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PAPER

Studies on the enantiomers of ZJM-289: synthesis and biological evaluation of antiplatelet, antithrombotic and neuroprotective activities†

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ZJM-289 is a potent racemic agent which inhibits both platelet aggregation and thrombosis superior to a known anti-ischemic stroke drug 3-*n*-butylphthalide (NBP). Herein, the enantiomers of ZJM-289, (*S*)-ZJM-289 and (*R*)-ZJM-289, were synthesized and evaluated for their biological activities. It was observed that the two enantiomers appeared to be almost as effective as ZJM-289 in inhibiting platelet aggregation *in vitro* and thrombus formation *in vivo*. Moreover, like ZJM-289, its enantiomers could regulate the ratio of thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$, and enhanced levels of nitric oxide (NO), cAMP and cGMP, suggesting that the anti-platelet and antithrombotic activities of the enantiomers and ZJM-289 are associated with both the arachidonic acid cascade and cGMP–NO signal pathway. Furthermore, it was found that oral administration of the enantiomers and ZJM-289 for three days significantly reduced the infarct size, brain water content and neurological deficit in rats after cerebral ischemia reperfusion. Importantly, the two enantiomers equally improved blood flow in the ischemic stroke model and modulated endothelial function through releasing moderate levels of NO, which might, at least partially, contribute to their neuroprotection. Collectively, the present study demonstrates that the two enantiomers are as potent as ZJM-289 in inhibition of platelet aggregation and thrombosis and in neuroprotection, and (*S*)-ZJM-289 shows somewhat better effects than (*R*)-ZJM-289 and ZJM-289 in a few cases. These findings may provide new insights into the development of therapeutic agents like ZJM-289 for the intervention of thrombosis-related ischemic stroke.

1. Introduction

Thromboembolic events are the major cause of morbidity and mortality worldwide.[1] Cerebral thrombosis involves the formation of a blood clot or thrombus inside a cerebral artery that restricts or blocks the flow of blood and can result in acute ischemic stroke, which accounts for 80% of all forms of stroke patients.[2] It has been generally accepted that the activation of platelets and the resultant aggregation play a major role in the pathogenesis of intravascular thrombosis.[3] Furthermore, platelet activation contributes significantly to ischemic microvascular occlusion and causes secondary injury after the initial ischemic insult.[4] Thus, platelet adhesion and aggregation have been identified as important targets for the development of antithrombotic drugs in the treatment of ischemic stroke.

Racemic 3-*n*-butylphthalide (NBP) is an anti-cerebral ischemic drug which was received by the State Food and Drug Administration (SFDA) of China in 2002.[5] A large variety of studies have demonstrated that NBP possesses multiple actions beneficial for ischemic stroke patients, such as improvement of brain microcirculation, inhibition of platelet aggregation and thrombosis, regulation of energy metabolism, and reduction in the brain infarct volume.[6] However, the clinical application of NBP is limited due to its moderate potency, and it has been found that the therapeutic effects of NBP could be improved by co-administration with other antiplatelet drugs.[7] Therefore, new derivatives of NBP with strong potency may be of great significance for the treatment of ischemic stroke.

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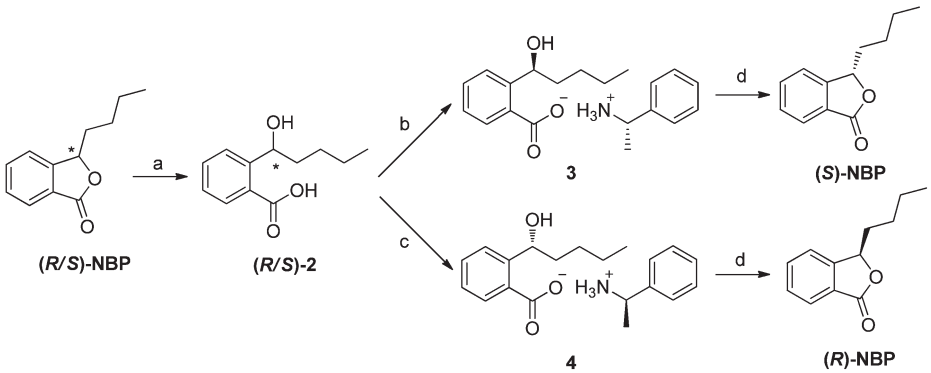


Scheme 1 Resolution of NBP. *Reagents and conditions*: (a) (i) 2 M NaOH, CH_3OH–H_2O, 50 °C, 0.5 h; (ii) 5% HCl, −10 to 0 °C; (b) (i) (*S*)-FEA, Et_2O, −20 to −10 °C; (ii) acetone, recrystallization repeated 2 times; (c) (i) (*R*)-FEA, Et_2O, −20 to −10 °C; (ii) acetone, recrystallization repeated 2 times; (d) (i) 2 M NaOH, r.t., 0.5 h; (ii) 3 M HCl, r.t., 0.5 h.

Recently, we have synthesized a series of nitric oxide (NO) releasing ring-opening derivatives of NBP that possessed marked activities of antiplatelet aggregation and antithrombosis both *in vitro* and *in vivo*. Among all the tested compounds, (*E*)-2-{[1-(2-diethylamino)acetoxy]pentyl}benzoic acid-{2-methoxy-4-[2-(4-nitrooxybutoxycarbonyl)vinyl]}phenyl ester hydrochloride (ZJM-289) showed the strongest inhibitory activity on the ADP- and TH-induced platelet aggregation *in vitro*, superior to NBP. In addition, ZJM-289 could reduce infarct volume and brain-water content in ischemic brains and promote functional recovery after ischemia-reperfusion through inhibition of iNOS and stimulation of the NO–sGC–cGMP pathway.[8] Furthermore, preliminary pharmacokinetic study demonstrated the ester bonds of ZJM-289 could be cleaved *in vivo* by esterases to generate NBP, substituted ferulic acid and NO, all of which function synergetically to improve the efficacy of NBP.[9]

It is estimated that about half of all therapeutic agents are chiral, but most of these drugs are administered in the form of the racemic mixture, *i.e.* a 50 : 50 mixture of its enantiomers.[10] However, chirality is one of the main features of biology, and many of the processes essential for life are stereoselective, implying that two enantiomers may work differently from each other in a physiological environment. Several reviews of the importance and value of developing a single enantiomer rather than a racemate have been documented.[11,12] Hence, we would like to investigate the two enantiomers of ZJM-289, (*S*)-ZJM-289 and (*R*)-ZJM-289, for their biological and pharmacological behavior. In this paper, we report the synthesis of the two enantiomers of ZJM-289 and their antiplatelet and antithrombotic activities as well as neuroprotective effects.

2. Results

2.1. Chemistry

Synthesis of the enantiomers of ZJM-289 started from the chemical resolution of racemic NBP.[13] As depicted in Scheme 1, NBP was subjected to saponification and sequential acidification to pH 3–4 using dilute HCl solution at −10 to 0 °C to form acid **2**. Treatment of **2** with the optically active base (*S*)-α-methylbenzylamine ((*S*)-FEA, ee > 98%) at −20 to −10 °C yielded the diastereoisomeric salt **3**. Scission of **3** with NaOH and finally lactonization under acid conditions to give (*S*)-NBP ($[\alpha]_D^{27}$ −66.6 (*c* 1.0, CH_3Cl), ee > 99%). (*R*)-NBP ($[\alpha]_D^{27}$ +68.4 (*c* 1.0, CH_3Cl), ee > 98%) was similarly obtained with (*R*)-α-methylbenzylamine ((*R*)-FEA, ee > 98%).

The (*S*)- or (*R*)-NBP was subjected to alkali hydrolysis and subsequent acidification to form the acid (*S*)- or (*R*)-**2**. Treatment of (*S*)- or (*R*)-**2** with chloroacetyl chloride yielded the acylated (*S*)- or (*R*)-**5**, which was esterified with phenol compound **6** in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to offer ester (*S*)- or (*R*)-**7**. The ester (*S*)- or (*R*)-**7** was condensed with diethylamine, followed by treatment with anhydrous ethereal HCl to provide the corresponding hydrochloride (*S*)- or (*R*)-ZJM-289, as depicted in Scheme 2. The racemic ZJM-289 was synthesized starting from 2-formylbenzoic acid by a reported six-step-reaction sequence.[14] All of the new compounds were purified by column chromatography and characterized by IR, ESI-MS, ^{1}H NMR, ^{13}C NMR, and HRMS, and individual compounds with chemical purity of >99% and optical purity (ee) of >97% (determined by chiral HPLC analysis) were used for subsequent experiments.

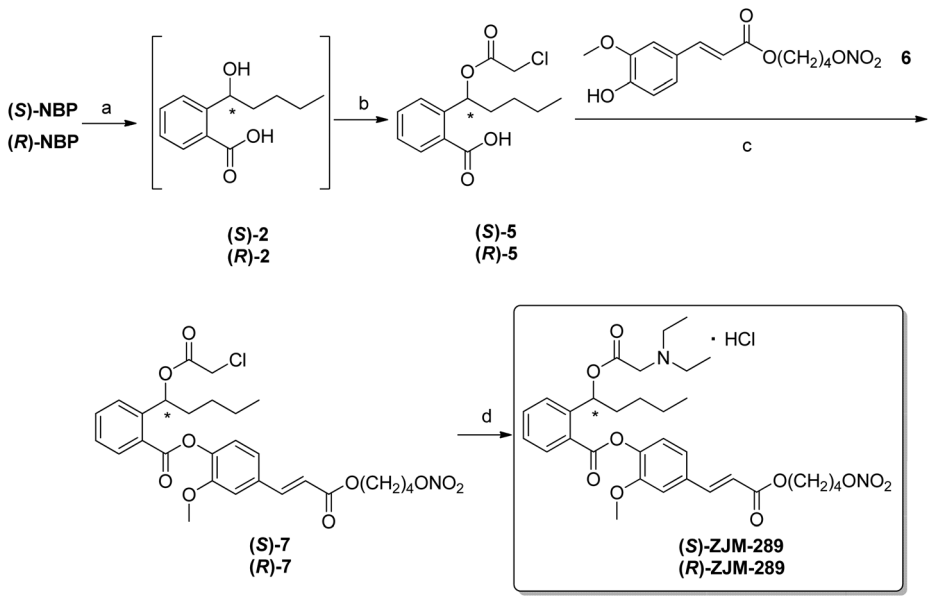
2.2. *In vitro* antiplatelet aggregation effects of (*S*)- and (*R*)-ZJM-289

The *in vitro* inhibitory activities of (*S*)- and (*R*)-ZJM-289 at concentrations ranging from 10 μM to 1.0 mM against adenosine diphosphate (ADP)-, thrombin (TH)- and arachidonic acid (AA)-induced platelet aggregation were evaluated using the Born's turbidimetric assay, and the IC_{50} values are listed in Table 1.

As shown in Table 1, the IC_{50} values of (*S*)- and (*R*)-ZJM-289 against ADP-, TH- and AA-induced platelet aggregation are 0.21, 0.44; 0.43, 0.42; and 0.09, 0.34 mM; respectively. The two enantiomers and ZJM-289 (0.24, 0.41, 0.16 mM) displayed almost comparable inhibitory effects on ADP- and TH-induced platelet aggregation while the IC_{50} value of (*S*)-ZJM-289 against AA-induced platelet aggregation was slightly lower than that of (*R*)-ZJM-289 or ZJM-289.

2.3. Antithrombotic activities of (*S*)- and (*R*)-ZJM-289 *in vivo*

We next investigated the effects of (*S*)- and (*R*)-ZJM-289 on the formation of thrombus in a rat extra-corporeal circulation of



Scheme 2 Synthesis of (*S*)- and (*R*)-ZJM-289. *Reagents and conditions*: (a) (i) 2 M NaOH, CH_3OH–H_2O, 50 °C, 0.5 h; (ii) 5% HCl, −10 to 0 °C; (b) $ClCH_2COCl$, Et_3N, DMAP, CH_2Cl_2, −10 to 0 °C, 5 h; (c) DCC, DMAP, CH_2Cl_2, r.t., 5 h; (d) (i) diethylamine, Et_3N, DMF, r.t., 8 h; (ii) HNO_3, Ac_2O, EtOAc, 0 °C, 12 h.

Table 1 The IC_{50} values of (*S*)- and (*R*)-ZJM-289 against rabbit platelet aggregation *in vitro*

	IC_{50} (mM)		
Compd.	ADP (10 μM)	TH (10 μM)	AA (1.0 mM)
NBP	>1.0[a]	>1.0[b]	0.10
ZJM-289	0.24	0.41	0.16
(*S*)-ZJM-289	0.21	0.43	0.09
(*R*)-ZJM-289	0.44	0.42	0.34

[a] A 51.3% inhibition of platelet aggregation was observed at maximal concentration of 1.0 mM. [b] A 31.3% inhibition of platelet aggregation was observed at maximal concentration of 1.0 mM.

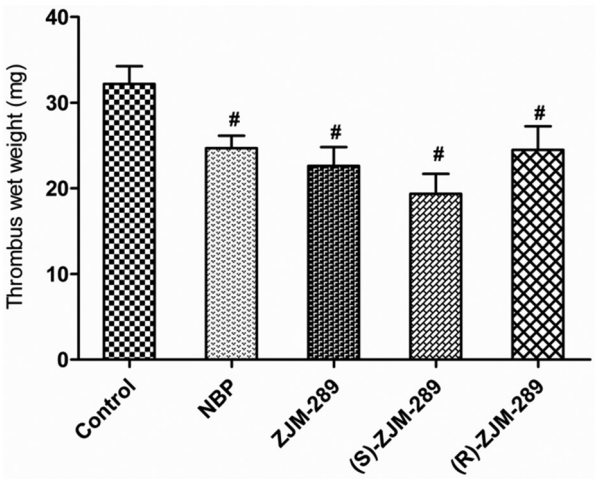


Fig. 1 Effects of (*S*)- and (*R*)-ZJM-289 on thrombus formation in rats. Male SD rats were randomized and treated orally with 0.1% CMC-Na, NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 for consecutive 5 days and the formation of thrombus in individual rats was induced. Subsequently, the thrombus weights were measured. Data are expressed as mean ± SD of individual groups of rats (n = 10) from two separate experiments. Control and experimental groups of rats were tested simultaneously. $^{\#}P < 0.05$ *vs.* control group, determined with one-way ANOVA followed by Tukey's *post hoc* test.

arteriovenous (A-V) cannula model. Male SD rats were randomized and treated orally with 0.1% CMC-Na (negative control), NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 at equimolar doses (2.63×10^{-4} mol kg^{-1}) for five days. The formation of thrombus in these rats was induced by a surgical procedure and the thrombus weights in individual rats were measured. As shown in Fig. 1, the thrombus wet weights in the rats receiving (*S*)-ZJM-289 (21.0 ± 8.8 mg), (*R*)-ZJM-289 (24.5 ± 7.8 mg) and ZJM-289 (22.6 ± 6.6 mg) were almost equal and less than that of the negative control group (32.2 ± 8.8 mg, $P < 0.05$), indicating that the two enantiomers and ZJM-289 have comparable antithrombotic activity in a rat model.

We further evaluated the antithrombotic effects of (*S*)- and (*R*)-ZJM-289 on a mouse model of thromboembolism. Male ICR mice were randomized and administered orally with vehicle (0.1% CMC-Na) alone, equimolar doses of NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289, respectively. Two hours after the treatment, the mice were injected intravenously with collagen and adrenaline to induce thromboembolism, and the development of hemiplegia and death in the rats were monitored within fifteen min. Apparently, treatment with the two enantiomers and ZJM-289 significantly reduced the onset of hemiplegia and death in mice. In comparison with (*S*)-ZJM-289 and ZJM-289, (*R*)-ZJM-289 displayed a relatively weak protective effect in this model (Table 2), but there was no significant difference between the three treatment groups.

Table 2 Effects of (*S*)- and (*R*)-ZJM-289 on death and hemiplegia induced by *i.v.* collagen–adrenaline in mice

Group	Dose (mg kg^{-1})	*n*	Number of death in 5 min and hemiplegia in 15 min	Protection rate (%)
Control	—	17	11	35.3
NBP	100	17	5	70.6[a]
ZJM-289	342	18	5	72.2[a]
(*S*)-ZJM-289	342	20	5	75.0[a]
(*R*)-ZJM-289	342	19	7	68.4[a]

[a] $P < 0.05$ *vs.* control group.

2.4. Neuroprotection of (*S*)- and (*R*)-ZJM-289 against focal cerebral ischemia/reperfusion (I/R)

The neuroprotection of (*S*)- and (*R*)-ZJM-289 against focal cerebral I/R was studied using a model of middle cerebral artery occlusion (MCAO). Male SD rats were randomized and treated orally with 0.1% CMC-Na (sham group and vehicle group), NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 at equimolar doses (2.63×10^{-4} mol kg^{-1}).

Since cerebral blood flow (CBF) has a close relation with brain injury, we examined whether preconditioning of the enantiomers were associated with an improvement of ischemic CBF. As shown in Fig. 2, MCAO caused more than 70% reduction in the regional cerebral blood flow (rCBF) compared with the pre-occlusion baseline values and this almost completely reversed by reperfusion in all groups. Compared with vehicle and NBP groups, the two enantiomers and ZJM-289 markedly increased rCBF during MCAO, and there was no significant difference between (*S*)-ZJM-289, (*R*)-ZJM-289 and ZJM-289 groups.

The neuroprotective effects of (*S*)- and (*R*)-ZJM-289 were also evaluated by examining neurological deficit, infarct volume and brain water content after three days of reperfusion. NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 were administered just after the onset of reperfusion and continued for three days in this study. Transient two hours of focal cerebral ischemia followed by reperfusion for three days induced brain infarct formation and neurological deficits. The two enantiomers and ZJM-289 appeared to be equally effective in reducing neurological deficits (Fig. 3a) and infarct volume (Fig. 3b) while there was a significant difference between (*S*)-ZJM-289 and NBP group ($P < 0.05$). As shown in Fig. 3c, I/R resulted in apparent brain edema (83.04 ± 1.26%, $P < 0.05$) compared with sham-operated group (78.82 ± 0.76%), which was also significantly improved in (*S*)-ZJM-289 (79.28 ± 0.95%), (*R*)-ZJM-289 (80.63 ± 1.08%) and ZJM-289 (80.29 ± 1.13%) groups. Compared with NBP group, (*S*)-ZJM-289 showed more intense decline in the brain water content.

To observe the effects of (*S*)- and (*R*)-ZJM-289 on cortical neuronal damage, HE staining was performed. As shown in Fig. 3d, in the sham group, the brain tissues were kept intact and no inflammatory cells infiltrated, cortical neurons kept eumorphism and arranged well, the cytoplasm was abundant and the nuclei were centered with clear staining. In contrast, in the vehicle group, cortical neurons were degenerated and necrotic, and their arrangement was disordered, and

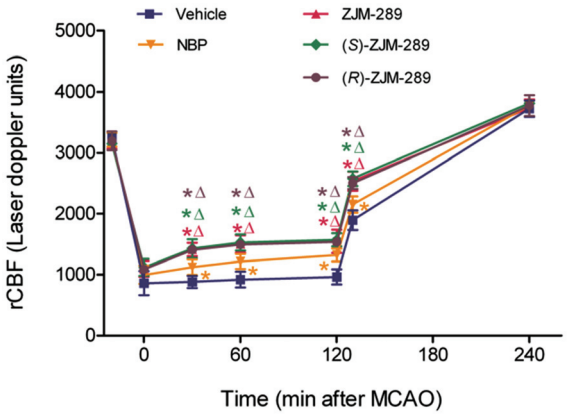


Fig. 2 Changes of cerebral blood flow in the ischemic cortex before, during and after MCAO measured by laser Doppler flowmetry. Male SD rats were randomized and treated orally with 0.1% CMC-Na, NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 2 h before MCAO. The animals were subjected to 2 h MCAO and 2 h reperfusion. Data are expressed as mean ± SD ($n = 6$). *$P < 0.05$ *vs.* vehicle group, △$P < 0.05$ *vs.* NBP group, determined with one-way ANOVA followed by Tukey's *post hoc* test.

accompanied with decreased density. However, treatment with (*S*)- and (*R*)-ZJM-289, as well as ZJM-289 obviously increased the amount of normal neurons, and significantly diminished the extent of damage, respectively.

2.5. Vascular modulation as another evidence of the neuroprotective effect of the enantiomers of ZJM-289

At three days after reperfusion, NO production in plasma was evaluated indirectly by measuring nitrite/nitrate (NO_x), and levels of ET-1, TXB_2, 6-keto-$PGF_{1\alpha}$, cGMP and cAMP in plasma were quantified by radioimmunoassay or enzyme-linked immunosorbent assay. In the present study, levels of NO_x were decreased significantly in the vehicle group compared to the sham group (Fig. 4a), which was in agreement with an earlier report where the plasma NO_x level was reduced in acute stroke.[15] Like ZJM-289, its enantiomers distinctly increased plasma NO_x concentration compared to vehicle and NBP groups. As a powerful biological marker of developing brain edema,[16] the plasma level of ET-1 was two-folder higher in the vehicle group (82.49 ± 20.66) as compared with sham group (39.99 ± 18.48). Treatment with (*S*)- and (*R*)-ZJM-289, significantly reduced plasma ET-1 level to 43.05 ± 15.06, 47.91 ± 11.14 and 47.34 ± 6.96 pmol L^{-1}, respectively (Fig. 4b), similar to ZJM-289. By contrast, NBP had no obvious effect on plasma NO_x and ET-1 levels. Furthermore, the ratio of TXB_2 : 6-keto-$PGF_{1\alpha}$ was increased significantly after focal cerebra I/R, and cAMP was decreased to a very low level, which might contribute to I/R injury. As shown in Fig. 4c and 4d, the two enantiomers and ZJM-289 could restore the dynamic balance between TXB_2 and 6-keto-$PGF_{1\alpha}$ and elevate plasma cAMP level to the baseline level. NBP decreased the ratio of TXB2 : 6-keto-$PGF_{1\alpha}$ and failed to improve the plasma cAMP level. Meanwhile, the enantiomers-, ZJM-289- and NBP-treated rats elevated the plasma cGMP level relative to the sham-operated rats, but without significant difference in these groups.

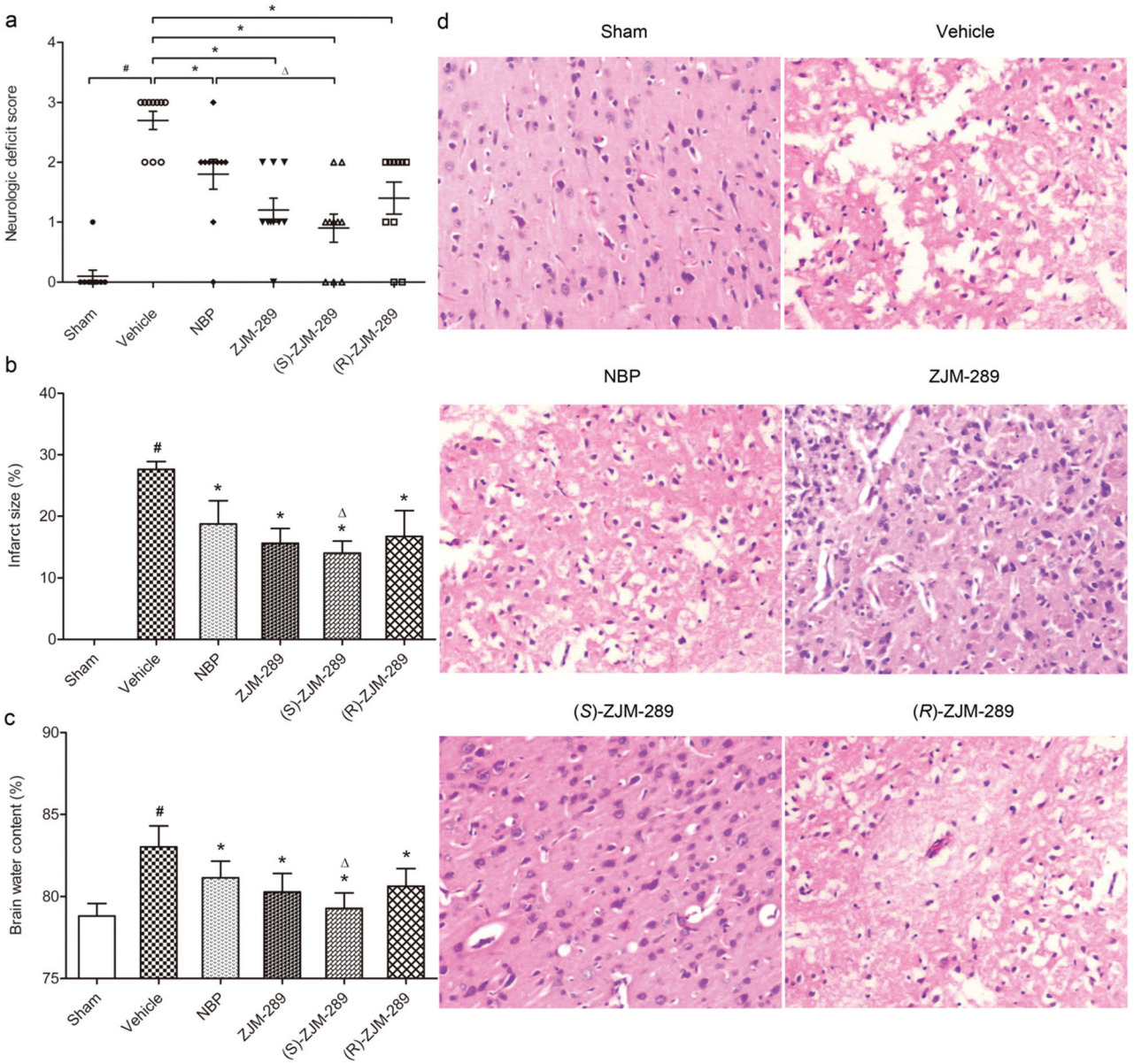


Fig. 3 Neuroprotective effects of (*S*)- and (*R*)-ZJM-289 following focal cerebral ischemia and reperfusion. Male SD rats received 2 h MCAO followed by 3 days reperfusion and treated orally with 0.1% CMC-Na, NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 just after the onset of reperfusion and continued for 3 days. Sham-operated rats were subjected to the same surgery, except MCAO, and treated orally with 0.1% CMC-Na. Data are expressed as mean ± SD (n = 10). $^{\#}P < 0.05$ *vs.* sham group, $^{*}P < 0.05$ *vs.* vehicle group, $\triangle P < 0.05$ *vs.* NBP group, determined with one-way ANOVA followed by Tukey's *post hoc* test. (a) Neurologic deficits were assessed at 3 days after reperfusion according to Longa's method. (b) Effects of (*S*)- and (*R*)-ZJM-289 on the brain infarct size. Animals were killed at 3 days after reperfusion and infarcted brain regions were visualized using TTC staining. (c) Effects of (*S*)- and (*R*)-ZJM-289 on the brain water content. After 3 days of reperfusion, the brain samples were weighed immediately after dissection (wet weight) and then dried at 105 °C for 24 h. (d) Cerebral sections were stained with hematoxylin and eosin and examined under light microscope at 3 days after reperfusion.

2.6. The enantiomers of ZJM-289 protected rat brain capillary endothelial cells (BCECs) against ischemia-like injury

BCECs are a primary target of ischemic brain insults and cerebral endothelial dysfunctions may contribute to postischemic secondary brain injury.[17] In this study, we investigated the protective effects of (*S*)- and (*R*)-ZJM-289 in rat BCECs. The BCECs in cultures were exposed to oxygen–glucose deprivation followed by reoxygenation (OGD/R), a model of ischemia-like injury, and treated with ZJM-289, (*S*)- and (*R*)-ZJM-289 for two hours before and during OGD/R. BCECs mortality was assessed by MTT and LDH assay (by measuring lactate dehydrogenase activity in the medium), respectively. For detection of cell apoptosis, cells were labeled with FITC-conjugated Annexin V (Annexin V-FITC) and propidium iodide (PI). The fluorescence was examined using a flow cytometry.

As shown in Fig. 5a, the viability of the BCECs exposed to OGD/R was reduced to 52.6 ± 5.6% compared with the

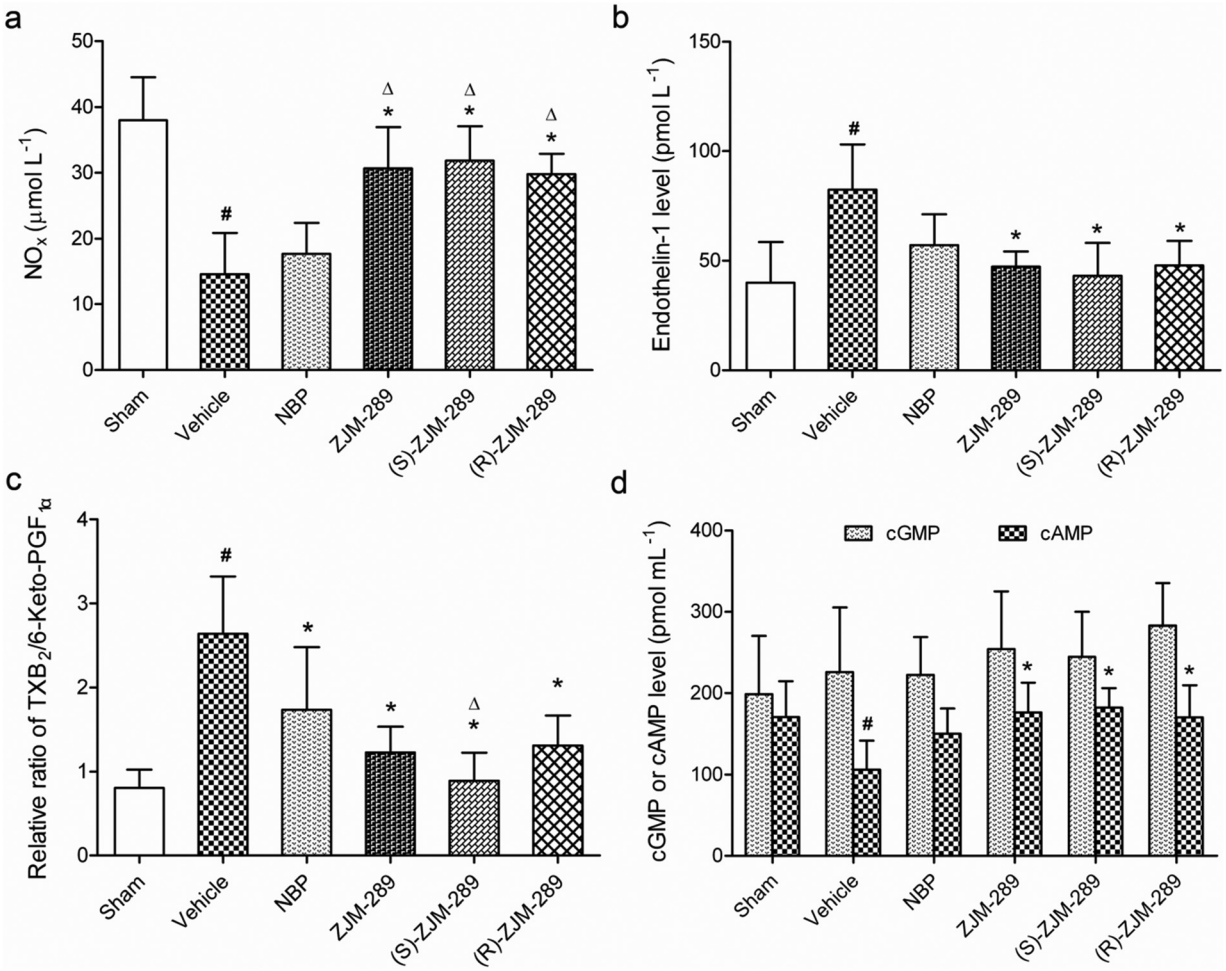


Fig. 4 Effects of (*S*)- and (*R*)-ZJM-289 on NO_x concentration (a), ET-1 level (b), the ratio of TXB_2 : 6-keto-$PGF_{1\alpha}$ (c) and cAMP : cGMP level (d) in plasma. Male SD rats received 2 h MCAO followed by 3 days reperfusion and treated orally with 0.1% CMC-Na, NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 just after the onset of reperfusion and continued for 3 days. Sham-operated rats were subjected to the same surgery, except MCAO, and treated orally with 0.1% CMC-Na. Data are expressed as mean ± SD (n = 10). $^{\#}P < 0.05$ *vs.* sham group, $^{*}P < 0.05$ *vs.* vehicle group, $^{\triangle}P < 0.05$ *vs.* NBP group, determined with one-way ANOVA followed by Tukey's *post hoc* test.

control. Pretreatment with (*S*)- and (*R*)-ZJM-289, as well as ZJM-289, significantly attenuated OGD/R-induced cell death and the cell viability was significantly increased to 81.2 ± 3.7, 70.4 ± 5.5 and 74.0 ± 4.1%, respectively. The cytoprotective effects of (*S*)- and (*R*)-ZJM-289 were also confirmed by LDH assay (Fig. 5b). Negative annexin V-FITC and PI labeling (Fig. 5c) showed a near absence of apoptosis and necrosis in control BCECs. The cultures subjected to OGD/R contained apoptotic (37.03 ± 2.07%) and necrotic (12.45 ± 1.49%) cells significantly ($P < 0.05$) more than the control cultures. The enantiomers equally and significantly decreased apoptotic and necrotic cells as ZJM-289, and the protective effect of (*S*)-ZJM-289 was stronger than that of NBP.

3. Discussion and conclusions

It is well-known that platelet activation contributes significantly to ischemic microvascular occlusion and causes secondary injury after the initial ischemic insult. Thus, inhibition of platelet aggregation in the cerebral microvessels is of great importance. We have previously reported ZJM-289 as a potent antiplatelet aggregation and antithrombotic agent. Since it is a racemic compound, we further synthesized the enantiomers of the ZJM-289, and simultaneously evaluated their antiplatelet, antithrombotic, and neuroprotective effects in the present study.

Compared with NBP, the two enantiomers and ZJM-289 displayed much stronger inhibitory activity on the ADP- and TH-induced platelet aggregation, but comparable effect on the AA-induced platelet activation. Additionally, the two enantiomers and ZJM-289 appeared to be almost equally effective in suppressing thrombus formation in the rat arteriovenous-shunt thrombosis model and in the mouse pulmonary embolization model, while (*R*)-ZJM-289 was somewhat less active.

Previous studies concluded that the ratio of TXA_2 : PGI_2 modulated vasomotion and thrombosis, which might contribute to the pathogenic consequences of ischemic stroke.[18] Therefore we examined the contents of TXB_2 and 6-keto-$PGF_{1\alpha}$, the stable metabolites of TXA_2 and PGI_2. Our results showed that the enantiomers of ZJM-289 significantly decreased the ratio of TXA_2 : PGI_2 in rats after I/R, which was in agreement with their

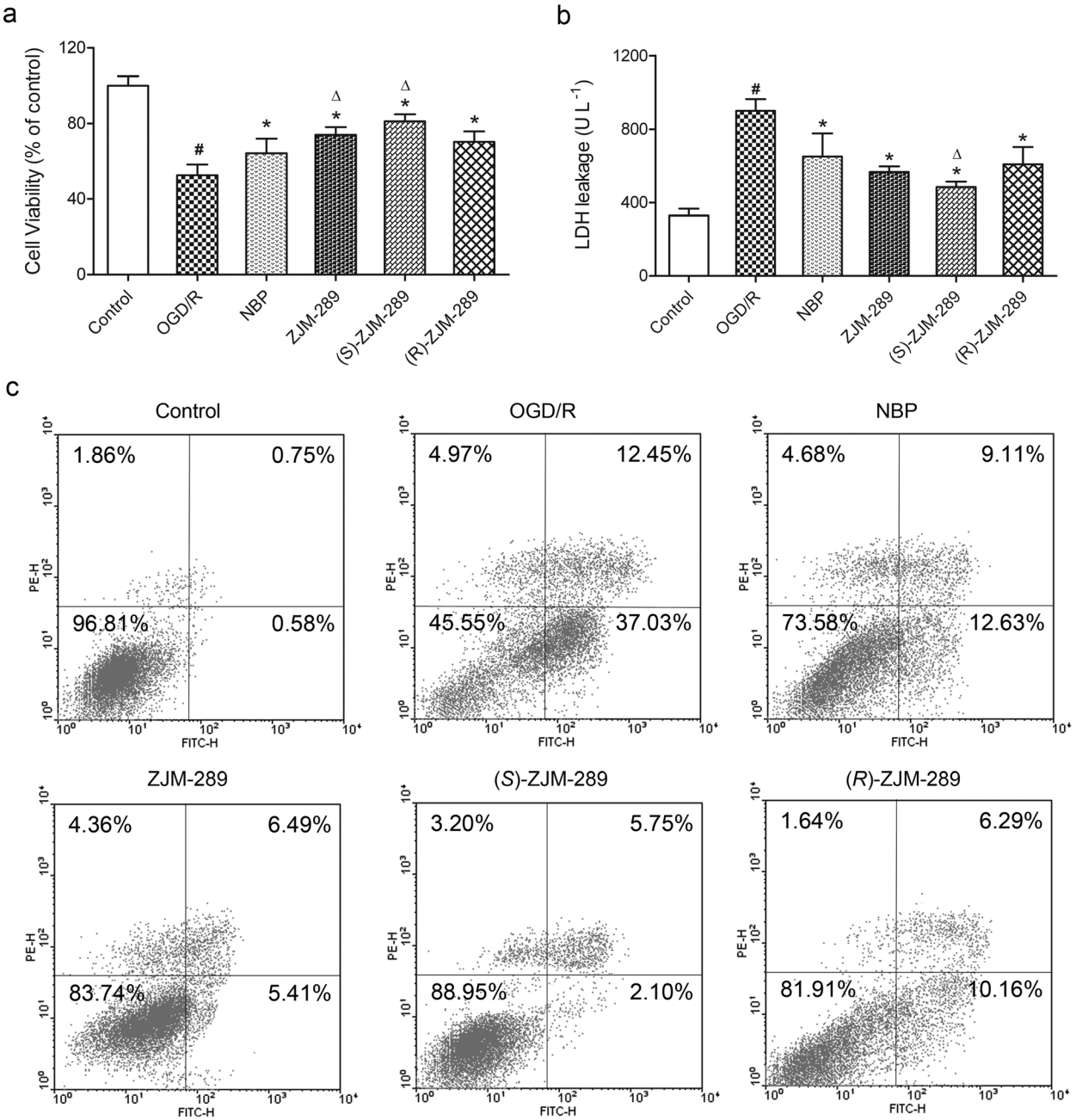


Fig. 5 (*S*)- and (*R*)-ZJM-289 protects primary cultured brain capillary endothelial cells (BCECs) against OGD/R-induced cytotoxicity. Primary cultures of rat brain capillary endothelial cells were prepared from 2-week-old rats and maintained in DMEM/F12 supplemented with 20% fetal bovine serum, heparin (100 U mL^{-1}) and basic fibroblast growth factor (1.5 ng mL^{-1}). BCECs were subjected to 2 h OGD followed by 24 h reoxygenation (OGD/R) and treated with 1 μM NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 2 h before and during OGD/R, respectively. (a) Effects of (*S*)- and (*R*)-ZJM-289 on the viability of OGD/R injured BCECs, determined using the MTT reduction assay ($n = 6$). (b) Cell death was assessed by testing LDH activity in the supernatant culture medium ($n = 6$). (c) Representative dot plots for quantification of mode of death ($n = 3$). The mean fluorescence from endothelial rich populations using FITC-conjugated Annexin V and propidium iodide (PI) were measured by flow cytometry. Data are expressed as mean ± SD. $^{\#}P < 0.05$ *vs.* control group, *P < 0.05 vs. OGD/R group, $\triangle P < 0.05$ *vs.* NBP group, determined with one-way ANOVA followed by Tukey's *post hoc* test.

inhibitory effects on AA-induced platelet aggregation and antithrombotic effects. It appears likely that the enantiomers of ZJM-289 exerted neuroprotective effects against I/R injury benefiting from their antiplatelet and antithrombotic activities.

Another important and favorable effect imparted by the enantiomers of ZJM-289 on ischemic stroke is associated with their releasing moderate levels of NO. Compared with NBP, the two enantiomers intensely increased the CBF in the ischemic region, probably due to the NO released by these compounds. In addition to its direct vasodilative effect, NO released by the two enantiomers may contribute to suppressing the overproduction of ET-1, a vasoconstrictor involved in severe ischemic brain injury,

subsequently enhancing CBF. By contrast, NBP had no such effects on levels of plasma NO and ET-1, leading to less augmentation of CBF. Furthermore, consistent with our previous observation that ZJM-289 stimulated NO–sGC–cGMP pathway, the enantiomers indeed elevated plasma cGMP level in the present experiments. As the second messenger, cGMP mediates various physiological responses such as inhibition of platelet adhesion, neurotransmission and many other processes.[19] Therefore, it seems reasonable to speculate that the neuroprotection of the two enantiomers, as well as their antiplatelet and antithrombotic actions, are partly mediated through cGMP pathways.

Furthermore, we found that pretreatment with the two enantiomers and ZJM-289 could protect BCECs against hypoxic-ischemic injury, which may be partially mediated by NO released from them. As a result, the two enantiomers and ZJM-289 could modulate endothelial function by maintaining NO level in ischemic cerebral vasculature, which contributes not only to their inhibition of platelet adhesion and aggregation, and decreased thrombus formation, but also to their neuroprotection.

In conclusion, (*S*)-ZJM-289, (*R*)-ZJM-289 and ZJM-289 have comparable inhibitory effects on platelet aggregation and thrombus formation *in vitro* and *in vivo*. Additionally, the two enantiomers and ZJM-289 appear to be equally effective in a rat model of focal cerebral I/R. And (*S*)-ZJM-289 is somewhat better than (*R*)-ZJM-289 in a few respects, which might be owing to their slight discrepancy in metabolism and pharmacokinetics properties, and the precise mechanisms remain to be investigated. The present findings may provide new insight into the development of novel antiischemic stroke agents like ZJM-289.

4. Experimental section

4.1 Chemistry

General. NO donor moieties **6** were prepared as described previously.[14] Melting points were determined using a capillary apparatus (RDCSYI). All of the synthesized compounds were purified by column chromatography (CC) on silica gel 60 (200–300 mesh) or thin layer chromatography (TLC) on silica gel 60 F254 plates (250 mm; Qingdao Ocean Chemical Company, China). Subsequently, they were routinely analyzed by IR (Shimadzu FTIR-8400S), ^{1}H NMR and ^{13}C NMR (Bruker ACF-300Q, 300 MHz), and MS (Hewlett-Packard 1100 LC/MSD spectrometer). The purity of test compounds was characterized by HPLC analysis (Shimadzu LC-2010A HPLC system) and high resolution mass spectrometry (Agilent technologies LC/MSD TOF). Target compounds (*S*)-ZJM-289 and (*R*)-ZJM-289 with a purity of >97% were used for subsequent experiments (see the ESI†).

(±)-2-(1-Hydroxypentyl)benzoic acid (2). To a stirred solution of NBP (1.90 g, 10.00 mmol) in CH_3OH–H_2O (20 mL, 1 : 1 v/v) was added NaOH (0.80 g, 20.00 mmol). The reaction mixture was heated at 50 °C for 0.5 h. The solvent was removed under reduced pressure and dissolved in water (20 mL), followed by acidification with 5% HCl to pH 3–4 at −10 to 0 °C. The mixture was extracted with cold Et_2O (10 mL × 3) and quickly used for the next step without any purification.

(−)-2-(1-Hydroxypentyl)benzoate (3). To a stirred solution of compound **2** (2.08 g, 10.00 mmol) in cold Et_2O was added (*S*)-FEA (1.21 g, 10.00 mmol, ee > 98%) dropwise such that the internal reaction temperature did not exceed −10 °C. The reaction mixture was allowed to stand for 5–10 min at −20 to −10 °C and then 6–10 h at RT and filtered to obtain the crude product as a white solid. The solid was recrystallized repeated 2 times from acetone to obtain compound **3** as white needle-like crystals (1.58 g, 48% yield for the two steps). mp 123–124 °C, $[\alpha]_D^{25}$ −8.94° (*c* 1.12, CH_3OH). IR (KBr): 1392, 1517, 1566, 1641, 3411 cm^{-1}. ^{1}H NMR ($(CD_3)_2CO$, 300 Hz, δ): 0.80–1.00 (m, 3H, CH_3), l.22–1.50 (m, 4H, CH_2CH_2), 1.64–1.88 (m, 5H, CH_2, CH_3), 3.62 (s, 1H, OH), 4.56 (dd, 1H, CH–NH_2, *J* = 7.2 Hz), 4.94 (dd, 1H, CH–OH, *J* = 3.4 Hz, 7.2 Hz), 7.14–7.66 (m, 8H, ArH), 7.70–7.84 (dd, 1H, ArH, *J* = 1.8 Hz, 7.2 Hz).

(+)-2-(1-Hydroxypentyl)benzoate (4). Compound **4** was synthesized in the same manner as compound **3**, starting from compound **2** (2.08 g, 10.00 mmol) and (*R*)-FEA (1.21 g, 10.00 mmol, ee > 98%), compound **4** was obtained as white needle-like crystals (1.51 g, 46% yield for the two steps). mp 123–124 °C, $[\alpha]_D^{25}$ +8.89° (*c* 1.05, CH_3OH). IR (KBr) and ^{1}H NMR spectral data were identical to those of compound **3**.

(−)-3-Butyl-1(3*H*)-isobenzofuranone ((*S*)-NBP). To a stirred solution of compound **3** (1.58 g, 4.83 mmol) in H_2O (20 mL) was added 2 M NaOH and stirred for 0.5 h at RT. The mixture was extracted with Et_2O (10 mL × 3). The water layer was acidified with 3 M HCl to pH 2 and stirred for 0.5 h at RT. The solution was then extracted with Et_2O (10 mL × 3). The combined organic layers were dried, filtered and evaporated to dryness to afford (*S*)-NBP as a light yellow oil (0.87 g, 94.8%). $[\alpha]_D^{27}$ −66.6° (*c* 1.00 $CHCl_3$). ee = 98.1%. ESI-MS: *m*/*z* 213 $[M + Na]^+$. ^{1}H NMR ($CDCl_3$, 300 Hz, δ): 0.93 (t, 3H, CH_3, *J* = 3.1 Hz), 1.37–1.45 (m, 4H, 2 × CH_2), 1.86–1.89 (m, 2H, CH_2), 5.48–5.49 (m, 1H, CH), 7.37–7.39 (m, 1H, ArH), 7.56–7.5 (m, 2H, ArH), 8.05 (d, 1H, ArH, *J* = 6.5 Hz).

(+)-3-Butyl-1(3*H*)-isobenzofuranone ((*R*)-NBP). Compound (*R*)-NBP was synthesized in the same manner as compound (*S*)-NBP, starting from compound **4** (1.51 g, 4.59 mmol), compound (*R*)-NBP was obtained as a light yellow oil (0.84 g, 96%). $[\alpha]_D^{27}$ +68.4° (*c* 1.00 $CHCl_3$). ee = 97.4%. IR (KBr), ESI-MS and ^{1}H NMR spectral data were identical to those of (*S*)-NBP.

(−)-2-(1-Hydroxypentyl)benzoic acid ((*S*)-2). Compound (*S*)-**2** was synthesized in the same manner as compound **2**. Starting from compound (*S*)-NBP (1.90 g, 10.00 mmol), compound (*S*)-**2** (2.08 g, 10.00 mmol) in cold Et_2O was used for the next step without any purification.

(−)-2-[1-(2-Chloroacetoxy)pentyl]benzoic acid ((*S*)-5). A solution of 2-chloroacetyl chloride (4.16 mL, 30.00 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise to a mixture of (*S*)-**2** (2.08 g, 10.00 mmol), Et_3N (4.17 mL, 30.00 mmol) and DMAP (1.0 mmol) in CH_2Cl_2 (150 mL) at −10 °C and the solution was left stirring at −10 °C for 5 h. The mixture was acidified with 1 M HCl to pH 2 and stirred for 1 h at RT. The organic layer was washed with water, dried, filtered, and evaporated to dryness. The residue was recrystallized from *n*-hexane to obtain (*S*)-**5** as white crystals (1.85 g, 65%, over two steps). mp 67–68 °C.

$[\alpha]_D^{27}$ −40.8° (*c* 1.00 $CHCl_3$). ESI-MS: *m/z* 283 $[M - H]^-$. IR (KBr): 1412, 1691, 1734, 2958, 3450 cm^{-1}. ^1H NMR ($CDCl_3$, 300 Hz, *δ*): 0.93 (t, 3H, CH_3, *J* = 4.2 Hz), 1.37–1.42 (m, 4H, 2 × CH_2), 1.88–1.91 (m, 2H, CH_2), 4.11 (m, 2H, $COCH_2Cl$), 6.78 (m, 1H, CH), 7.36–7.42 (m, 1H, ArH), 7.56–7.62 (m, 2H, ArH), 8.08 (d, ArH, *J* = 8.1 Hz), 10.89 (brs, 1H, COOH). ^{13}C NMR ($CDCl_3$, 300 Hz, *δ*): 172.0, 166.5, 140.8, 133.1, 130.3, 130.0, 127.1, 125.7, 74.8, 41.0, 36.3, 27.8, 22.4, 13.8.

(+)-2-[1-(2-Chloroacetoxy)pentyl]benzoic acid ((*R*)-5). Compound (*R*)-**5** was synthesized in the same manner as compound (*S*)-**5**. Starting from compound (*R*)-NBP (1.90 g, 10.00 mmol), compound (*R*)-**5** was obtained as a white crystals (1.79 g, 63%, over two steps). mp 67–68 °C. $[\alpha]_D^{27}$ +39.6° (*c* 1.00 $CHCl_3$). IR (KBr), ESI-MS, ^1H NMR and ^{13}C NMR spectral data were identical to those of (*S*)-**5**.

(+)-(*E*)-2-(1-Chloroacetoxypentyl)benzoic acid {4-[2-(4-nitrooxybutoxycarbonyl)vinyl]}phenyl ester ((*S*)-7). To a stirred solution of acylated compound (*S*)-**5** (1.42 g, 5.0 mmol) and compound **6** (1.56 g, 5.0 mmol) in anhydrous CH_2Cl_2 (60 mL) was added DMAP (0.2 mmol) and the solution was left stirring at 0 °C for 10 min. DCC (1.13 g, 5.5 mmol) was then added and the solution was left stirring at RT for 6 h. The solution was then filtered, and the filtrate washed sequentially with 1 M HCl, a saturated aqueous solution of $NaHCO_3$, water, and brine. The solution was then dried, filtered, and evaporated to dryness. The residue was purified by flash chromatography (petroleum ether–EtOAc 5 : 1 v/v) to obtain compound (*S*)-**7** as a colourless waxy solid (2.27 g, 83%), mp 60–62 °C. $[\alpha]_D^{27}$ +6.6° (*c* 1.00 $CHCl_3$). ESI-MS: *m/z* 595 $[M + NH_4]^+$. ^1H NMR ($CDCl_3$, 300 Hz, *δ*): 0.86 (t, 3H, CH_3, *J* = 6.9 Hz), 1.26–1.52 (m, 4H, 2 × CH_2), 1.85–1.94 (m, 6H, 4 × CH_2), 4.00 (m, 2H, $COCH_2Cl$), 4.26 (t, 2H, CH_2ONO_2, *J* = 5.8 Hz), 4.53 (t, 2H, OCH_2, *J* = 6.0 Hz), 6.42 (d, 1H, CH, *J* = 15.9 Hz), 6.68 (m, 1H, CH), 7.26–7.30 (m, 2H, ArH), 7.43 (m, 1H, ArH), 7.55–7.63 (m, 4H, ArH), 7.70 (d, 1H, CH, *J* = 16.0 Hz), 8.15 (d, 1H, ArH, *J* = 8.0 Hz). ^{13}C NMR ($CDCl_3$, 300 Hz, *δ*): 166.7, 166.6, 164.9, 152.3, 144.0, 143.6, 133.3, 132.3, 130.8, 129.4, 129.4, 127.7, 127.3, 126.4, 122.4, 122.4, 118.0, 74.8, 72.6, 63.6, 41.0, 36.3, 27.9, 25.1, 23.7, 22.4, 13.9. HRMS for $C_{27}H_{30}NO_9ClNa$ ($[M + Na]^+$) calcd: 570.1507, Found: 570.1516.

(−)-(*E*)-2-(1-Chloroacetoxypentyl)benzoic acid {4-[2-(4-nitrooxybutoxycarbonyl)vinyl]}phenyl ester ((*R*)-7). Compound (*R*)-**7** was synthesized in the same manner as compound (*S*)-**7**. Starting from compound (*R*)-**5** (1.42 g, 5.00 mmol), compound (*R*)-**7** was obtained as a colourless waxy solid (2.16 g, 80%). mp 60–62 °C. $[\alpha]_D^{27}$ −10.6° (*c* 1.10 $CHCl_3$). ESI-MS, ^1H NMR, ^{13}C NMR and HRMS spectral data were identical to those of (*S*)-**7**.

(+)-(*E*)-2-[1-(Diethylaminoacetoxy)pentyl]benzoic acid {2-methoxy-4-[2-(4-nitrooxybutoxycarbonyl)vinyl]}phenyl ester hydrochloride ((*S*)-ZJM-289). To a stirred solution of (*S*)-**7** (1.09 g, 2.00 mmol) and Et_3N (1.1 mL, 8.00 mmol) in DMF (10 mL) was added diethylamine (0.6 mL, 6.00 mmol) and the solution was left stirring at RT for 8 h. The solution was then filtered, and the filtrate was reconstituted in EtOAc (30 mL) and water (30 mL). The organic layer was washed with water and brine. The solution was then dried, filtered, and evaporated to dryness. The residue was purified by flash chromatography (petroleum ether–EtOAc 3 : 1 v/v), to which EtOAc (2.0 mL) and 1 M ethereal HCl (2.0 mL) was added dropwise at 0 °C. The solution was left stirring at RT for 1 h and filtered. The residue was purified the title compound as a white solid (1.01 g, 82%, for two steps). $[\alpha]_D^{27}$ +7.5° (*c* 1.10 $CHCl_3$). ee = 99.5%. mp 106–108 °C. ESI-MS: *m/z* 615 $[M + H]^+$. IR (KBr): 759, 1254, 1630, 1747, 2861, 2956, 3444 cm^{-1}. ^1H NMR ($CDCl_3$, 300 Hz, *δ*): 0.86 (t, 3H, CH_3, *J* = 6.6 Hz), 1.37 (m, 7H, 2 × CH_2 and CH_3), 1.48 (t, 3H, CH_3, *J* = 7.2 Hz), 1.78 (m, 6H, 2 × CH_3), 3.26–3.41 (m, 4H, 2 × NCH_2), 3.89 (s, 3H, OCH_3), 4.00 (m, 2H, $COCH_2N$), 4.27 (t, 2H, CH_2ONO_2, *J* = 5.4 Hz), 4.53 (t, 2H, $COOCH_2$, *J* = 5.7 Hz), 6.42 (d, 1H, CH, *J* = 15.9 Hz), 6.76 (m, 1H, CH), 7.19 (m, 3H, ArH), 7.44–7.65 (m, 3H, ArH), 7.68 (d, 1H, CH, *J* = 16.0 Hz), 8.21 (d, 1H, ArH, *J* = 7.8 Hz), 12.73 (brs, 1H, NH^+). ^{13}C NMR ($CDCl_3$, 300 Hz, *δ*): 166.4, 164.5, 164.4, 151.5, 144.2, 142.6, 141.4, 133.5, 133.4, 131.2, 128.1, 127.1, 126.1, 123.3, 121.3, 118.2, 111.4, 75.3, 72.7, 63.6, 55.9, 48.6, 48.5, 48.2, 36.1, 27.9, 25.1, 23.7, 22.3, 13.9, 10.1, 10.0. HRMS for $C_{32}H_{43}N_2O_{10}$ ($[M + H]^+$) calcd: 615.2918, found: 615.2931.

(−)-(*E*)-2-[1-(Diethylaminoacetoxy)pentyl]benzoic acid {2-methoxy-4-[2-(4-nitrooxybutoxycarbonyl)vinyl]}phenyl ester hydrochloride ((*R*)-ZJM-289). Compound (*R*)-ZJM-289 was synthesized in the same manner as compound (*S*)-ZJM-289. Starting from compound (*R*)-**7** (1.09 g, 2.00 mmol), compound (*R*)-ZJM-289 was obtained as a white solid (1.03 g, 84%, for two steps). mp 106–108 °C. $[\alpha]_D^{25}$ −7.1° (*c* 1.12 $CHCl_3$). ee = 98.6%. IR (KBr), ESI-MS, ^1H NMR, ^{13}C NMR and HRMS spectral data were identical to those of (*S*)-ZJM-289.

4.2 Platelet aggregation *in vitro*

Blood samples were withdrawn from rabbit carotid artery and mixed with 3.8% trisodium citrate (9 : 1 v/v), followed by centrifuging at 500 rpm for 10 min. The supernatants were collected and used as platelet rich plasma (PRP). Additional samples were centrifuged at 3000 rpm for 10 min and the supernatants were collected as platelet poor plasma (PPP). The effect of individual compounds on the ADP-, TH- and AA-induced platelet aggregation was measured by Born's turbidimetric method using a Platelet-Aggregometer (LG-PABER-I Platelet-Aggregometer, Beijing). Briefly, PRP (240 μL) was pre-treated in duplicate with vehicle, different concentrations of NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 for 5 min and exposed to 10 μM of ADP, 0.5 U mL^{-1} of TH, or 1.0 mM of AA incubated at 37 °C for 5 min, respectively. The formation of platelet aggregation was monitored longitudinally by optical density. Compounds under study or vehicle alone were added to the PRP samples 5 min before addition of the aggregating agent. The antiplatelet aggregatory activity of NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 was evaluated as percent inhibition of platelet aggregation compared to positive controls that had been pre-treated with vehicle alone and exposed to the inducer samples. The IC_{50} values of these compounds were determined by nonlinear regression analysis or the percent inhibition at the maximal concentration tested (1.0 mM) was calculated.

4.3 Antithrombotic activity assay in rats

The antithrombotic activities of NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 were tested in male SD rats, as previously described.[20] Briefly, after anesthesia, rats were subjected to surgical exposure of their neck areas and inserted with an 8 cm polyethylene tube connecting the left jugular vein and right carotid artery. The saline-filled shunt was assembled by connecting two cannulae with a 6 cm slightly curved Tygon tubing containing a 6 cm long cotton thread. The rats were maintained on extracorporeal circulation for 15 min, during which a thrombus formed and adhered to the cotton thread. The shunt was then removed and the thread with the associated thrombus was withdrawn and immediately weighed. The wet weight of thrombus was determined by subtracting the weight of thread from the total wet weight. Antithrombotic efficacy was indicated by decreases in the weight of thrombus formed in 15 min of exposure to flowing blood.

4.4 Effect of compounds on the thrombosis in mice

Male Swiss mice (30–35 g, from ICR animal colony) were randomized and treated orally with vehicle alone or compounds at indicated concentrations for two hours, respectively. Subsequently, the mice were injected intravenously with a mixture of 1.0 mg mL^{-1} of collagen and 44.5 μg mL^{-1} of adrenaline, and observed for thrombosis-related death for 5 min and hemiplegia for 15 min. The antithrombotic effects of NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 were calculated as percent protection relative to controls injected with vehicle alone. The experimental protocols of animal studies were approved by the Animal Research Care Committee of China Pharmaceutic University.

4.5 Neuroprotection against focal cerebral ischemia/reperfusion (I/R)

4.5.1 Ischemic/reperfusion (I/R) model and drug administration. Male Sprague–Dawley rats (250–300 g) were purchased from B&K Universal Group Limited, Shanghai, China. Animals were kept under standard laboratory conditions and maintained in a 12 h light–12 h dark cycle with free access to food and water. Animals care and treatment were conducted in accordance with the institutional guidelines. Rats were anesthetized with chloral hydrate (300 mg kg^{-1}, i.p.) and subjected to middle cerebral artery occlusion (MCAO) as described previously with slight modification.[21] Briefly, the right common carotid artery, internal carotid artery (ICA), and external carotid artery (ECA) were exposed. A 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co., Ltd., Beijing, China) with a rounded tip was inserted into the ICA through the ECA stump and gently advanced to occlude the MCA. After 2 h of MCAO, the suture was withdrawn to restore blood flow (reperfusion). Sham-operated rats were manipulated in the same way, but the MCA was not occluded. Core body temperatures were monitored with a rectal probe and maintained at 37 °C ± 0.5 °C during the whole procedure.

Rats were divided randomly into 6 groups: (1) sham group, (2) vehicle group (I/R), (3) I/R + NBP (50 mg kg^{-1}, i.g.) group, (4) I/R + ZJM-289 (172 mg kg^{-1}, i.g.) group, (5) I/R + (*S*)-ZJM-289 (172 mg kg^{-1}, i.g.) group, (6) I/R + (*R*)-ZJM-289 (172 mg kg^{-1}, i.g.) group. The drugs were administered daily, starting at the onset of reperfusion and continued for 3 days.

4.5.2 Neurologic scoring. Neurologic scores were evaluated by a blinded observer at 3 days after reperfusion, according to Longa's method.[21]

4.5.3 Measurement of infarct size. At 3 days after reperfusion, rats were decapitated and the brains were rapidly removed onto ice and sliced into six 2 mm-thick coronal sections. Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride for 30 min at 37 °C followed by overnight immersion in 10% formalin PBS. The infarcted tissue remained unstained (white), whereas normal tissue was stained red. The infarct zone was demarcated and analyzed by image processing software. The infarcted area was numerically integrated across each section and was expressed as a percentage of the total area of whole brain.

4.5.4 Brain-water content. The brain samples were weighed immediately after dissection (wet weight) and then dried at 105 °C for 24 h. The percent of water content was calculated with the formula (wet weight − dry weight)/wet weight × 100%.

4.5.5 Histopathology. The ipsilateral hemisphere of ischemic brain samples was harvested and post-fixed in formalin, and cut coronally at 2 mm intervals from the frontal pole. Paraffin-embedded coronal slices sectioned at 5 μm, and stained with hematoxylin–eosin. Histopathological evaluation was performed by a pathologist blinded to the treatment group.

4.5.6 Measurement of regional cerebral blood flow (rCBF). rCBF during I/R was monitored continuously by laser-Doppler flowmetry (BIOPAC MP 150, CA, USA) after treatment with NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289. At the ischemic hemisphere, a 2 × 2 mm cranial window was drilled through the skull 2.0 mm lateral to the midline and 3.0 mm caudal to bregma. The laser-Doppler probe was mounted on a micromanipulator and placed over the brain surface. The analog output from the probe was fed into an analog-to-digital converter and displayed on a chart recorder. rCBF was expressed as absolute laser-Doppler units.

4.5.7 Measurement of NO, ET-1, TXB_2, 6-keto-$PGF_{1\alpha}$, cGMP and cAMP. Carotid artery blood samples were collected in tubes containing EDTA and centrifuged at 3000 rpm for 10 min, at 4 °C. NO production was evaluated indirectly by measuring nitrite/nitrate (NO_x), the stable metabolites of NO. NO_x content in plasma was measured using colorimetric total NO assay kit (JianCheng Bioengineering Institute, Nanjing, China) as described previously.[22] Plasma cGMP and cAMP levels were determined using a commercial enzyme immunoassay (R&D Systems, Minneapolis, MN, USA).

Two milliliters of carotid artery blood were collected in tubes containing 40 μL aprotinin and 30 μL EDTA. These samples were immediately centrifuged at 3000 rpm for 10 min. Collected plasma was stored at −80 °C until batched assays were performed. Plasma ET-1 levels were evaluated with ET radioimmunoassay kit (Beijing Purevalley Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

Three milliliters of carotid artery blood were mixed with 200 μL indomethacin-EDTA·2Na and immediately centrifuged at 3500 rpm for 15 min. Collected plasma was stored at −80 °C until assayed. The levels of TXB_2 and 6-keto-$PGF_{1\alpha}$ were measured by radioimmunoassay according to the manufacturer's instructions (Beijing Purevalley Biotechnology Co., Ltd, Beijing, China).

4.6 The enantiomers of ZJM-289 protected rat BCECs against OGD/R injury

4.6.1 Rat BCECs culture. Primary cultures of BCECs were prepared from 2-week-old Sprague-Dawley rats as described previously with some modifications.[23] Briefly, the isolated cerebral gray matter was digested by trypsin (0.05%) at 37 °C for 30 min, then filtered through 145 μm nylon mesh. The filtrate was filtered through 75 μm nylon mesh and the matter was collected on the nylon mesh. Then the matter was digested by collagenase type II (0.1%) at 37 °C for 30 min. Cells were collected by centrifugation at 1500 rpm for 5 min and resuspended in DMEM–F12 (1 : 1) with 20% (v/v) fetal bovine serum, 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin, 100 U L^{-1} heparin and 1.5 ng mL^{-1} basic fibroblast growth factor. Cells were seeded in gelatin coated 96- or 6-well plates and cultured in a humidified incubator in air with 5% CO_2 (Thermo Scientific 3110, OH, USA).

4.6.2 Oxygen glucose deprivation and reoxygenation (OGD/R) treatment. BCECs were rinsed twice with phosphate buffered saline (PBS), and OGD was induced with Earle's solution without glucose (pH 7.4, 143 mM NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, and 2.4 mM HEPES). The cultures were introduced into an anaerobic chamber flushed with 5% CO_2 and 95% N_2 for 15 min. The chamber was tightly sealed and placed in an incubator at 37 °C for 2 h. Controls were incubated with Earle's solution containing 5.6 mM glucose and maintained in an incubator with 5% CO_2 atmosphere at 37 °C. After the deprivation period, cultures were returned back to the normal culture medium under normoxic conditions for 24 h, corresponding to the reoxygenation period. NBP, ZJM-289, (*S*)- and (*R*)-ZJM-289 were applied to BCECs 2 h before and during OGD/R. The vehicle-treated cultures received 0.1% DMSO.

4.6.3 Cell viability and apoptosis assessment. At 24 h post-OGD, BCECs death was measured by the MTT assay and cell viability of the vehicle-treated control group not exposed to either OGD or drugs was defined as 100%. To confirm neuron death, lactate dehydrogenase (LDH) activity in the medium 24 h after OGD was determined colorimetrically according to the protocols of an LDH kit (JianCheng Bioengineering Institute, Nanjing, China).

For detection of cell apoptosis, cells were stained with FITC-conjugated Annexin V and propidium iodide (PI). The fluorescence was then examined using a flow cytometry (BD FACSCanto, NJ, USA), which discriminated intact cells ($FITC^-/PI^-$), apoptotic cells ($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$).

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