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Research paper

Design, synthesis and evaluation of novel potent angiotensin II receptor 1 antagonists

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ABSTRACT

A series of new angiotensin II (Ang II) receptor 1 antagonists were designed, synthesized and evaluated. All compounds showed nanomolar affinities for the angiotensin II type 1 receptor in radioligand binding assays and could reduce blood pressure significantly in spontaneously hypertensive rats(SHRs). From which, compound **2b** displayed higher affinity binding to angiotensin II type 1 receptor at the same order of magnitude to irbesartan with an IC_{50} value of 1.26 ± 0.08 nM in radioligand binding assays. **2b** showed an efficient and long-lasting effect in reducing blood pressure, the maximal reducing responses were 40.62 ± 4.08 mmHg of MBP at 15 mg/kg and 28.39 ± 2.09 mmHg at 10 mg/kg in SHRs, 39.56 ± 4.83 mmHg at 15 mg/kg and 29.05 ± 2.20 mmHg at 10 mg/kg in RHRs, the significant antihypertensive effect lasted beyond 12 h both in SHRs and in RHRs. In the single-dose pharmacokinetic experiments, compound **2b** could be absorbed efficiently and metabolized smoothly in Wistar rats after oral administration. The values of C_{max}, T_{max}, AUC_{0-72} and MRT_{0-72} were 885.61 ± 432.7 ng/mL, 5.67 ± 1.51 h, 6110.28 ± 7398.33 ng/mL h and 7.87 ± 2.30 h at 10 mg/kg, 2945.55 ± 1543.67 ng/mL, 4.33 ± 0.82 h, 26473.62 ± 12217.16 ng/mL h and 10.24 ± 6.94 h at 15 mg/kg, 5759.03 ± 1331.75 ng/mL, 5 ± 1.10 h, 89488.44 ± 18413.15 ng/mL·h and 12.89 ± 2.0 h at 30 mg/kg respectively. The $T_{1/2}$ values of the three groups were similar, about 9–10 h. Compound **2b** was distributed into tissues rapidly and extensively after oral administration. The level of it was the highest in the liver, followed by in spleen, kidney, and the lowest in brain. The acute toxicity assays of **2b** proved its low acute toxicity with an LD_{50} value of 1551.71 mg/kg, and no toxicity reaction appeared at dose of 1200.00 mg/kg. These encouraging results make compound **2b** an effective, long-lasting and safe anti-hypertensive drug candidate and worthy of further investigation.

1. Introduction

Hypertension, defined as raised blood pressure greater than or to 140 mmHg systolic or 90 mmHg diastolic, is a serious health problem associated with an increased risk of death, stroke, and metabolic syndromes including insulin resistance and lipid abnormalities [1,2]. It is recognized as one of the leading risk factors for human morbidity and mortality [3,4]. As the population ages and the prevalence of contributing factors such as obesity, sedentary lifestyle and smoking rise, hypertension has currently affected approximately one billion adults globally and it may increase to 1.56 billion by the year 2025 [5]. Despite global awareness of hypertension, its consequences and the availability of effective therapeutics, an estimated 32% of hypertensive patients remain untreated [6].

The rennin angiotensin system (RAS) plays a central role in the pathophysiology of hypertension, electrolyte balance and blood volume [7]. Angiotensin II (Ang II), an octapeptide formed within the RAS from angiotensin I by angiotensin converting enzyme (ACE), is the biologically active component of the rennin-

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angiotensin system and is responsible for most of the peripheral effects of this system and it is one of the most powerful vasoconstrictors [8,9]. Angiotensin II receptor blockers (ARBs) are the newest class of approved antihypertensive agents and have rapidly become established as one of the leading therapeutic drugs in the management of hypertension [10,11]. At present, many efficient and safe ARBs have been widely used in the treatment of hypertension, such as losartan, valsartan, irbesartan, candesartan, telmisartan and so on.

Irbesartan, a potent, long-acting angiotensin II receptor 1 antagonist, decreases blood pressure effectively in a dose-dependent manner in patients with mild-to-moderate hypertension [12,13]. Here a series of new compounds (compound **1a**–**c**, **2a**–**d**) (Fig. 1) were designed and synthesized taking irbesartan as lead compound. The dominant conformations of compound **1b**, **2b** and irbesartan were shown as Fig. 2 from computer software Spartan 10. According to their overlay conformations, it is indicated that they align well with each other. And the affinities of this series of compounds to AT_1 receptor were tested by specific radioligand binding assay *in vitro*. The anti-hypertension effects of them were evaluated in spontaneously hypertensive rats (SHRs) *in vivo*. The anti-hypertension effects in renal hypertensive rats (RHRs), plasma pharmacokinetics, tissue distribution in Wistar rats and acute toxicity in ICR mouse of compound **2b** were further investigated.

2. Results and discussion

2.1. Chemistry

The preparation of compounds **1a**–**c** and **2a**–**d** was performed by means of a multistep procedure described in Schemes 1 and 2.

The synthesis of **1a**–**c** was accomplished starting from the commercially available 1-amino-cyclopentanecarboxamide (**3**), which reacted with different acyl chlorides in DCM using Et_3N as base to give amide **4a**–**d**. **4a**–**d** were cyclized to produce spiroazacyclic compounds **5a**–**d** with 10 M KOH in MeOH. 4-Methylindole **6a** was *N*-protected by acylation with benzoyl chloride to give (4-methyl-1H-indol-1-yl) (phenyl) methanone **7a**, which was brominated with NBS to produce (4-bromomethyl-1H-indol-1-yl) (phenyl) methanone **8a**. **8a** was substituted by **5a**–**d** and then de-protected in the presence of K_2CO_3 to generate indole compounds **9a**–**c** which were then coupled with 2-fluoro-benzonitrile to give benzonitrile **10a**–**c**. The target compounds **1a**–**c** were acquired after the formation of tetrazole using **10a**–**c** with sodium azide and chlorotributylstannane in DMF. At each stage of the synthetic sequence the product was isolated, purified by column chromatography and characterized by NMR and mass spectrum techniques.

The preparation of **2a**–**d** was similar to **1a**–**c**.

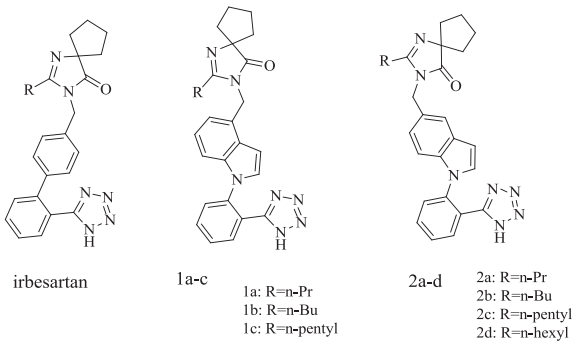


Fig. 1. Chemical structure of irbesartan and compounds **1a**–**c**, **2a**–**d**.

2.2. Biological evaluation

2.2.1. Radioligand binding assays

Immunohistochemical staining showed that smooth muscle actin was expressed in cytoplasm (Fig. 3), which indicated that the obtained cells were vascular smooth muscle cells. The radioligand binding assays showed that all the compounds have nanomolar affinity for the AT_1 receptor subtype (Table 1, Fig. 4). Generally, compounds **1b** and **2b** had much stronger inhibitory ability than compounds **1a**, **1c**, **2a**, **2c** and **2d** in binding assays which indicated that compounds **1b** and **2b** might have better antihypertensive activity *in vivo*. And among all the compounds, compound **2b** showed higher affinity to the angiotensin AT_1 receptor with an IC_{50} value of 1.26 ± 0.08 nM than irbesartan whose IC_{50} value was 1.46 ± 0.34 nM.

2.2.2. In vivo anti-hypertension effects of the compounds

In spontaneously hypertensive rats (SHRs), the effects of compounds **1a**–**c**, **2a**–**d** (15 mg/kg) and irbesartan (15 mg/kg) on the mean blood pressure (MBP) *in vivo* after oral administration were shown in Fig. 5. The results indicated that all the new compounds, especially compound **1b** and **2b,** could decrease blood pressure significantly under the dose of 15 mg/kg compared with the negative control group. The maximal response of compound **1b** at 15 mg/kg was observed at 3 h after oral administration. It lowered 40.57 ± 2.90 mmHg of MBP and the significant ($p < 0.01$) antihypertensive effect sustained for at least 12 h. Among these compounds, **2b** has the strongest antihypertensive effect, whose maximal response lower 40.62 ± 4.08 mmHg MBP at 15 mg/kg and 28.39 ± 2.26 mmHg at 10 mg/kg at 4 h after oral administration in SHRs (Fig. 6a). The antihypertensive effects in RHRs were tested to be coincident with which in SHRs (Fig. 6b). No influence of these compounds to the heart rates of rats was observed. These results suggested that the anti-hypertensive effects of compound **1b** and **2b** were superior to irbesartan, especially **2b**, which were worthy of further investigation.

2.2.3. Pharmacokinetic characteristics of compound 2b

The HPLC chromatograms of compound **2b** in plasma and liver tissues were showed in Fig. 7 and Fig. 8 respectively. The concentrations of compound **2b** in rat plasma samples obtained from 6 male Wistar rats orally administered with **2b** solution at 10–30 mg/kg were quantified. The pharmacokinetic parameters were shown in Table 2. The mean peak plasma concentration-time curve of **2b** was shown in Fig. 9. The maximum concentration (C_{max}) value ranged from 855 ng/mL at 10 mg/kg to 5759.03 ng/mL at 30 mg/kg. The area under the concentration-time curve from 0 to 72 h ($AUC_{0\sim72}$) ranged from 6110.28 ng/mL·h to 89488.44 ng/mL·h. The elimination half-lives ($T_{1/2}$) ranged from 9 h to 13 h. Dose dependency of $AUC_{0\sim\infty}$ and C_{max} (10–30 mg/kg) was assessed and both $AUC_{0\sim\infty}$ and C_{max} met the dose proportionality criteria(Fig. 10).

The compound levels in heart, liver, spleen, kidney and brain of Wistar rats at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 h post oral administration of compound **2b** at 10–30 mg/kg were shown in Fig. 11. The pharmacokinetic parameters were shown in Table 3. It could be found that **2b** was extensively distributed in rat tissues. It was accumulated especially in liver which exhibited the highest concentration ranged from 720.73 ng/g at 10 mg/kg to 1995.52 ng/g at 30 mg/kg, followed by spleen ranged from 348.02 ng/g at 10 mg/kg to 1881.59 ng/g at 30 mg/kg. The level of compound **2b** was lowest in the brain, which ranged from 37.16 ng/g to 196.47 ng/g. As compound **2b** was detectable in brain, it was likely that **2b** was able to penetrate the blood-brain barrier (BBB). For all tissues, compound **2b** showed continuous increase at 0–4 h and decrease from 6 to 72 h post administration.

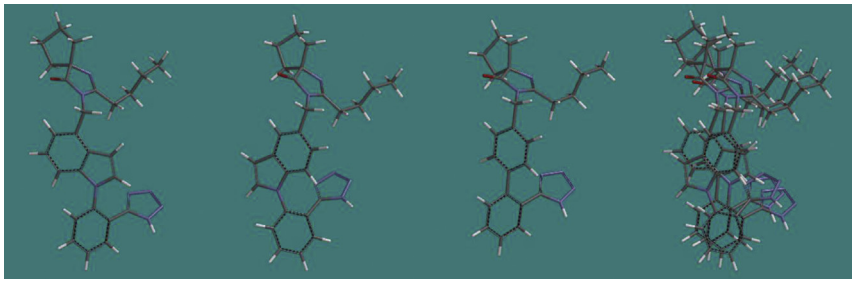
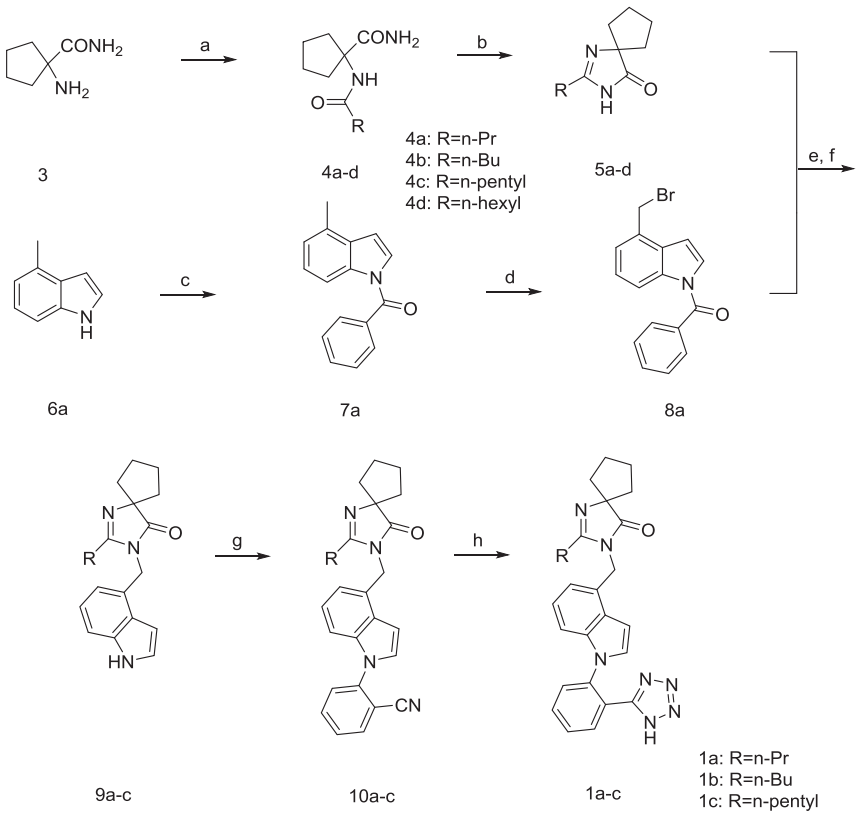


Fig. 2. Energy-minimized conformations of **1b**, **2b**, irbesartan and their overlay conformations.



Scheme 1. Reagents and reaction conditions: (a) RCOCl, Et_3N, DCM; (b)KOH, MeOH, reflux; (c) benzoyl chloride, Et_3N, DMAP, CH_2Cl_2; (d) NBS, AIBN, CCl_4, reflux; (e) NaH, THF, 50 °C; (f) NaOH, H_2O, CH_3OH, reflux; (g) 2-fluorobenzonitrile, K_2CO_3, DMF, reflux; (h) NaN_3, Bu_3SnCl, DMF, reflux.

These results showed that compound **2b** could be absorbed efficiently and metabolized gradually in plasma and in tissues of Wistar rats.

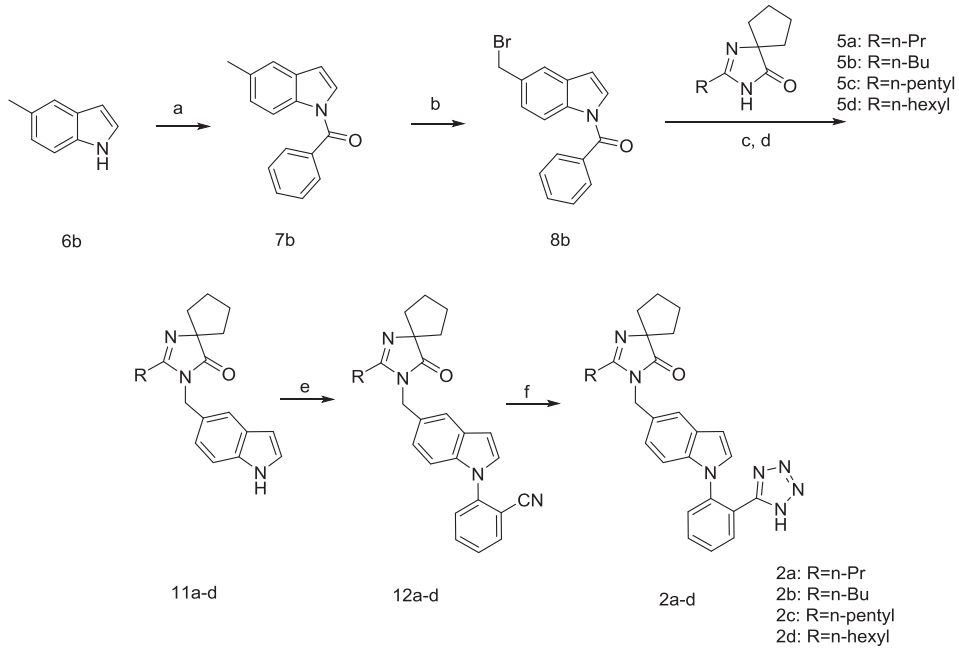
2.2.4. Acute toxicity test of compound 2b

The acute toxicity assay showed that compound **2b** had low acute toxicity with the LD_{50} value of 1551.71 mg/kg and the 95% confidence interval was 1348.88–1745.07 mg/kg (Table 4). There was no death and obvious untoward reaction appeared at dose of 1200 mg/kg after oral administration, and no significant change in the weight of survival mice after 2 weeks' observation.

3. Conclusions

In this work, a series of novel angiotensin II receptor 1 antagonists were designed, synthesized and evaluated. All the compounds showed nanomolar affinities for the angiotensin II type 1 receptor in radioligand binding assays and could reduce blood pressure significantly in SHRs. In the binding assay, compound **2b** showed stronger inhibitory ability against Ang II than irbesartan. In antihypertensive effect tests *in vivo*, **2b** showed an efficient and long-lasting effect in reducing blood pressure in SHRs and RHRs at 15 mg/kg. The pharmacokinetic experiments showed that **2b** could be absorbed efficiently and metabolized smoothly in Wistar rats after oral administration. With single-dose **2b** 10, 20 and 30 mg/kg, the mean plasma exposure (C_{max} and AUC_{0-72}) was increased in a dose-dependent manner. It was well distributed in all tissues including in brain tissues which suggested that **2b** could cross the BBB and play important role in the control of central nervous system blood pressure. Besides, compound **2b** has low acute toxicity.

In summary, compound **2b** could be preliminary considered as an effective candidate with high performance for novel antihypertension drug and deserved for further investigation.



Scheme 2. Reagents and conditions: (a) benzoyl chloride, Et_3N, DMAP, CH_2Cl_2; (b) NBS, AIBN, CCl_4, reflux; (c) NaH, THF, 50 °C; (d) NaOH, H_2O, CH_3OH, reflux; (e) 2-fluorobenzonitrile, K_2CO_3, DMF, reflux; (f) NaN_3, Bu_3SnCl, DMF, reflux.

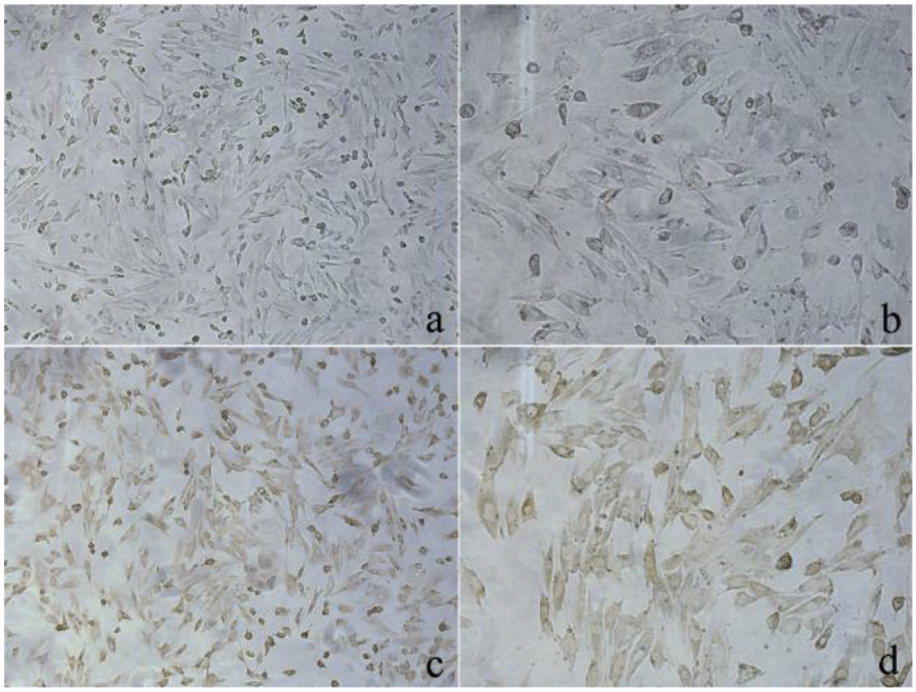


Fig. 3. The morphology of cultured VSMCs detected by phase-contrast microscope. (a) Untreated VSMCs, ×100. (b) Untreated VSMCs, ×200. (c) SM α-actin immunocytochemical staining of cultured VSMCs, ×100. (d) SM α-actin immunocytochemical staining of cultured VSMCs, ×200.

4. Experimental section

4.1. Chemistry

All chemical reagents were of highest commercially available quality and applied without further purification. Yields of the purified products were not optimized. Melting points (MP) were measured on an electro thermal melting point apparatus and were not accurated. ^{1}H NMR spectra were measured on a Bruker 400 MHz spectrometer using TMS (Me_4Si) as internal standard. ESI-MS spectra were recorded on a Micromass triple quadrupole mass spectrometer. Column chromatography (CC) was performed using silica gel H (300–400). All final compounds had a purity level greater than 95% based upon HPLC, and ^{1}H NMR.

Table 1
IC_{50} and Ki value of the tested compounds (**1a**–**c**, **2a**–**d**) and irbesartan.

Compound	IC_{50} ± SEM (nM)	Ki (nM)
1a	7.75 ± 0.16	5.61 ± 0.13
1b	3.05 ± 0.16	2.21 ± 0.12
1c	6.15 ± 0.20	4.67 ± 0.15
2a	7.04 ± 0.19	5.07 ± 0.15
2b	1.26 ± 0.17	0.91 ± 0.12
2c	5.33 ± 0.43	3.42 ± 0.30
2d	6.15 ± 0.20	4.67 ± 0.15
Irbesartan	1.46 ± 0.34	1.05 ± 0.25

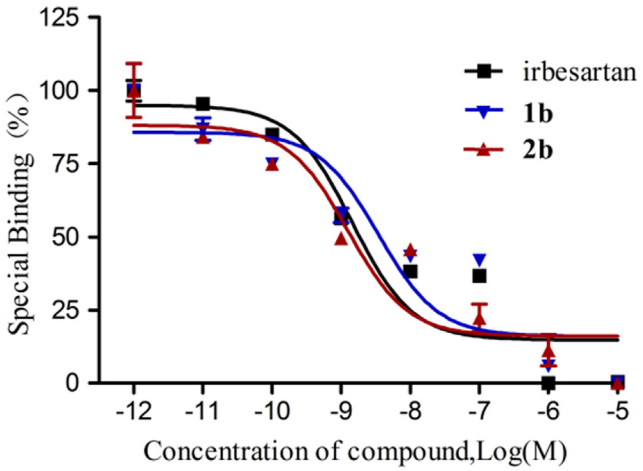


Fig. 4. Inhibitory effects of compounds **1b**, **2b** and irbesartan (10^{-5} – 10^{-12} M) on specific binding of ^{125}I-Ang II to AT_1 receptors in VSMCs.

*4.1.1. General procedure for the synthesis of **5a**–**d***

1-amino-cyclopentanecarboxamide (**3**) (8.0 g, 62.5 mmol) was acylated with alkyl acyl chloride (93.7 mmol) and triethylamine (17.3 mL, 125.0 mmol) in 50 mL dichloromethane(DCM) at 0 °C. After the reaction was completed, the resulting mixture was added with 30 mL water, and extracted with DCM (25 mL × 3). The organic layer was dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was dissolved in 50 mL MeOH and then 50 mL 10 M KOH was added slowly. The mixture was refluxed for 3 h. After cooled to room temperature, the mixture was added 50 mL H_2O and extracted with DCM (30 mL × 4). The organic layer was dried over $MgSO_4$, the solvent was removed under reduced pressure. The resulting residue was purified by CC to give **5a**–**d**.

*4.1.1.1. 2-Propyl-1,3-diazaspiro[4.4]non-1-en-4-one (**5a**).* The above general procedure was followed by using butyryl chloride as acylation reagent to give **5a** as oil. Yield: 87.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 10.7 (s, 1H), 2.34 (t, 2H), 1.94-1.50 (m, 8H), 1.35 (m, 2H), 0.92 (t, 3H). MS(ESI): $[M+H]^+$ calcd 181.1; found181.2.

*4.1.1.2. 2-Butyl-1,3-diazaspiro[4.4]non-1-en-4-one (**5b**).* The above general procedure was followed using pentyl chloride as acylation reagent to give **5b** as oil. Yield: 89.5%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 10.7 (s, 1H), 2.33 (t, 2H), 1.93-1.50 (m, 10H), 1.35 (m, 2H), 0.92 (t, 3H). MS(ESI): $[M+H]^+$ calcd 195.1; found 195.2.

*4.1.1.3. 2-Pentyl-1,3-diazaspiro[4.4]non-1-en-4-one (**5c**).* The above general procedure was followed using hexanoyl chloride as acylation reagent to give **5c** as oil. Yield: 91.2%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 10.7 (s, 1H), 2.32 (t, 2H), 1.92-1.49 (m, 10H), 1.34 (m, 4H), 0.91 (t, 3H). MS(ESI): $[M+H]^+$ calcd 209.2; found 209.3.

*4.1.1.4. 2-Hexyl-1,3-diazaspiro[4.4]non-1-en-4-one (**5d**).* The above general procedure was followed using heptanoyl chloride as acylation reagent to give **5d** as oil. Yield: 81.1%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 10.7 (s, 1H), 2.33 (t, 2H), 1.95-1.45 (m, 12H), 1.35 (m, 4H), 0.92 (t, 3H). MS(ESI): $[M+H]^+$ calcd 223.2; found 223.3.

*4.1.2. N-benzoyl-4-methylindol (**7a**)*

7a was prepared by the general procedure described by Dhanoa [14].

*4.1.3. N-benzoyl-5-methylindol (**7b**)*

7b was prepared by the general procedure described by Dhanoa [14].

*4.1.4. (4-(bromomethyl)-1H-indol-1-yl)(phenyl)methanone(**8a**)*

8a was prepared by the general procedure described by Dhanoa [14].

*4.1.5. (5-(bromomethyl)-1H-indol-1-yl)(phenyl)methanone(**8b**)*

8b was prepared by the general procedure described by Dhanoa [14].

*4.1.6. General procedure for the synthesis of **9a**–**c**, **11a**–**d***

A solution of an appropriate spiroazacyclic compound **5a**–**d** (5.03 mmol) and NaH (0.12 g, 5.53 mmol, 60%) in 100 mL anhydrous THF was stirred for 30 min at 50 °C. After cooled to rt, the solution was added dropwise the mixture of an appropriate bromide **8a**–**b** (6.04 mmol) in anhydrous THF (50 mL). The solution was stirred for 3 h at 50 °C. The resulting mixture was poured into 30 mL ice water, and extracted with ethyl acetate (EA) (50 mL × 3). The organic layer was dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was dissolved in 10 mL methanol and then added NaOH (0.8 g, 20 mmol) in 10 mL water slowly. The mixture was refluxed for 3 h, and the solvent was removed in vacuo. The residue was dissolved in 15 mL H_2O and extracted with DCM (10 mL × 4). The organic layer was dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was purified by CC to give the product as oil.

*4.1.6.1. 3-((1H-indol-4-yl)methyl)-2-propyl-1,3-diazaspiro[4.4]non-1-en-4-one(**9a**).* **9a** was synthesized according to the above general procedure. Yield: 79.2%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ:8.39 (brs, 1H), 7.36-7.34 (d, 1H), 7.36-6.83 (m, 3H), 6.56-6.55 (d, 1H), 4.98 (s, 2H), 2.26 (t, 2H), 2.01-1.96 (m, 8H), 1.84-1.51 (m, 2H), 0.84 (t, 3H). MS(ESI): $[M+H]^+$ calcd 310.2; found 310.4.

*4.1.6.2. 3-((1H-indol-4-yl)methyl)-2-butyl-1,3-diazaspiro[4.4]non-1-en-4-one (**9b**).* **9b** was synthesized according to the above general procedure. Yield: 76.8%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.35 (s, 1H), 7.37-7.34 (m, 1H), 7.26-6.84 (m, 3H), 6.56-6.55 (m, 1H), 4.94 (s, 2H), 2.28 (t, 2H), 2.01-1.96 (m, 8H), 1.52 (m, 2H), 1.28 (m, 2H), 0.84 (t, 3H). MS(ESI): $[M+H]^+$ calcd 324.2; found 324.2.

*4.1.6.3. 3-((1H-indol-4-yl)methyl)-2-pentyl-1,3-diazaspiro[4.4]non-1-en-4-one(**9c**).* **9c** was synthesized according to the above general procedure. Yield: 71.3%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.39 (brs, 1H), 7.36-7.34 (d, 1H), 7.26-6.84 (m, 3H), 6.56-6.55 (m, 1H), 4.98 (s, 2H), 2.27 (t, 2H), 2.01-1.96 (m, 8H), 1.29-1.26 (m, 6H), 0.82 (t, 3H). MS(ESI): $[M+H]^+$ calcd 338.2; found 338.5.

*4.1.6.4. 3-((1H-indol-5-yl)methyl)-2-propyl-1,3-diazaspiro[4.4]non-1-en-4-one (**11a**).* **11a** was synthesized according to the above general procedure. Yield: 77.1%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ:

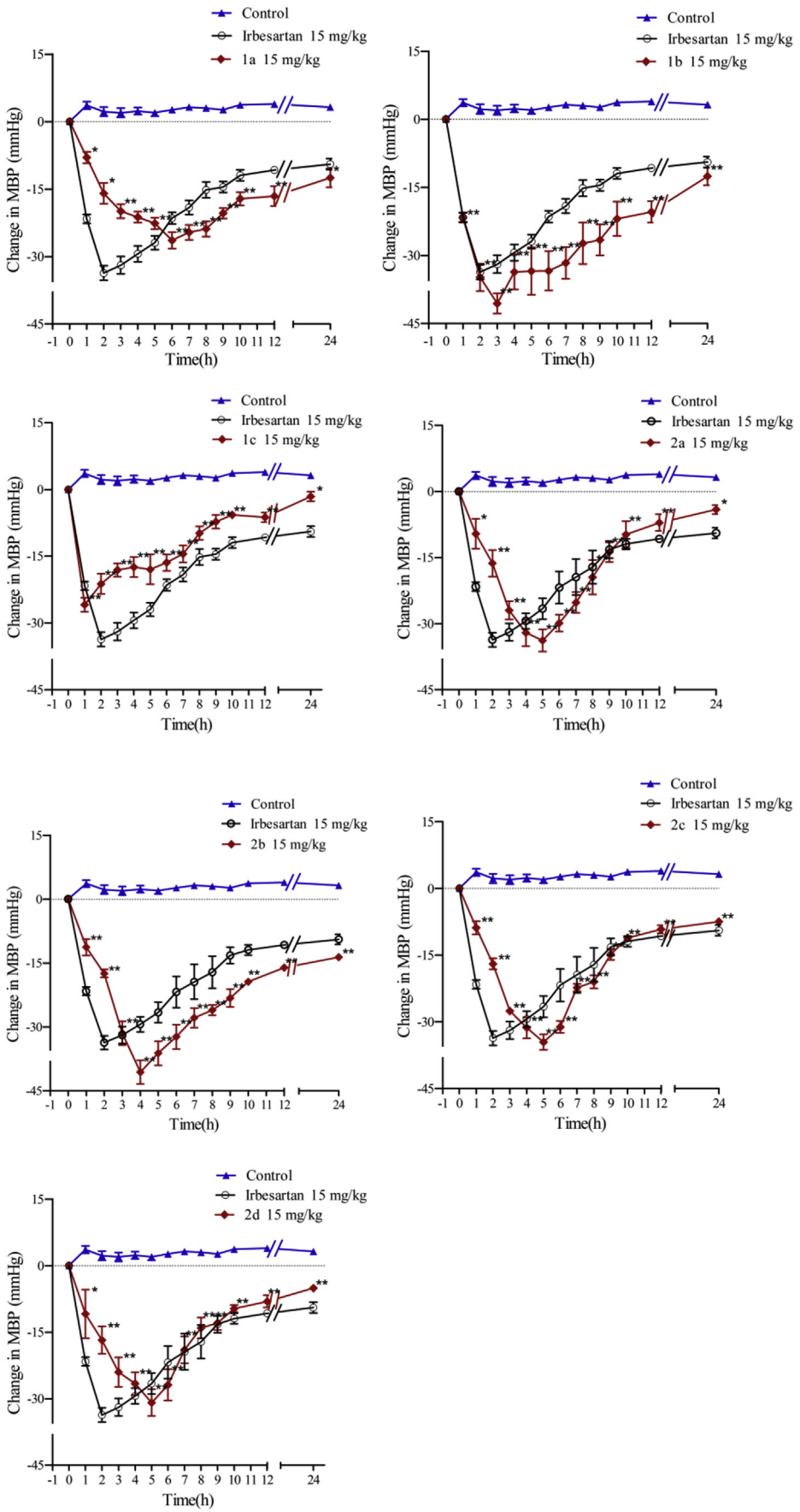


Fig. 5. Effects of compound **1a**–**c**, **2a**–**d** and irbesartan on mean blood pressure (MBP) in spontaneously hypertensive rats. *and** Significant difference from negative control, $p < 0.05$ and $p < 0.01$, respectively.

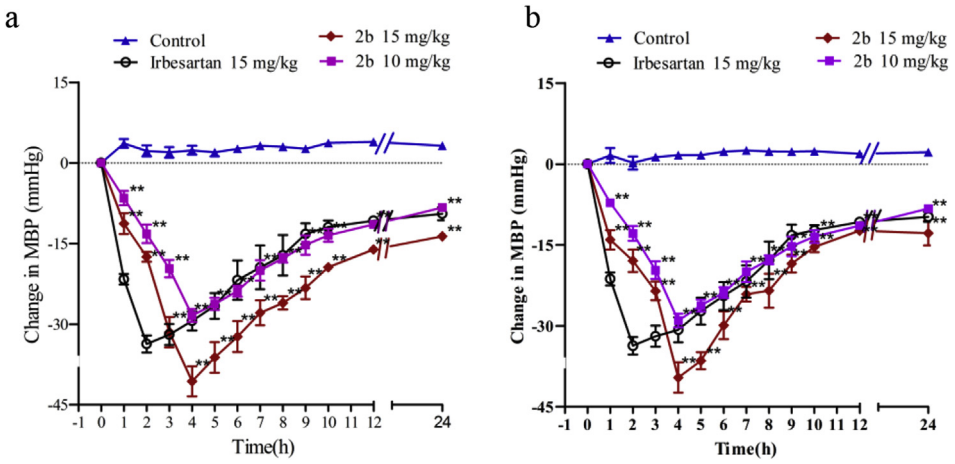


Fig. 6. (a) Effects of compound **2b** (15 mg/kg, 10 mg/kg) and irbesartan (15 mg/kg) on mean blood pressure (MBP) in SHRs. (b) Effects of compound **2b** (15 mg/kg, 10 mg/kg) and irbesartan (15 mg/kg) on mean blood pressure (MBP) in RHRs. *and** Significant difference from negative control, $p < 0.05$ and $p < 0.01$, respectively.

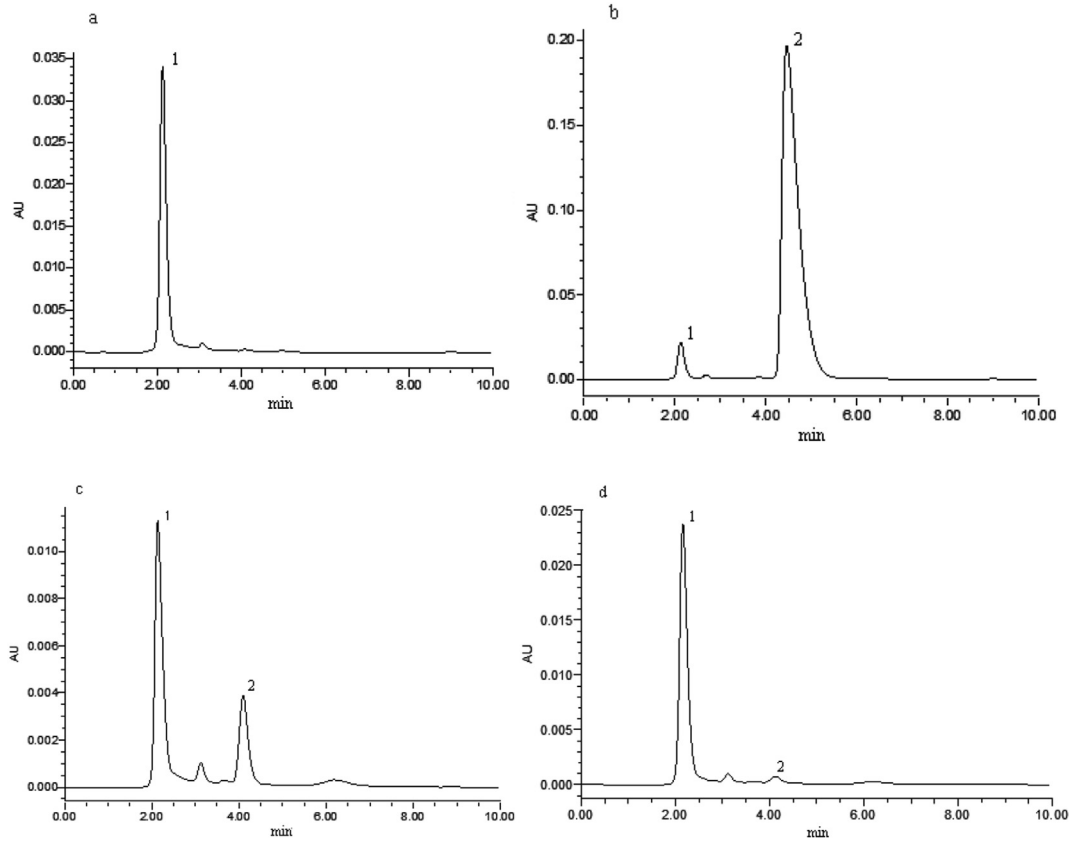


Fig. 7. HPLC Chromatogram of compound **2b** in plasma of Wistar rats. a, Blank plasma; b, Blank plasma spiked with **2b**; c, A plasma sample from a subject after an oral of **2b** at 4 h at 10 mg/kg; d, A plasma sample from a subject after an oral of **2b** at 24 h at 10 mg/kg; 2- compound **2b** ($t_R = 4.2$ min).

8.91 (brs, 1H), 7.50 (s, 1H), 7.40-7.37 (m, 1H), 7.28-7.26 (m, 1H), 7.07-7.04 (m, 1H), 6.59-6.55 (m, 1H), 4.83 (s, 2H), 2.43 (t, 2H), 2.08-1.91 (m, 8H), 1.59-1.51 (m, 2H), 0.84 (t, 3H)。MS(ESI): $[M+H]^+$ calcd 310.2; found 310.4.

*4.1.6.5. 3-((1H-indol-5-yl)methyl)-2-butyl-1,3-diazaspiro[4.4]non-1-en-4-one(**11b**).* **11b** was synthesized according to the above general procedure. Yield: 77.4%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.90 (brs, 1H), 7.50 (s, 1H), 7.40-7.37 (m, 1H), 7.28-7.26 (m, 1H), 7.07-7.04 (m, 1H), 6.59-6.55 (m, 1H), 4.84 (s, 2H), 2.42 (t, 2H), 2.08-1.91 (m, 8H), 1.63-1.59 (m, 2H), 1.39-1.33 (m, 2H), 0.88 (t, 3H). MS(ESI): $[M+H]^+$ calcd 324.2; found 324.2.

*4.1.6.6. 3-((1H-indol-5-yl)methyl)-2-pentyl-1,3-diazaspiro[4.4]non-1-en-4-one (**11c**).* **11c** was synthesized according to the above general procedure. Yield: 76.3%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ:8.90 (brs, 1H), 7.50 (s, 1H), 7.40-7.37 (m, 1H), 7.28-7.26 (m, 1H), 7.07-7.04 (m, 1H), 6.59-6.55 (m, 1H), 4.84 (s, 2H), 2.42 (t, 2H), 2.01-

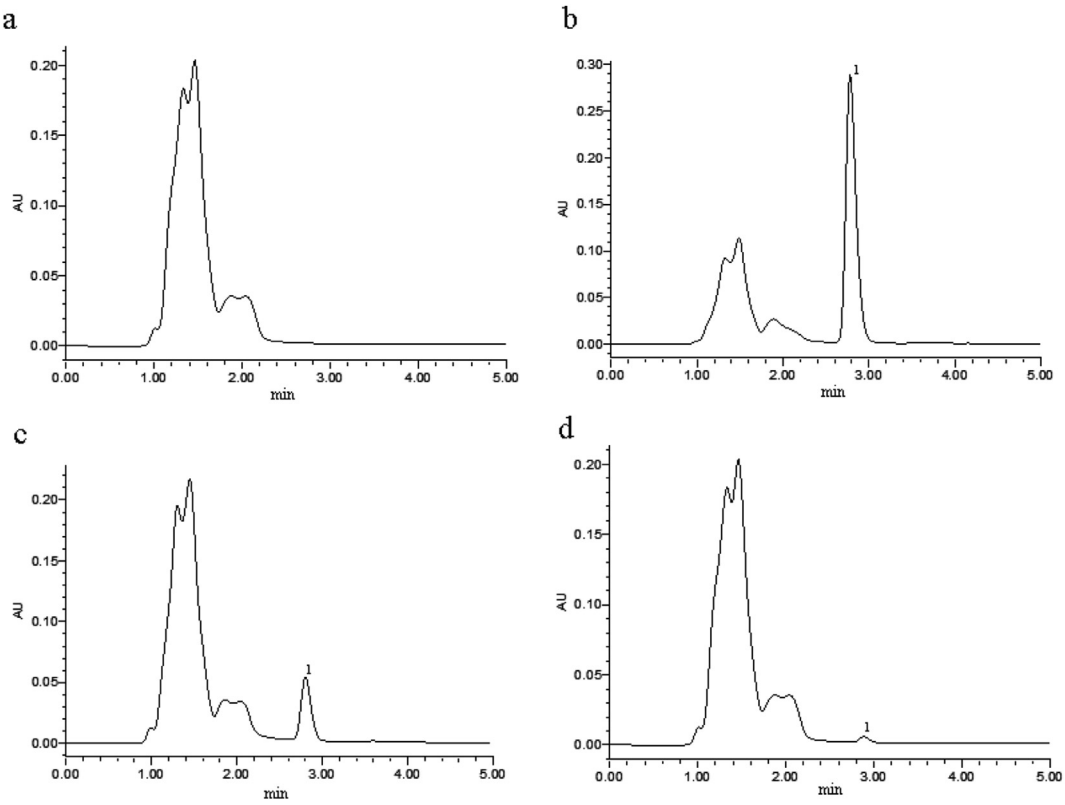


Fig. 8. HPLC Chromatogram of compound **2b** in liver tissue of Wistar rats. a, Blank liver tissue; b, Blank liver tissue spiked with **2b**; c, A liver tissue sample from a subject after an oral of **2b** at 4 h at 10 mg/kg; d, A liver tissue sample from a subject after an oral of **2b** at 24 h at 10 mg/kg; 1-compound **2b** (t_R = 2.8 min).

Table 2
Pharmacokinetic parameters of compound **2b** in plasma of male Wistar rats after oral administration (10 mg/kg, 15 mg/kg, 30 mg/kg) (n = 6).

Dose (mg/kg)	Pharmacokinetic parameters						
	$AUC_{(0-72)}$ (ng/mL h)	$AUC_{(0-\infty)}$ (ng/mL h)	$MRT_{(0-72)}$ (h)	$MRT_{(0-\infty)}$ (h)	$T_{1/2}$ (h)	T_{max}(h)	C_{max}(ng/mL)
10	6110.28 ± 7398.33	6687.54 ± 8119.11	7.87 ± 2.30	6.61 ± 3.97	9.93 ± 0.51	5.67 ± 1.51	855.61 ± 432.7
15	23364.97 ± 10681.56	26070.54 ± 10505.84	10.24 ± 6.94	13.66 ± 14.83	9.03 ± 1.20	4.33 ± 0.86	2945.55 ± 1543.67
30	89488.44 ± 18413.15	88846.69 ± 17500.03	12.89 ± 2.0	16.06 ± 5.24	9.93 ± 3.40	5.00 ± 1.10	5759.03 ± 1331.75

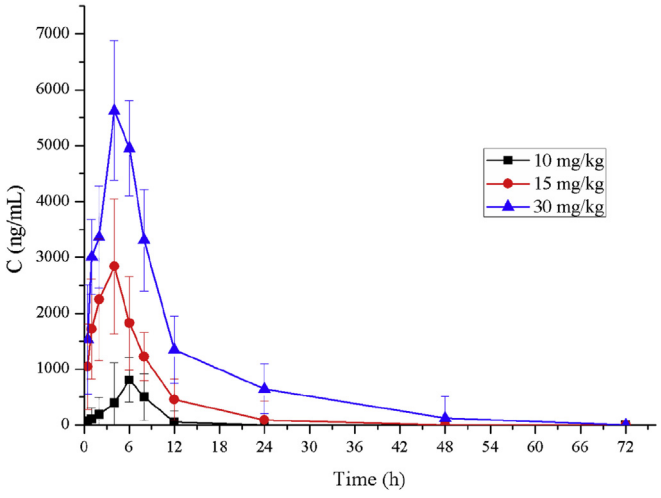


Fig. 9. Observed plasma concentrations of **2b** in rats after a signal oral administration (10 mg/kg, 15 mg/kg, 30 mg/kg). Data are average values of 6 experiments (Mean ± SD).

1.96 (m, 8H), 1.64-1.26 (m, 6H), 0.82 (t, 3H). MS(ESI): $[M+H]^+$ calcd 338.2; found 338.5.

*4.1.6.7. 3-((1H-indol-5-yl)methyl)-2-hexyl-1,3-diazaspiro[4.4]non-1-en-4-one(**11d**).* **11d** was synthesized according to the above general procedure. Yield: 74.8%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.90 (brs, 1H), 7.51 (s, 1H), 7.40-7.36 (m, 1H), 7.28-7.25 (m, 1H), 7.06-7.04 (m, 1H), 6.59-6.54 (m, 1H), 4.83 (s, 2H), 2.41 (t, 2H), 2.01-1.96 (m, 8H), 1.65-1.26 (m, 8H), 0.80 (t, 3H). MS(ESI): $[M+H]^+$ calcd 352.2; found 352.2.

*4.1.7. General procedure for the synthesis of **10a–c**, **12a–d***

A solution of an appropriate indole compound **9a–c** or **11a–d** (2 mmol) in 20 mL DMF was added K_2CO_3 (552 mg, 4 mmol) and 2-fluorobenzonitrile (266 mg, 2.2 mmol). The mixture was stirred-under reflux for 5 h under nitrogen. The resulting mixture was diluted with water 60 mL and extracted with EA (30 mL × 4). The organic layer was washed with saturated salt water (40 mL × 4) and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was purified by CC to give a yellow solid.

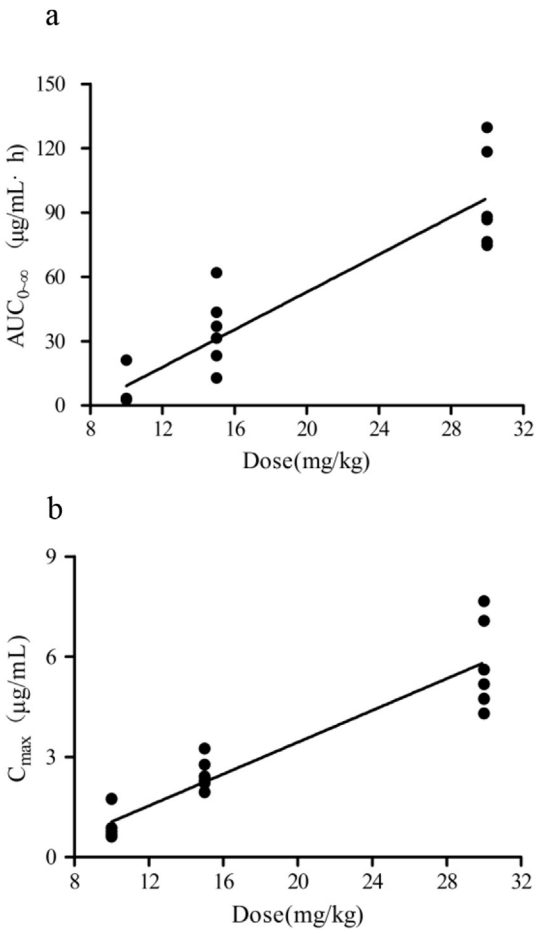


Fig. 10. Compound **2b** $AUC_{(0-\infty)}$ (y = 4.38x − 34.67; R^2 = 0.85) (a) and C_{max} (y = 0.26x − 1.56, R^2 = 0.96) (b) versus dose (mg/kg) following a signal oral administration of male Wistar rats (n = 6).

*4.1.7.1. 2-(4-((4-oxo-2-propyl-1,3-diazaspiro[4.4]non-1-en-3-yl) methyl)-1H-indol-1-yl) benzonitrile (**10a**).* **10a** was synthesized according to the above general procedure. Yield: 85.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.98-7.92 (m, 1H), 7.83-7.78 (m, 1H), 7.72 (m, 1H), 7.66 (d, 1H), 7.33 (d, 1H), 7.01 (t, 1H), 6.87 (d, 1H), 6.73 (d, 1H), 6.66 (d, 1H), 4.94 (s, 2H), 2.27 (t, 2H), 1.93-1.80 (m, 6H), 1.76-1.63 (m, 2H), 1.54-1.40 (m, 2H), 0.80 (t, 3H)。MS(ESI): $[M+H]^+$ calcd 411.2; found 411.5.

*4.1.7.2. 2-(4-((2-butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl) methyl)-1H-indol-1-yl)benzonitrile (**10b**).* **10b** was synthesized according to the above general procedure. Yield: 87.1%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 7.85 (d, 1H), 7.74 (t, 1H), 7.58 (d, 1H), 7.51 (t, 1H), 7.43 (d, 1H), 7.27 (d, 1H), 7.19 (t, 1H), 6.94 (d, 1H), 6.80 (d, 1H), 5.01 (s, 2H), 2.33 (t, 2H), 2.07–1.83 (m, 8H), 1.52 (m, 2H), 1.28 (m, 2H), 0.82 (t, 3H). MS(ESI): $[M+H]^+$ calcd 425.2; found 425.3.

*4.1.7.3. 2-(4-((4-oxo-2-pentyl-1,3-diazaspiro[4.4]non-1-en-3-yl) methyl)-1H-indol-1-yl)benzonitrile (**10c**).* **10c** was synthesized according to the above general procedure. Yield: 89.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.94 (d, 1H), 7.77 (t, 1H), 7.70 (t, 1H), 7.61 (d, 1H), 7.32 (d, 1H), 7.05-6.96 (m, 1H), 6.87 (d, 1H), 6.73 (d, 1H), 6.64 (d, 1H), 4.94 (s, 2H), 2.29 (t, 2H), 1.92-1.80 (m, 6H), 1.74–1.62 (m, 2H), 1.49-1.38 (m, 2H), 1.20-1.10 (m, 4H), 0.77 (t, 3H). MS(ESI): $[M+H]^+$ calcd 439.2; found 439.6.

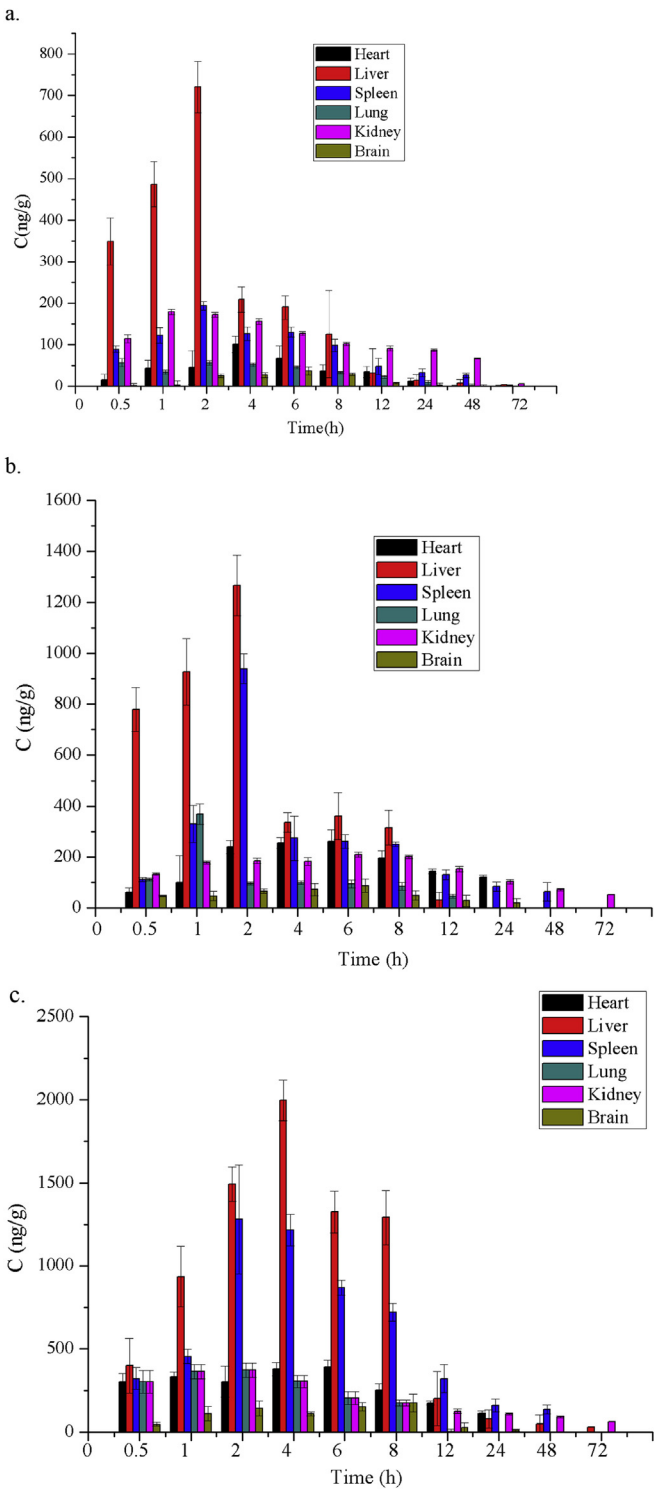


Fig. 11. Observed tissues concentrations of **2b** in Wistar rats after a signal oral administration (10 mg/kg, 15 mg/kg, 30 mg/kg). Data are average values of 6 experiments (Mean ± SD) (a, 10 mg/kg; b, 15 mg/kg; c, 30 mg/kg).

*4.1.7.4. 2-(5-((4-oxo-2-propyl-1,3-diazaspiro[4.4]non-1-en-3-yl) methyl)-1H-indol-1-yl)benzonitrile (**12a**).* **12a** was synthesized according to the above general procedure. Yield: 87.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.94 (d, 1H), 7.77 (t, 1H), 7.69 (t, 1H), 7.62 (d, 1H), 7.32 (d, 1H), 7.01 (t, 1H), 6.87 (d, 1H), 6.72 (d, 1H), 6.63 (d, 1H), 4.94 (s, 2H), 2.28 (t, 2H), 1.95-1.78 (m, 6H), 1.75-1.61 (m, 2H),

Table 3
Pharmacokinetic parameters of compound **2b** in tissues of male Wistar rats after oral administration (10 mg/kg, 15 mg/kg, 30 mg/kg) (n = 6) .

Tissue	Dose (mg/kg)	Pharmacokinetic parameters						
		$AUC_{(0-72)}$ (ng/mL·h)	$AUC_{(0-\infty)}$ (ng/mL h)	$MRT_{(0-72)}$ (h)	$MRT_{(0-\infty)}$ (h)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)
Heart	10	825.30 ± 325.98	962.11 ± 270.31	7.68 ± 2.01	12.00 ± 3.80	16.92 ± 3.07	4 ± 1.27	104.79 ± 14.76
	15	5835.75 ± 189.17	6699.84 ± 170.61	16.93 ± 0.13	23.85 ± 0.13	15.85 ± 1.91	4.67 ± 1.63	282.74 ± 16.16
	30	7359.74 ± 242.03	8757.78 ± 342.77	16.18 ± 0.79	28.07 ± 3.07	20.18 ± 3.13	4.8 ± 1.10	400.68 ± 35.21
Liver	10	3073.19 ± 416.59	3319.04 ± 361.25	4.93 ± 0.98	3.58 ± 2.86	2.33	20.08	720.7 ± 61.76
	15	4889.25 ± 652.86	23517.36 ± 5041.71	4.77 ± 0.56	4.77 ± 0.56	1.80 ± 0.71	2	1226.45 ± 118.86
	30	14473.39 ± 1846.79	15623.11 ± 2006.10	6.30 ± 1.13	5.49 ± 2.83	2.12 ± 0.26	4	1995.52 ± 122.77
Spleen	10	9474.46 ± 357.51	11742.48 ± 403.88	20.25 ± 0.32	30.42 ± 1.92	17.85 ± 1.36	2	348.02 ± 20.19
	15	11936.29 ± 1625.20	12046.8 ± 1663.33	9.71 ± 1.84	13.08 ± 3.88	7.57 ± 0.8	1.5 ± 1.23	2362.87 ± 69.41
	30	18867.22 ± 796.59	21045.84 ± 2156.90	13.27 ± 0.67	20.38 ± 3.15	13.36 ± 4.58	2	1881.59 ± 326.57
Lung	10	744.90 ± 114.67	745.02 ± 114.48	10.63 ± 1.90	10.63 ± 1.90	3.04 ± 0.86	2	62.06 ± 6.55
	15	1346.97 ± 71.89	1346.98 ± 71.89	6.64 ± 0.31	3.64 ± 0.31	2.46 ± 0.08	1	369.40 ± 39.65
	30	2361.07 ± 122.06	2412.15 ± 142.36	4.85 ± 0.29	4.10 ± 2.02	2.35 ± 0.03	2 ± 1.23	395.93 ± 31.93
Kidney	10	4335.85 ± 131.16	10849.31 ± 815.64	21.03 ± 0.13	25.30 ± 9.31	26.95 ± 6.95	1.17 ± 0.41	179.86 ± 6.7
	15	5729.83 ± 201.60	7691.91 ± 1045.48	19.37 ± 0.25	29.40 ± 4.03	22.58 ± 6.01	2.33 ± 0.81	210.02 ± 10.18
	30	22361.07 ± 1220.06	29445.3 ± 2458.1	24.85 ± 0.28	24.10 ± 2.0	22.35 ± 0.03	2 ± 1.23	395.93 ± 31.93
Brain	10	345.51 ± 64.23	345.52 ± 64.23	8.10 ± 1.90	8.10 ± 1.90	2.73 ± 0.06	6.67 ± 1.03	37.16 ± 9.63
	15	1181.40 ± 295.76	1181.40 ± 295.76	11.60 ± 3.20	11.60 ± 3.20	2.59 ± 0.09	4.83 ± 2.04	93.32 ± 21.89
	30	1789.98 ± 180.40	1791.54 ± 177.72	9.03 ± 0.50	9.02 ± 0.51	2.99 ± 1.53	5.83 ± 3.37	196.47 ± 39.95

Table 4
The lethal dose (LD_{50}) of compound **2b** determined by acute toxicity test (n = 10).

Dose(mg/kg)	Log(dose)	Mortality (%)	LD_{50} and 95% confidence interval (mg/kg)
1200.00	3.08	0	1551.71 (1343.88–1745.08)
1500.00	3.18	50	
1875.00	3.27	60	
2343.75	3.37	80	
2929.69	3.47	100	

1.53-1.44 (m, 2H), 0.81 (t, 3H). MS(ESI): $[M+H]^+$ calcd 411.2; found 411.5.

*4.1.7.5. 2-(5-((2-butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl) methyl)-1H-indol-1-yl)benzonitrile (**12b**).* **12b** was synthesized according to the above general procedure. Yield: 83.4%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 7.84-7.82 (m, 1H), 7.74-7.72 (m, 1H), 7.56 (d, 1H), 7.52-7.51 (m, 2H), 7.43-7.41 (m, 1H), 7.28-7.27 (m, 1H), 7.07-7.05 (m, 1H), 6.71 (d, 1H), 4.79 (s, 2H), 2.32 (t, 2H), 2.06-1.83 (m, 8H), 1.56-1.51 (m, 2H), 1.30-1.26 (m, 2H), 0.85 (t, 3H). MS(ESI): $[M+H]^+$ calcd 425.2; found 425.3.

*4.1.7.6. 2-(5-((4-oxo-2-pentyl-1,3-diazaspiro[4.4]non-1-en-3-yl) methyl)-1H-indol-1-yl)benzonitrile (**12c**).* **12c** was synthesized according to the above general procedure. Yield: 81.6%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.93 (d, 1H), 7.77 (t, 1H), 7.67 (t, 1H), 7.61 (d, 1H), 7.35 (s, 1H), 7.26 (d, 1H), 7.03-6.77 (m, 2H), 6.57 (s, 1H), 4.72 (s, 2H), 2.32 (t, 2H), 1.92-1.75 (m, 6H), 1.70-1.61 (m, 2H), 1.51-1.42 (m, 2H), 1.23-1.13 (m, 4H), 0.78 (t, 3H)。 MS(ESI): $[M+H]^+$ calcd 439.2; found 439.6.

*4.1.7.7. 2-(5-((2-hexyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl) methyl)-1H-indol-1-yl)benzonitrile (**12d**).* **12d** was synthesized according to the above general procedure. Yield: 79.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 7.93 (d, 1H), 7.83-7.57 (m, 3H), 7.35 (s, 1H), 7.26 (d, 1H), 7.04-6.78 (m, 2H), 6.57 (s, 1H), 4.71 (s, 2H), 2.32 (t, 2H), 1.94-1.74 (m, 6H), 1.71-1.60 (m, 2H), 1.51-1.39 (m, 2H), 1.25-1.06 (m, 6H), 0.79 (t, 3H). MS(ESI): $[M+H]^+$ calcd 453.2; found 453.6.

*4.1.8. General procedure for the synthesis of **1a–c**, **2a–d***

A solution of an appropriate benzonitrile compound **10a–c** or **12a–d** (0.26 mmol) in 10 mL DMF was added sodium azide (102 mg, 1.57 mmol) and chlorotributylstannane (0.4 mL, 1.57 mmol). The solution was refluxed for 10 h under nitrogen. Then the resulting mixture was poured into 10 mL ice water and the pH was adjusted to 5 by addition of 1 M hydrochloric acid solution. The mixture was extracted with DCM (30 mL × 3) and washed with saturated salt water (50 mL × 5). The organic phase was gathered and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was purified by CC to give white solid.

*4.1.8.1. 3-((1-(2-(1H-tetrazol-5-yl)phenyl)-1H-indol-4-yl)methyl)-2-propyl-1,3-diazaspiro[4.4]non-1-en-4-one (**1a**).* **1a** was synthesized according to the above general procedure. Yield: 64.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.98-7.92 (m, 1H), 7.83-7.78 (m, 1H), 7.72 (t, J = 7.3 Hz, 1H), 7.66 (d, J = 7.8 Hz, 1H), 7.33 (d, J = 3.3 Hz, 1H), 7.01 (t, J = 7.8 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 6.73 (d, J = 7.2 Hz, 1H), 6.66 (d, J = 3.3 Hz, 1H), 4.94 (s, 2H), 2.27 (t, J = 7.4 Hz, 2H), 1.93-1.80 (m, 6H), 1.76-1.63 (m, 2H), 1.54-1.40 (m, 2H), 0.80 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 185.97, 161.86, 154.69, 137.72, 136.76, 132.61, 131.61, 130.31, 129.15, 128.99, 128.96, 126.75, 123.28, 122.52, 117.99, 109.75, 101.57, 76.31, 41.48, 37.34, 37.34, 30.19, 25.94, 25.94, 18.40, 13.84. HRMS (ESI): m/z calculated for $C_{26}H_{27}N_7O$ $[M+H]^+$: 454.2355; found 454.2342.

*4.1.8.2. 2-Butyl-3-((1-(2-(1H-tetrazol-5-yl)phenyl)-1H-indol-4-yl) methyl)-1,3-diazaspiro[4.4]non-1-en-4-one (**1b**).* **1b** was synthesized according to the above general procedure. Yield: 62.1%. ^{1} H NMR (400 MHz, DMSO-d_6, ppm) δ 7.94 (d, J = 7.5 Hz, 1H), 7.77 (t, J = 7.4 Hz, 1H), 7.69 (t, J = 7.5 Hz, 1H), 7.62 (d, J = 7.8 Hz, 1H), 7.31 (d, J = 3.2 Hz, 1H), 7.00 (t, J = 7.7 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 6.73 (d, J = 7.2 Hz, 1H), 6.64 (d, J = 3.1 Hz, 1H), 4.94 (s, 2H), 2.29 (t, J = 7.5 Hz, 2H), 1.94-1.78 (m, 6H), 1.74-1.61 (m, 2H), 1.47-1.37 (m, 2H), 1.28-1.16 (m, 2H), 0.75 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 186.02, 161.98, 155.12, 137.61, 136.81, 132.20, 131.59, 130.40, 129.06, 128.94,

128.89, 126.78, 124.06, 122.43, 117.99, 109.82, 101.41, 76.34, 41.49, 37.32, 37.32, 28.06, 27.04, 25.94, 25.94, 21.98, 14.04. HRMS (ESI): *m/z* calculated for $C_{27}H_{29}N_7O$ $[M+H]^+$: 468.2512; found 468.2505.

*4.1.8.3. 3-((1-(2-(1H-tetrazol-5-yl)phenyl)-1H-indol-4-yl)methyl)-2-pentyl-1,3-diazaspiro[4.4]non-1-en-4-one (**1c**).* **1c** was synthesized according to the above general procedure. Yield: 62.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.95 (d, J = 6.7 Hz, 1H), 7.78 (t, J = 7.1 Hz, 1H), 7.70 (t, J = 7.4 Hz, 1H), 7.62 (d, J = 7.8 Hz, 1H), 7.32 (d, J = 3.3 Hz, 1H), 7.05-6.97 (m, 1H), 6.87 (d, J = 8.3 Hz, 1H), 6.73 (d, J = 7.2 Hz, 1H), 6.64 (d, J = 3.2 Hz, 1H), 4.94 (s, 2H), 2.29 (t, J = 7.6 Hz, 2H), 1.92-1.80 (m, 6H), 1.74-1.62 (m, 2H), 1.49-1.38 (m, 2H), 1.20-1.10 (m, 4H), 0.77 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 186.04, 162.01, 155.00, 137.65, 136.80, 132.34, 131.62, 130.39, 129.08, 128.97, 128.94, 126.79, 123.81, 122.46, 118.03, 109.80, 101.48, 76.34, 41.49, 37.32, 37.32, 31.05, 28.32, 25.95, 25.95, 24.62, 22.15, 14.26. HRMS (ESI): *m/z* calculated for $C_{28}H_{31}N_7O$ $[M+H]^+$: 482.2668; found 482.2659.

*4.1.8.4. 3-((1-(2-(1H-tetrazol-5-yl)phenyl)-1H-indol-5-yl)methyl)-2-propyl-1,3-diazaspiro[4.4]non-1-en-4-one (**2a**).* **2a** was synthesized according to the above general procedure. Yield: 67.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 7.95 (d, J = 7.6 Hz, 1H), 7.77 (t, J = 7.5 Hz, 1H), 7.69 (t, J = 7.5 Hz, 1H), 7.62 (d, J = 7.8 Hz, 1H), 7.32 (d, J = 3.3 Hz, 1H), 7.01 (t, J = 7.7 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 6.72 (d, J = 7.2 Hz, 1H), 6.64 (d, J = 3.2 Hz, 1H), 4.94 (s, 2H), 2.28 (t, J = 7.3 Hz, 2H), 1.95-1.78 (m, 6H), 1.75-1.61 (m, 2H), 1.53-1.44 (m, 2H), 0.81 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 186.02, 161.78, 155.22, 137.57, 136.79, 132.08, 131.58, 130.42, 129.06, 128.90, 128.87, 126.73, 124.30, 122.41, 117.86, 109.83, 101.35, 76.34, 41.48, 37.34, 37.34, 30.19, 25.94, 25.94, 18.40, 13.86. HRMS (ESI): *m/z* calculated for $C_{26}H_{27}N_7O$ $[M+H]^+$: 454.2355; found 454.2345.

*4.1.8.5. 2-Butyl-3-((1-(2-(1H-tetrazol-5-yl)phenyl)-1H-indol-5-yl)methyl)-1,3-diazaspiro[4.4]non-1-en-4-one (**2b**).* **2b** was synthesized according to the above general procedure. Yield: 65.7%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.93 (d, J = 6.3 Hz, 1H), 7.76-7.55 (m, 3H), 7.35 (s, 1H), 7.25 (s, 1H), 7.00-6.79 (m, 2H), 6.55 (s, 1H), 4.71 (s, 2H), 2.32 (t, 2H), 1.95-1.68 (m, 6H), 1.73-1.56 (m, 2H), 1.55-1.39 (m, 2H), 1.30-1.22 (m, 2H), 0.79 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 186.13, 161.82, 155.56, 137.52, 136.07, 131.72, 131.58, 130.89, 129.00, 129.00, 128.86, 128.65, 124.84, 121.21, 118.95, 110.72, 103.38, 76.27, 43.30, 37.25, 37.25, 28.08, 27.08, 25.93, 25.93, 22.04, 14.11. HRMS (ESI): *m/z* calculated for $C_{27}H_{29}N_7O$ $[M+H]^+$: 468.2512; found 468.2500.

*4.1.8.6. 3-((1-(2-(1H-tetrazol-5-yl)phenyl)-1H-indol-5-yl)methyl)-2-pentyl-1,3-diazaspiro[4.4]non-1-en-4-one (**2c**).* **2c** was synthesized according to the above general procedure. Yield: 68.1%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 7.93 (d, J = 7.5 Hz, 1H), 7.77 (t, J = 7.1 Hz, 1H), 7.68 (t, J = 7.3 Hz, 1H), 7.61 (d, J = 7.7 Hz, 1H), 7.35 (s, 1H), 7.26 (d, J = 3.1 Hz, 1H), 7.03-6.78 (m, 2H), 6.57 (s, 1H), 4.71 (s, 2H), 2.32 (t, J = 7.6 Hz, 2H), 1.92-1.75 (m, 6H), 1.70-1.61 (m, 2H), 1.51-1.42 (m, 2H), 1.23-1.13 (m, 4H), 0.78 (t, J = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 186.10, 161.98, 155.56, 137.74, 136.02, 132.42, 131.68, 130.75, 129.12, 129.02, 128.90, 128.80, 128.77, 121.35, 119.02, 110.67, 103.67, 76.24, 43.30, 37.24, 37.24, 31.11, 28.33, 25.93, 25.93, 24.68, 22.22, 14.27. HRMS (ESI): *m/z* calculated for $C_{28}H_{31}N_7O$ $[M+H]^+$: 482.2668; found 482.2671.

*4.1.8.7. 2-Hexyl-3-((1-(2-(1H-tetrazol-5-yl)phenyl)-1H-indol-5-yl)methyl)-1,3-diazaspiro[4.4]non-1-en-4-one (**2d**).* **2d** was synthesized according to the above general procedure. Yield: 61.1%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 7.93 (d, J = 7.4 Hz, 1H), 7.83-7.57 (m, 3H), 7.35 (s, 1H), 7.26 (d, J = 3.1 Hz, 1H), 7.04-6.78 (m, 2H), 6.57 (s, 1H), 4.71 (s, 2H), 2.32 (t, J = 7.5 Hz, 2H), 1.94-1.74 (m, 6H), 1.71-1.60 (m, 2H), 1.51-1.39 (m, 2H), 1.25-1.06 (m, 6H), 0.79 (t, J = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 186.10, 161.91, 155.61, 137.73, 136.02, 132.42, 131.66, 130.73, 129.12, 129.01, 128.90, 128.79, 128.77, 121.33, 119.02, 110.66, 103.65, 76.25, 43.29, 37.24, 37.24, 31.28, 28.46, 28.33, 25.92, 25.92, 24.91, 22.35, 14.30. HRMS (ESI): *m/z* calculated for $C_{29}H_{33}N_7O$ $[M+H]^+$: 496.2825; found 496.2815.

4.2. Biological evaluation

4.2.1. Radioligand binding assay

Primary vascular smooth muscle cells (VSMCs) were obtained from thoracic aorta of SPF SD rats (Slac laboratory animal, Shanghai, China) and cultured by the tissue explants methods. The inverted phase contrast microscope was used to observe cell growth, immunohistochemical staining was used to identify cells, and trypan blue staining was used to detect cell viability [15,16]. At first, thoracic aorta was cut into tissues at the size of 1 mm × 1 mm after processing, and then transferred to cell culture flasks. DMEM with 10% fetal bovine serum and 1% mixture of penicillin and streptomycin were appropriately added when the tissues adhered to the bottom surface uniformly. Culture flask was inverted and incubated in 37 °C, 5% CO_2 for 3–5 h. Tissues were immersed into the culture medium completely when attaching on the bottom. The cells were extended when growing to covering the entire sidewall. All the cells were incubated in 37 °C, 5% CO_2. And Morphological changes was observed using an inverted phase contrast microscope and identified by immunohistochemical methods with stained smooth muscle-α-actin.

3–6 generations of VSMCs were used for experiments. The new compounds and irbesartan (Shanghai Zhongkang Weiye Biological Technology Co., Ltd. Shanghai, China) were dissolved in DMSO and diluted to different concentrations (10^{-10} – 10^{-4} M) with PBS before experiments. ^{125}I-Ang II (Zhongshan Hospital, Fudan University, China) was dissolved with PBS and diluted to 0.2 nM. VSMCs (10^6 cells/well, 500 μL) were seeded into 24-well plates in 37 °C, 5% CO_2. After the cells adhered to the wall, 500 μL ^{125}I-Ang II and 10 μL compound **1a**–**c**, **2a**–**d** were added respectively to the final concentrations of 10^{-12} – 10^{-6} M and then cells were cultivated in 4 °C for 150 min [17]. After that, cells were washed 3 times with PBS and digested for 10 min with 0.1 M NaOH. The cells bound by ^{125}I-Ang II were counted by γ-counter (SN-682, Ri Huan Company, Shanghai). The half inhibition constant (IC_{50} value) of the combination of these compounds with membrane protein were estimated by the nonlinear portion of the competition curves. The K_i value was calculated from the formula $K_i = IC_{50}/(1 + [L]/k_d)$, [L] was the concentration of radioligand present in tubes [18].

4.2.2. In vivo study of anti-hypertensive effect

Spontaneous hypertensive rats (SHRs) (250 ± 20 g, Beijing Vital River Laboratory Animal Co., Ltd. Beijing, China) and renal hypertensive rats (RHRs) (250 ± 20 g, Department of Pharmaceutical Science and Technology, Donghua University, Shanghai, China) were used to evaluate the effects on systolic blood pressure (SBP) and diastolic blood pressure (DBP) of new compounds. 54 male SHRs were divided into nine experimental groups randomly: negative control group, positive control group, compound **1a**, **1b**, **1c**, **2a**, **2b**, **2c** and **2d** groups. Each compound was suspended in a 0.5% solution of sodium carboxymethyl cellulose and administered orally at the dose of 15 mg/kg. Irbesartan (15 mg/kg) was taken as positive control group. The negative control group was administered the same volume of sodium carboxymethyl cellulose solution. Another 6 male SHRs was used to test the antihypertensive effect of compound **2b** at 10 mg/kg. Besides, 24 male RHRs were divided into four groups randomly: negative control group, positive control

group and compound **2b** (15 mg/kg and 10 mg/kg) groups. Then blood pressure and heart rates were monitored before and after the administration by a biological signal analysis system (MPA - 2000, Alcott Biotech, China). Six determinations were made in every session of blood pressure measurements and the means of the six values were taken as the systolic blood pressure level and diastolic blood pressure level, respectively. The mean arterial pressure (MBP) was calculated by the formula: MBP = (SBP − DBP)/3 + DBP, and all the results were expressed as mean ± SD. A probability level of less than 0.05 was considered significant.

4.2.3. Pharmacokinetic characteristics of compound 2b

High performance liquid chromatography (HPLC) method was used to analyze the drug concentration of compound **2b** in plasma and in tissues. The method was developed, validated and operated on two separate but similar Waters-brand systems. The flow rate was 1.0 mL/min and the injection volume was 20 μL. Separations were performed at ambient temperature on a reverse phase Wondasilicon C18-WR column (4.6 × 150 mm, 5 μm). For the analysis of drug concentration in plasma, Mobile Phases A was 0.02 mol/L potassium dihydrogen phosphate solution (55%) and Mobile Phases B was acetonitrile solution (45%). For the analysis of drug concentration in tissues, Mobile Phases A was acetonitrile (60%) and Mobile Phases B was pure water (40%). Microsoft Excel program, Origin 8.0 and DAS 2.0 software were used to calculate the pharmacokinetic parameters.

18 male Wistar rats (180 ± 20 g, Shanghai Slac Laboratory Animal Company, Shanghai, China) were randomly divided into three groups and administrated with **2b** at dose of 10 mg/kg, 5 mg/kg and 2 mg/kg, respectively. Blood samples were collected from each rat by retro-orbital puncture at a predetermined time interval of predose, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h into the polypropylene microfuge tubes containing heparin. Plasma was separated by centrifuging the blood samples at 4000 × g at 4 °C for 10 min. Then 200 μL plasma samples for analysis were placed into a tube followed by 100 μL of internal standard. Acetonitrile (200 μL) was added to precipitate proteins and the tube was vortexed for 1 min. Precipitated proteins were separated by centrifugation for 5 min at 12,000 × g at 4 °C. The supernatant was filtered by needle filter of 0.45 μm and the resulting 20 μL filtrate was taken to analyze in HPLC. Linearity for **2b** was tested by extracting plasma standards spiked at nominal concentrations of 0.1, 0.5, 1, 5, 10 μg/mL. The calibration line was generated by least squares linear regression of the peak height ratio (PHR) of analyte/internal standard against nominal concentration with a weighting of concentration^{-2} [19,20].

198 Wistar rats (180 ± 20 g, Shanghai Slac Laboratory Animal Company, Shanghai, China) were used to investigate the tissue distribution of compound **2b**. Animals were orally administrated with **2b** at 10 mg/kg, 15 mg/kg and 30 mg/kg respectively. Heart, liver, spleen, lung, kidney and brain samples were collected at 0 (prior to dosing), 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h post-dosing (n = 6). 0.25 g samples from different tissues were obtained after being rinsed with normal saline solution and blotted with filter paper. The samples were grinded into tissue fluid (0.25 g/1 mL normal saline solution) with High-speed Homogenizer (PRO200, Bio-gen series, USA). The tissue fluid was centrifuged at 10,000 × g for 10 min. 500 mL supernate was collected and added with 2 mL methanol, and then it was vortexed for 60 s and centrifuged at 12,000 × g for 5 min. Supernatant was obtained and dried in gentle stream of nitrogen flow at 50 °C. The residue was spun for 2 min after being reconstituted with 100 μL methanol to get supernatant sample. 20 μL supernatant samples were taken to analyze through HPLC [21,22].

4.2.4. Acute toxicity of compound 2b

To detect the safety of compound **2b**, the acute toxicity assay was carried out with 50 normal ICRs (20 ± 2 g, Academia Sinica, China). The LD_{50} value was delivered via intragastric administration (ig) at doses of 2929.69, 2343.75, 1875, 1500, 1200 mg/kg. Survival was assessed daily for two weeks. The lethal dose (LD_{50}) and 95% confidence limits were determined from logistic regression analysis (GLM) curve fitting of the 14 days' mortality data. Survival rats were observed continuously and recorded systematically for the physical signs of toxicity including weight, mobility, aggressiveness and respiratory movements. At the 15th day, the survivals were dissected to examine the pathological changes of organs [23].

4.2.5. Statistics

Results were expressed as means ± standard error of the means. Data were analyzed by one-way analysis of variance. When overall statistical significance was achieved ($P < 0.05$), anova one way and *t*-test statistical were used to compare each of the doses to the vehicle control. Probability values less than 0.05 were considered to be significant. Binding isotherms from competition studies were obtained using the nonlinear regression program GraphPad Prism 5 software (Network of Science Software of China) and pharmacokinetic parameters were acquired by DAS.2.0 software. And the completed animal research here adhered to the "Principles of Laboratory Animal Care" and was approved by IACUC.

Notes

The authors declare no competing financial interest.

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Abbreviations

RAS	renin angiotensin system
AII	angiotensin II
ARBs	angiotensin II receptor blockers
ACE	angiotensin converting enzyme
SHR	spontaneously hypertensive rat
RHR	renal hypertensive rats
ICR	Institute for Cancer Research
VSMCs	Vascular Smooth Muscle Cells
MBP	mean blood pressure
BBB	blood-brain barrier
LD_{50}	median lethal dose
IC_{50}	half maximal inhibitory concentration
C_{max}	maximum concentration
$AUC_{0\sim72}$	area under the concentration-time curve
$T_{1/2}$	elimination half-lives
DCM	methylene chloride
DMF	N,N-dimethylformamide
NMR	nuclear magnetic resonance
MP	melting points
TMS	Me_4Si
CC	column chromatography
DCM	dichloromethane
ESI	electrospray ionization
MS	mass spectrometry
DMSO	dimethylsulfoxide

THF	tetrahydrofuran
EA	ethyl acetate
HRMS	high-resolution mass spectrometry
DMAP	4-(dimethylamino)pyridine
AIBN	azodiisobutyronitrile
NBS	bromosuccinimide
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
HPLC	High Performance Liquid Chromatography.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.07.023.

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