ang II. The resulting VSMCs were washed thrice with PBS and digested for 10 min with 0.1 M NaOH. The binding rate of the cells combined with ^{125}I-Ang II was estimated using γ-counter. The IC_{50} and half inhibitory rates of **1a** are calculated using the non-linear part of the competitive curve[6].

Activity of blood pressure in vivo

Compound **1a** was dissolved in DMSO and oleic acid in the ratio 1 : 4. Next, 18 spontaneously hypertensive rats were selected randomly and divided into three groups, that is, blank control group, losartan group and compound **1a** group with 1 ml dose (10 mg/kg) in each rat. Non-invasive arteriacaudalis blood pressure measurement was used to measure BP in rats during the normal state before administration, and also at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 24 h respectively. SBP, DBP and heart rate were recorded at every time point. Average blood pressure (MBP) = (SBP – DBP)/3 + DBP.

Acute toxicity test

Acute toxicity test of **1a** was measured in 60 normal ICR mice (30 male mice, 30 female mice, 20 ± 2 g, Chinese Academy of Sciences). They were randomly divided into six groups, with half the number of male mice and female mice in each group. The dosages for each group were 5949.9, 4164.9, 2915.5, 2040.8, 1428.6 and 1000.0 mg/kg separately. The mice were dosed once per day. Survival rate was calculated after two weeks. The mortality within 14 days was analysed by logistic regression. LD_{50} values and 95% confidence interval were calculated using the fitting analysis. The physiological indicators of toxicity, including skin changes, flexibility, aggression and breathing exercises, were observed and systematically recorded. On the 15th day, the surviving mice were dissected and their organs were examined for pathological changes.

Pharmacokinetic experiments

The plasma concentration was detected in Wistar rats using HPLC. A high performance liquid chromatograph was used to detect the concentration of drugs in the blood plasma. The principal component of this system is equipped with a four-element liquid phase pump, including Alliance 2489 separation module, on-line degassing device, automatic sampler and 600 nm ultraviolet absorption detector. Instrumental control, data acquisition and data processing were carried out using Waters software. The flow rate was 0.8 ml/min, and sample volume was 20 µl. The C_{18} reverse-phase column (150 mm × 4.6 mm, 5 µm) was used for separation at room temperature. The mobile phase A was 0.1% formic acid solution containing 2 mm ammonium acetate (20%), while mobile phase B was methanol (80%). A standard curve for **1a** was established (1, 5, 10, 50, 100 and 500 µg/ml).

Tissue distribution of drugs

Tissue distribution studies were conducted in 66 Wistar rats (half of the male and female rats, 200 ± 20 g, Shanghai SLAC Laboratory Animal Co Ltd). They were randomly divided into 11 groups, and dosage used was 10 mg/kg. The rats were killed at 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h separately. The heart, liver, spleen, lung, kidney and brain were removed and cleaned with physiological saline. Next, 0.2 g of tissue was immersed in 1 ml saline for further experiments. A tissue homogenizer was used to homogenize the tissue into liquid. Then 3 ml of methanol was added to deposit the protein, vortexed for 1 min and centrifuged for 20 min at 6000 rpm at 4°C. The supernatant was collected for further experiments.

Statistical analysis

The results are shown as the average value ± standard deviation and analysed by one-way ANOVA. The difference between the comparative groups was statistically significant when the *P*-value was less than 0.05 (ref. 26). The results of binding experiment and antihypertensive activity experiment were calculated using the nonlinear regression program (GraphPad Prism 5.0 software).

Results and discussion

*Characterization method of **1a***

The resulting product was not optimized. The chemical composition of the compound was identified by ^{1}H NMR and ^{13}C NMR (Figure 1).

The total amount of the obtained final product, viz. **1a**, was 83 mg; and the yield was 73.8%, and the melting point was 189–193°C. ^{1}H NMR (400 MHz, DMSO-d_6): 8.53 (d, J = 8.53 Hz, 1 h), 8.12 (dd, J = 8.9, 2.2 Hz, 1 h), 7.71 (d, J = 9.0 Hz, 1 h), 7.67 (s, 1 h), 7.57, 7.51 (m, 1 h), 7.49 (d, J = 3.3 Hz, 1 h), 7.44 (d, J = 7.8 Hz, 1 h), 7.29 (s, 1 h), 7.14 (d, J = 8.3 Hz, 1 h), 7.01 (t, J = 7.8 Hz, 1 h), 6.63 (d, J = 3.1 Hz, 1H), 6.39 (d, J = 7.2 Hz, 1H), 5.90 (s, 2H), 2.90 (t, J = 7.5 Hz, 2H), 1.73 (m, 2H), 1.34 (m, 2H), 0.84 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): 169.01, 160.58, 143.09, 142.03, 140.71, 137.09, 136.03, 135.75, 130.98, 130.65, 129.20, 128.25, 128.17, 126.51, 122.37, 118.12, 117.14, 115.13, 111.20, 110.58, 100.88, 45.56, 29.06, 27.06, 22.22, 14.12.

^{125}I-Ang II radio ligand receptor binding experiment

Compound **1a** and losartan are competitive with regard to binding with ^{125}I–Ang II (Figure 2 and Table 1). The **1a** had a higher affinity to bind with AT1 receptor (**1a**: IC_{50} = 4.05 ± 2.11 nM, Ki = 2.93 ± 1.53 nM; losartan: IC_{50} = 12.23 ± 3.42 nM, Ki = 8.86 ± 2.49 nM). The radioactive receptor binding experiment showed that **1a** had specific binding capacity to the AT1 receptor.

Antihypertensive activity experiment

Antihypertensive activity experimental results revealed that (Figure 3), at the dose of 10 mg/kg, compound **1a** showed good activity, began to reduce BP after 1 h and reached maximum hypotensive value at 50 mmHg in 5 h (Figure 3). The activity persisted for more than 12 h. The antihypertensive effect was obvious until 24 h. However, at the dose of 10 mg/kg, losartan reached maximum hypotensive value at 45 mmHg in 3 h and antihypertensive activity in 12 h. The pressure drop in losartan for 24 h was less than that in compound **1a**. In addition, **1a** did not affect the heart rate of rats. Compound **1a** was more effective in lowering BP at the same dose, and was more stable and long-lasting.

Acute toxicity test

In the acute toxicity test, LD_{50} value of **1a** was 2897.34 mg/kg, and 95% confidence interval was 2466.84–3418.04 mg/kg (Table 2). No abnormalities were found in the ICR mice, but individual mice died at the highest dose. The weight of mice did not change significantly after two weeks. The mice did not show obvious attack behaviours, indicating that **1a** has low acute toxicity and high biosafety.

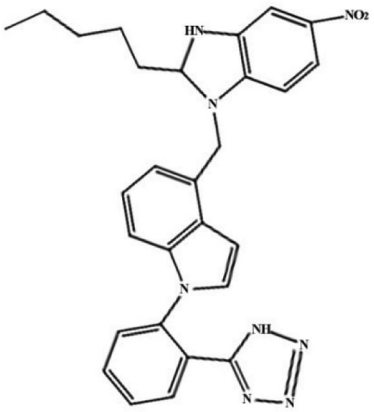

Figure 1. Chemical structure of compound **1a**.

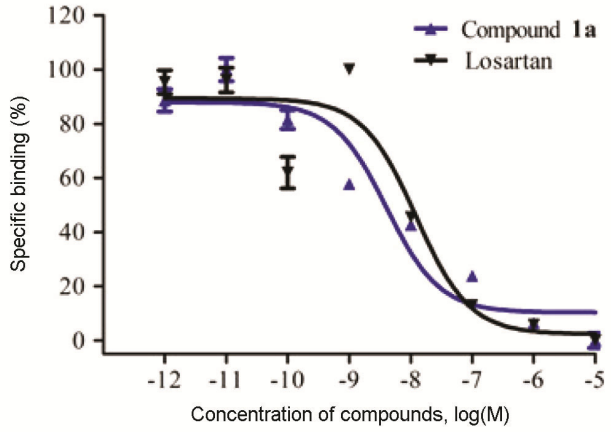

Figure 2. Inhibitory effects of compounds **1a** and losartan (10^{-5}–10^{-12} M) on specific binding of ^{125}I–Ang II to AT1 receptors in vascular smooth muscle cells.

Blood concentration experiment

GraphPad Prism software, DAS 2.0 was used to estimate the pharmacokinetic parameters. Figure 4 shows average blood concentration versus time curve. After dosing

Table 1. IC_{50} and Ki values of the tested compound **1a** and losartan

Drugs	IC_{50} ± SEM (nM)	Ki (nM)
Compound **1a**	4.05 ± 2.11	2.93 ± 1.53
Losartan	12.23 ± 3.42	8.86 ± 2.49

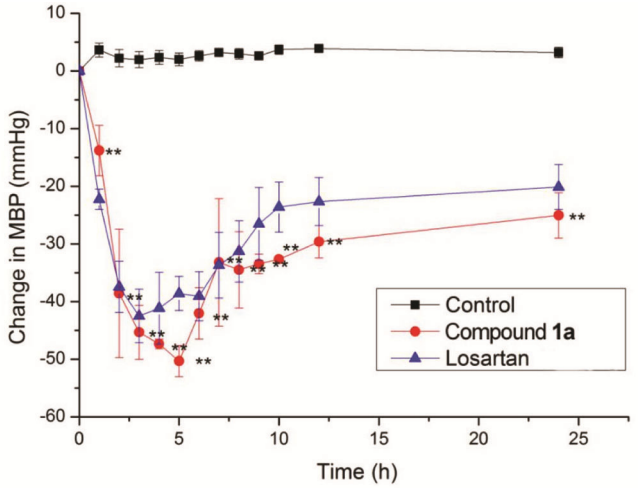

Figure 3. Effects of compound **1a** (10 mg/kg) and losartan (10 mg/kg) on mean blood pressure (MBP) in SHRs. *,**indicate significant difference from negative control, $P < 0.05$ and $P < 0.01$ respectively.

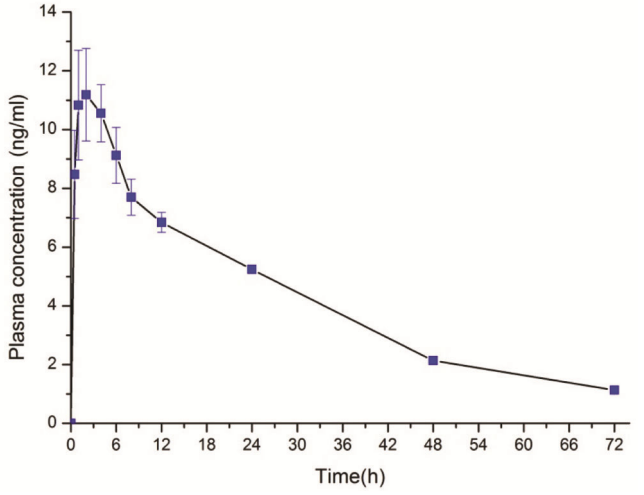

Figure 4. Plasma concentration of compound **1a** (10 mg/kg) observed in rats by signal oral administration ($n = 6$).

Table 2. Lethal dose (LD_{50}) of compound **1a** determined by acute toxicity test (n = 10)

Dose (mg/kg)	Log (dose)	Mortality (%)	LD_{50} and 95% confidence interval
1000	3	0	2897.34
1428.57	3.08	9	(2466.84–3418.04)
2040.82	3.25	28	
2915.45	3.32	56	
4164.93	3.57	84	
5949.90	3.62	98	

Table 3. Pharmacokinetic parameters of compound **1a** (10 mg/kg) in tissues of Wistar rats observed by signal oral administration (n = 6)

Parameter	Heart	Liver	Spleen	Lung	Kidney	Brain
T_{max} (h)	0.4 ± 0.31	0.4 ± 0.22	3.8 ± 0.40	0.9 ± 0.27	0.49 ± 0.14	1.8 ± 0.52
C_{max} (ng/g)	8.41 ± 0.43	49.67 ± 0.85	13.73 ± 0.14	9.23 ± 0.61	20.38 ± 0.38	5.7 ± 0.52
Ke (h^{-1})	0.052 ± 0.01	0.06 ± 0.03	0.072 ± 0.01	0.062 ± 0.04	0.05 ± 0.006	0.042 ± 0.01
$T_{1/2}$ (h)	9.01 ± 0.83	10.74 ± 0.06	8.82 ± 0.04	12.53 ± 2.05	15.92 ± 0.06	16.5 ± 0.01
AUC_{0-72} (ng/g/h)	199.037 ± 0.68	273.03 ± 2.01	283.28 ± 0.81	215.93 ± 7.92	201.93 ± 7.82	124.92 ± 2.71

The parameters time to reach peak drug concentration (T_{max}), peak drug concentration (C_{max}), elimination rate constant (Ke), mean apparent elimination half-life ($T_{1/2}$) and concentration-time curve (AUC) was used.

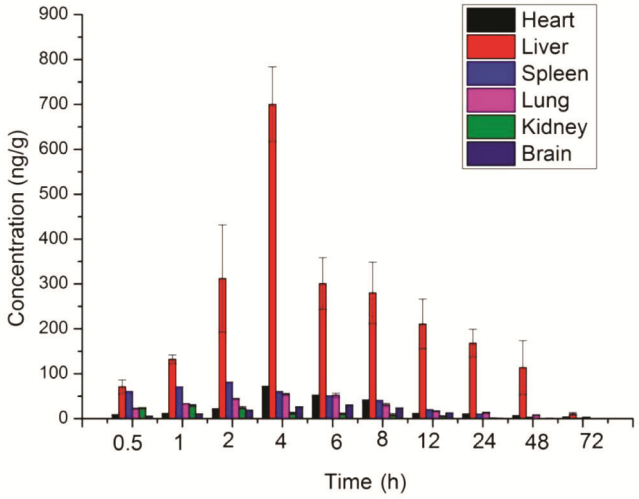

Figure 5. Tissue concentration of compound **1a** (10 mg/kg) in heart, liver, spleen, lung, kidney and brain observed by a signal oral administration (n = 6).

10 mg/kg of **1a** for 2 h, the serum concentration of Wistar rats approached the peak level. Thereafter, the blood concentration gradually decreased and presence of drug could still be detected after 72 h. The results showed that **1a** can be absorbed quickly and metabolized slowly in the body.

Tissue distribution experiment

The main parameters of each organization were shown in Figure 5 and Table 3. Compound **1a** in the liver showed the highest concentration, followed by that in the kidney, spleen, lung, heart and brain. The lowest drug concentration was in brain tissue. Compound **1a** is mainly metabolized by the liver. The drug residue could be observed even after 72 h.

Conclusion

In this study, a novel type of angiotensin II receptor antagonist, **1a** has been synthesized as an antihypertensive compound. Experiments were conducted to verify the antagonistic activity of **1a** at the cellular level of AT1 receptor. The hypotensive effect of **1a** in animals was verified by oral administration of spontaneous hypertensive rats. Acute toxicity experiments showed that **1a** had a good biological safety. Results of the pharmacokinetic experiments showed that **1a** can be uniformly distributed in the body.

In conclusion, **1a** for AT1 receptor has excellent antagonist effects. In addition, it can reduce BP quickly and smoothly. The antihypertensive activity lasted for more than 12 h. Compound **1a** can be absorbed quickly in the body and also can be distributed to the main tissues; so it plays a protective role to the heart, brain, kidney and other vital organs. Therefore, **1a** is a novel potential drug for treating hypertension.

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