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5d, a novel analogue of 3-n-butylphthalide, protects brains against nervous injury induced by ischemia/reperfusion through Akt/Nrf2/NOX4 signaling pathway

Running title, 5d protects brains against nervous injury

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Abbreviations: I/R, ischemia/reperfusion; MCAO, middle cerebral artery occlusion;

OGD/R, oxygen-glucose deprivation and recovery; Nrf2, nuclear-factor-E2-related factor; Keap-1, kelch-like ECH-associated protein-1; ARE, antioxidant response element; PI3K/Akt, phosphatidylinositol 3-kinase/Akt; ROS/RNS, reactive oxygen species/reactive nitrogen species; MDA, malonaldehyde; LDH, Lactate dehydrogenase; rCBF, regional cerebral blood flow; NBP, racemic 3-*n*-butylphthalide; TTC, 2,3,5-triphenyltetrazolium chloride; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered salin; H&E, hematoxylin and eosin; NeuN, neuron-specific nuclear; TUNEL, dUTP nick-end labeling; DAB, diaminobenzidine

Abstract

Oxidative stress has been shown to play a vital role in the pathogenesis of the ischemia/reperfusion (I/R) and may represent a target for treatment. 3-Butyl-2-benzothiophen-1(3H)-one (**5d**), a novel analogue of the racemic 3-*n*-butylphthalide (NBP), exerts enhanced free radical scavenging, anti-platelet and anti-thrombotic effects, as well as preventive neuroprotection in a rat model of ischemia/reperfusion (I/R). Nevertheless, it is still unknown about the therapeutic effect of **5d** against ischemic stroke and underlying mechanism(s) involved. **5d** (30 mg/kg and 90 mg/kg) significantly reduces brain damage after I/R *in vivo*, and protects primary cultured cortical neurons against oxygen-glucose deprivation and recovery (OGD/R)-induced cytotoxicity *in vitro*. Besides, **5d** induces Nrf2 nuclear localization through PI3K/Akt signaling pathway, and lowers the intracellular ROS

levels by lowering NADPH oxidase activity. However, Nrf2 siRNA transfection before OGD/R could decrease the neuronal cell viability and increase the expression of NOX4 elicited by **5d** treatment. These results suggest that the therapeutic effect of **5d** against ischemic stroke is due to the decrease of NADPH oxidase activity by promoting Nrf2 nuclear localization through PI3K/Akt signaling pathway. These findings point to that **5d** might be an effective candidate for the treatment of ischemic stroke.

Key words: 5d; Neuroprotection; Nrf2; NADPH oxidase; ROS

Introduction

Cerebral ischemia has high mortality and is a leading cause of disability worldwide (Rosamond et al., 2008; Donnan et al., 2008). Oxidative stress has been shown to play a critical role in ischemia/reperfusion (I/R) injury through increased formation of reactive oxygen species/reactive nitrogen species (ROS/RNS) leading to lipid peroxidation and DNA damage (Love, 1999; Lewen et al., 2000; Allen and Bayraktutan, 2009). Although many treatment strategies have implemented antioxidants to promote neuroprotection during I/R, their clinical efficacy has proven disappointing (Lo et al., 2003; Hacke et al., 2008).

Nuclear-factor-E2-related factor (Nrf2) has been reported to regulate an expensive set of antioxidant/detoxification genes that act in synergy to remove ROS/RNS through sequential enzymatic reactions (Shih et al., 2003; Satoh et al., 2006; Tanito et al., 2007). Under basal conditions, Nrf2 is localized mainly in the

cytoplasm where it binds to the Kelch-like ECH-associating protein (Keap-1) and is degraded by the proteasome pathway. Once the cell is exposed to oxidative or electrophilic stress Nrf2 is dissociated from Keap-1 and translocates to the nucleus where, by binding to the antioxidant response element (ARE) in the promoter of antioxidant genes, it induces their expression.

It is well known that NADPH oxidase is a major complex that produces detrimental oxygen derived free radicals during the ischemic period (Suh et al., 2008). It can be expressed and activated by various stressors such as ischemic injury (Suh et al., 2008; Chen et al., 2009), redox stress in amyotrophic lateral sclerosis (Wu et al., 2006; Li et al., 2008), and Alzheimer disease (Zekry et al., 2003; Di Virgilio, 2004; Block, 2008). But there is few data to show the relationship between Nrf2 and NADPH oxidase after ischemic stroke.

3-butylbenzo[*c*]thiophen-1(3*H*)-one (**5d**) is a novel analogue of the racemic 3-*n*-butylphthalide (NBP) (Fig.1). As the parent compound, NBP was approved by the State Food and Drug Administration (SFDA) of China as a new drug for the treatment of ischemic stroke (Zhang et al., 2006). However, it is often administered with antioxidant and/or anti-platelet drug(s) to improve its therapeutic effect (Zhu and Tan, 2010). Given that a number of drug molecules containing sulfur exhibit a better efficacy in several aspects, especially for antioxidation, anti-free radicals, and neuroprotection (Iudin et al., 2011; Zenkov et al., 2007), we therefore designed and synthesized a novel class of compounds by bioisosteric replacement of the oxygen atom at the 2-position of phthalide, the scaffold of NBP, with sulfur and, meanwhile,

introduction of various lengths of alkyl, including straight/branch chain alkyl or arylalkyl groups, into 3-position of phthalide. Through a series of screening experiments, we finally found that **5d** exerted enhanced free radical scavenging, anti-platelet aggregation, and anti-thrombotic potency, as well as potent preventive neuroprotection in a rat model of I/R (Wu et al, 2012). Nevertheless, much is still unknown about the therapeutic potential of **5d** against ischemic stroke.

In this context, we used an *in vivo* middle cerebral artery occlusion (MCAO) model and an *in vitro* oxygen-glucose deprivation and recovery (OGD/R) model to investigate whether **5d** could induce a therapeutic effect on ischemic injury and, if so, whether the relevant mechanisms involved is concerned with Nrf2 and NADPH oxidase.

Material and Methods

Chemicals and Reagents

5d (purity > 99%) was synthesized by the Center of Drug Discovery, China Pharmaceutical University, and the molecular structure was shown in Fig.S-1 (Wang et al., 2011; Wu et al., 2012). The compound was dissolved in normal saline. Nuclear and cytoplasmic protein extraction kit, TUNEL apoptosis detection kit, and 8-OH-dG kits were obtained from Beyotime Institute of Biotechnology (Haimen, China). Lactate dehydrogenase (LDH) kit was purchased from JianCheng Bioengineering Institute (Nanjing, China). The primary antibodies used were polyclonal antibodies against Nrf2, NOX4, β-actin (Bioworld Technology Inc., USA), Lamin B (Santa Cruz

Biotechnology, CA, USA), NeuN (Beijing Biosynthesis Biotechnology Co., Ltd., Fuzhou, China), ERK, p-ERK, p38, p-p38, JNK, p-JNK, Akt, p-Akt (Cell signaling Technology, Beverly, MA, USA). The goat anti-rabbit IgG-HRP secondary antibody was supplied by Bioworld Technology Inc. (MN, USA). PI3K inhibitor LY294002 and ERK inhibitor U0126 were purchased from Beyotime Institute of Biotechnology (Haimen, China). 2,3,5-triphenyltetrazolium chloride (TTC), NBP, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were purchased from Sigma Chemical Co., (St. Louis, MO, USA).

Animals and treatments

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. All protocols and procedures used in the study observed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Utilization Committee of China Pharmaceutical University.

Middle cerebral artery occlusion (MCAO) and drug administration

Rats were anesthetized i.p. with 350 mg/kg chloral hydrate (Sinopharm Chemical Reagent, Beijing, China) and subjected to a procedure of the middle cerebral artery occlusion (MCAO), as described previously (Wu, et al., 2012). During the experiment, the regional cerebral blood flow (rCBF) was monitored at each time point (0 h, 0.5 h and 1 h after the onset of middle cerebral artery occlusion and 0.5 h, and 1 h after

reperfusion) using a laser Doppler flowmetry (LDF 100C, BIOPAC Systems) in several animals, as described in detail previously (Nito et al., 2004). The control rats received a sham surgery. The core body temperature of individual rats was monitored with a rectal probe and maintained at 37 ± 0.5 ℃ during the whole procedure.

Rats were divided randomly into five groups: (1) Sham group, (2) Model group, (3) I/R + **5d** (30 mg/kg, i.g.) group, (4) I/R + **5d** (90 mg/kg, i.g.) group, (5) I/R + NBP (80 mg/kg, i.g.) group. The drugs were administered daily, starting at the onset of reperfusion and continued for 3 days.

Neurological deficit evaluation

The neurological deficit of the rats was evaluated by a blinded observer at 0, 24 h, 48 h and 72 h after reperfusion, according to Longa's method (Longa et al., 1989).

Infarction measurement

At 3 d after reperfusion, rats were decapitated and the brains were rapidly removed onto ice and sliced into six 2-mm-thick coronal sections TTC staining (Wu et al, 2012). Infarct area was expressed as a percentage of the total area of whole brain.

Hematoxylin-eosin (H&E) and TUNEL assessment

At 3 d after reperfusion, rats were deeply anesthetized with chloral hydrate and perfused with heparinized (10 U/ml) saline followed by 4% paraformaldehyde in PBS. The brains were rapidly removed, frozen, cut across region of the hippocampus, and mounted. 20-μm sections were used for regular H&E staining; 6-μm sections were stained for dUTP nick-end labeling (TUNEL) assay using an apoptosis detection kit (Beyotime Institute of Biotenchnology Co., Ltd. Haimen, China) according to the

manufacturer's protocol.

Immunohistochemistry staining

Free-floating brain slices (6 μm) were washed in PBS to remove cryopreservative. The tissues were incubated for 10 min at room temperature in 3% H_2O_2 solution to reduce endogenous peroxide, blocked in normal goat serum, and then incubated overnight at 4°C with rabbit polyclonal antibodies (1:100; Beijing Biosynthesis Biotechnology Co., Ltd., Fuzhou, China). Then the samples incubated with biotin-conjugated secondary antibody (Fuzhou Maxim Biotechnology Co., Ltd., Beijing, China), followed by an streptavidin-peroxidase complex (Fuzhou Maxim Biotechnology Co., Ltd., Beijing, China) with diaminobenzidine (DAB) as substrate. All sections were analyzed using a Microscope (OLYMPUS CKX41, Tokyo, Japan).

Intracellular reactive oxygen species (ROS) measurement

Oxidant generation was evaluated in cortical neurons using fluorescence microsphere 2',7'-dichlorofluorescin (DCF), as described previously (Bejma et al., 2000). 1×10^6 cortical neurons were added in assay medium and incubated at 37°C for 15 min. This allowed DCF-DA to be cleaved by intracellular esterase to derive free DCF. The rate of oxidant production was followed at the excitation wavelength of 488 nm and emission wavelength of 525 nm for 30 min using a Hitachi F-2000 fluorescence spectrometer.

Malondialdehyde (MDA) and 8-OH-dG examination

At 3 d after reperfusion, the rats were sacrificed and their brains were rapidly removed, rinsed with 0.9% cold saline, and blotted with filter paper before being kept

at −70 °C. After quantification of protein concentrations, the content of MDA in brain tissues was determined using commercial analysis kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's protocol.

To examine DNA oxidative damage, the brain tissues were homogenized for DNA extraction using a DNA isolation kit according to the manufacturer's protocol (Jiancheng Institute of Biotechnology, Nanjing, China). The 8-OH-dG level was determined using an ELISA kit (Jiancheng Institute of Biotechnology, Nanjing, China) and the optical density was measured at 450 nm. The data, expressed as pg of 8-OH-dG per μg of DNA, was calculated on the basis of a linear calibration curve generated with 8-OH-dG standard solution.

Primary cortical neuron culture

Primary cortical neurons were generated from fetus of Sprague-Dawley rats. The cortices were isolated and cultured as described by Zhao et al., 2012. Briefly, whole cerebral cortices were dissected, incubated for 15 min in 0.25% trypsin at 37 °C and mechanically dissociated using Pasteur pipette. Cells were seeded at a density of 1.5×10^5 cells/cm^2 onto poly-D-lysine-coated 96- or 6-well plates and were maintained in a humidified incubator in air with 5% CO_2 (Thermo Scientific 3110, OH, USA). After 24 h, the culture medium was changed to neurobasal medium supplemented with 2% B27. Medium replacement was performed every 3 d. After 15 d in culture, the cells form extensive axonal and dendritic networks and are ready for the experiments.

Oxygen-glucose deprivation and recovery (OGD/R) treatment

Neurons 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 ℃ for 2 h. Controls were incubated with Earle's solution contained 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 recovery period. **5d** and NBP were applied to cortical cell cultures 2 h before OGD and during 6 h of re-oxygenation. The vehicle-treated cultures received 0.1% DMSO.

Cell viability assessment

At 24 h post-OGD, neuronal cell 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 a LDH kit (JianCheng Bioengineering Institute, Nanjing, China).

Transient transfection of small RNA interference (siRNA)

Primary neuronal cells were grown on 24-well plates (1×10^5 cells/well) and 60-mm dishes ($1\text{-}2 \times 10^6$ cells/well) that were coated in advance with poly-D-lysine in Neurobasal medium containing B-27. The siRNA construct used was obtained as mismatched siRNA control (Negative Control, Santa Cruz Biotechnology, Santa Cruz, CA), siRNA against Nrf2 (siNrf2, Santa Cruz Biotechnology, Santa Cruz, CA),

siRNA against Akt (siAkt, Dharmacon, Lafayette, CO). Cells were transfected with 10-50 nM siRNA using lipofectamineTM 2000 (Invitrogen, Carlsbad, CA) based on the manufacturer's instruction. At 24 h after transfection, the cells were treated with 10 μM **5d** for 24 h and examined by either Western blot analysis or MTT assay.

Western blot analysis

The neurons were extracted with a cell lysis buffer (Beyotime, Nanjing, China). The total protein concentration was measured and adjusted to equal concentrations across different samples. The protein was separated on a 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The PVDF membranes were incubated with the indicated primary antibodies overnight at 4 °C, and then incubated with the secondary antibodies conjugated to horse-radish peroxidase. The proteins were visualized by a Keygen ECL system (Kaiji, Nanjing, China) and scanned with a Clinx Chemi-Scope chemiluminescence imaging system (Gel Catcher 2850, China). The relative optical densities of the specific proteins were determined by a ChemiScope analysis program.

Statistical analysis

All data are expressed as mean ± SD. Statistical analyses were performed with one-way ANOVA followed by Turkey's *post hoc test* for multiple comparison tests. Significant differences were accepted when $p < 0.05$.

Results

5d protects primary cultured cortical neurons against OGD/R-induced

cytotoxicity

To investigate the effect of **5d** on cell viability, primary cultured neurons were exposed to different doses (0.1-10 μM) of **5d** for 24 h and subjected to a MTT assay. As shown in Fig.1A, treatment with **5d** at different concentrations showed no influence on the cell viability under basal conditions, indicating that **5d** had no toxic effect. To examine the neuroprotective effects of **5d** against OGD/R-induced cytotoxicity, various concentrations of **5d** were added to the culture medium 2 h before OGD and during 6 h of re-oxygenation. As shown in Fig. 1B, the viability of the cells exposed to OGD/R was reduced to 44.5% compared with the control. Treatment with **5d** (90.3%) or NBP (80.5%) at 10 μM significantly attenuated OGD/R-induced cell death. The cytoprotective effects of **5d** were also confirmed by LDH assay. Compared with the control, cells exposed to OGD/R had a significant increase (from 16.6% to 42.2%) in LDH release, which was decreased in **5d** or NBP-treated cells (Fig. 1C). Importantly, the protective effect of NBP (23.0%) at 10 μM was less than that of **5d** (18.0%) at the same dosage.

5d decreases the level of ROS generation and the content of MDA and 8-OH-dG after I/R

It is well established that, after cerebral I/R, the ROS production is dramatically increased and leads to more severe tissue damage by propagating DNA and lipids peroxidation (Madamanchi et al., 2005; Schreibelt et al., 2007). In order to investigate the effect of **5d** on ROS generation, we pre-loaded neuronal cells with the ROS-sensitive fluorophore DCF-DA, prior to treatment with **5d**, and then measured

DCF-DA fluorescence. As shown in Fig.4D, ROS production was rapidly initiated after OGD/R injury. The addition of **5d** or NBP resulted in a significant attenuation of this effect. Moreover, at the dosage of 10 μM, the effect of **5d** was superior to that of NBP.

MDA is a product of lipid peroxidation, whereas 8-OH-dG is a product of DNA oxidation. In this study, it is observed that the content of MDA and 8-OH-dG were markedly up-regulated in the vehicle-treated animals, which could be reversed by **5d** or NBP treatment (Fig.4E-4F). Nevertheless, the antioxidant effect of **5d** was superior to that of NBP. These results indicated that **5d** exerted stronger neuroprotective effect than NBP following I/R through its antioxidant activity, which might be related to the introduction of sulfur in its molecular structure (Iudin et al., 2011; Zenkov et al., 2007).

5d induces Nrf2 nuclear localization by promoting the dissociation between Nrf2 and Keap-1

Nrf2 is a key transcription factor that regulates antioxidant genes as an adaptive response to oxidative stress or pharmacological stimuli (Thompson et al. 2012). To investigate the effect of **5d** on the Nrf2 signaling, the cellular locations of Nrf2 and kelch-like ECH-associated protein 1 (Keap-1) were evaluated after I/R. Immunoblot analysis revealed that the expression of Nrf2 in the nuclear fraction was significantly higher in **5d**-treated groups than in the Model group. On the contrary, Keap-1 expression in the cytosolic fraction decreased in **5d**-treated groups compared with the Model group (Fig.3). Moreover, **5d** also reinforced the translocation of Nrf2 to the

nucleus compared with the OGD/R group in vitro (Fig.4A). These results demonstrated that **5d** promoted the dissociation between Nrf2 and Keap-1 and induced Nrf2 nuclear localization. In order to better clarify the mechanistic link between **5d** and the translocation of Nrf2, cells were transfected with Nrf2-siRNA. Fig.4C represented the effective down-regulation of Nrf2 obtained with siRNA in cultured cortical neurons. Moreover, Nrf2 down regulation led to a marked decrease of cell viability elicited by **5d** (Fig.4D)

5d promotes the translocation of Nrf2 through PI3K/Akt signaling pathway after I/R

To further elucidate the upstream signaling pathway involved in **5d**-mediated Nrf2 activation, PI3K/Akt, and three MAPK cascade, p38, JNK, and ERK were evaluated after I/R, all of which are major signaling enzymes involved in cellular protection against oxidative stress. The expression of p-p38, p-JNK and p-Akt were significantly increased in the model group, while **5d** markedly down-regulated the expression of p-JNK and p-p38 and up-regulated that of p-Akt (Fig.5). These results indicated that **5d** might regulate the expression of Akt to cope with the nervous injury following I/R.

To verify whether Akt pathway participated in **5d**-induced translocation of Nrf2 to the nuclear, the inhibitor of LY294002, a PI3K inhibitor, was added 30 min before OGD/R. As shown in Fig.6A-6B, pretreatment with LY294002 abolished **5d**-induced translocation of Nrf2 to the nuclear. To further confirm these observations, cells were transfected with siAkt. Consequently, Akt-downregulation significantly decreased the

expression of nuclear Nrf2 elicited by **5d** treatment (Fig.6C-6E). These results indicated that PI3K/Akt was involved in **5d**-induced translocation of Nrf2 to the nuclear.

5d modulates NADPH oxidase through the activation of Nrf2 after I/R

Transient cerebral ischemia is well known to generate ROSs via NADPH oxidase in neuronal cells of damaged brain tissue. NADPH oxidase is one of the most important sources of superoxide free radicals in the acute phase of ischemic damage (Suh et al., 2008). Although NADPH oxidase activity is very important for ROS generation, which can trigger neuronal cell death after ischemic brain injury, its regulatory mechanism during brain ischemic injury is still unclear. We hypothesized that the activation of Nrf2 causes inhibition of ROS production via NADPH oxidase. Kim et al demonstrated that NOX4 (one of the membrane-bound subunits) is, by far, the major source of oxidative stress and neurodegeneration on ischemic stroke. In our study, NOX4 expression was increased in the brains of rats subjected to I/R compared with the Sham controls. **5d** administration significantly inhibited the upregulation of NOX4 caused by I/R, compared with the vehicle-treated groups (Fig.8A-8B). To confirm the effect of Nrf2 on the downregulation of NOX4, we subjected a primary neuronal culture to OGD/R with Nrf2-specific siRNA transfection. As shown in Fig.8C-8D, siRNA transfection against Nrf2 mRNA in cortical primary neurons enhanced the expression of NOX4 elicited by **5d** treatment.

Discussion

The development of antioxidant drugs is essential for the treatment of ischemic stroke. Clinical use of potential antioxidant treatment has been prevented owing to inefficiency or/and serious side effects. In order to discover novel antioxidant drugs against cerebral ischemia, we have focused our studies on **5d**, an analogue of NBP. Our previous study reported the preventive neuroprotection of **5d** against I/R injury. However, there is still a need for a deeper scientific investigation of the therapeutic effects of **5d** against ischemic stroke and the mechanisms involved.

In the present study, **5d** exerted neuroprotection against I/R-induced neuronal injury as manifested by the increase of rCBF, decrease of brain infarction, and reduction of neurological deficit (Fig.S-2). Compared with 24h in the previous text, the timeline of neurological deficit scores are extended to 72h in this study. This is because in the previous experiment, **5d** was administrated to the rats for 7 successive days before ischemia, whereas the administration was started at the onset of reperfusion and continued for 3 days in this study. The aim of the former text is to investigate the preventive effect of **5d** against cerebral ischemia, while the focus of the latter is the therapeutic effect of **5d** against I/R. Indeed, in the present study, the timeline of neurological deficit scores were extended to 14 days and the results showed that at72h after I/R, the neurological function of rats in the Model group began to recover spontaneously and the recovery rate of nerve function in NBP and **5d**-treated group was significantly higher than that in the Model group (data not shown). Besides the timeline, it also needs to explain why the Sham data have a score of zero (24h) in the earlier data set but in this manuscript reach that point only after 48

hours. Actually, at 24h after I/R, the neurological deficit scores of all groups in this study generally higher than those in the previous one, indicating that the extent of surgical injury in this experiment was generally greater than that in the previous test. In the previous study, the neurological deficit scores of all rats in the Sham group were "0", whereas there were 2 rats in this test whose neurological deficit scores in the Sham group were "1". This is the reason for the difference of the point that a score of "0" reached in the two manuscripts. It is worth mentioning that to reduce the infarct size induced by MCAO, **5d**'s therapeutic effect shown in this study is similar to its preventive effect in the previous text. Moreover, in this study, H&E staining, NeuN-immunohistochemical staining and TUNEL staining confirmed that **5d** rescued neuronal loss after ischemia (Fig.S-3). These therapeutic effects were also similar to the preventive effects found in the previous studies. All the data above suggests that as a candidate compound, **5d** exerts both preventive and therapeutic effects against cerebral ischemia.

Nrf2 is a transcription factor that regulates expansive set of antioxidant genes acting in synergy to remove ROS through sequential enzymatic reactions (Ishii et al., 2000; Shih et al., 2003). In this study, **5d** combated against oxidative stress as evidence by the suppression of ROS generation and down-regulation of MDA and 8-OH-dG content. The neuroprotection of **5d** was stronger than that of NBP, which was consistent with **5d**' design idea of introducing sulfur in the molecular structure to improve its antioxidant activity. This was the first study to report that **5d** significantly induced the translocation of Nrf2 to the nucleus. Under normal conditions, the Nrf2

inhibitor Keap-1 bands and retains Nrf2 in the cytoplasm where it can be targeted for ubiquitin mediated degradation (Cullinan et al. 2004). However, under certain conditions, Keap1 or Nrf2 may be chemically modified through phosphorylation (Motohashi and Yamamoto 2004), deacetylation (Tkachev et al. 2011), and S-nitrosylation (Um et al. 2011). These chemical modifications enhance Nrf2 disassociation from Keap-1, thus facilitating Nrf2 nuclear translocation and subsequent Nrf2-dependent gene expression. In this context, **5d** decreased the expression of Keap-1 in the cytosolic fraction. This result demonstrated that the induction of Nrf2 translocation by **5d** might through inhibiting the ubiquitin ligase activity of Keap-1 or promoting the dissociation between Nrf2 and Keap-1. In order to clarify the involvement of Nrf2 in **5d**-mediated neuroprotection, we silenced Nrf2 gene expression by siRNA (Fig.). Data obtained confirmed that Nrf2 played a fundamental role in neuroprotection induced by **5d** (Fig.).

A number of studies have shown that Nrf2 activation can be achieved through induction of protein kinase such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 (PI3K) (Gines et al., 2003; Nakazawa et al., 2003; Kraft et al., 2006). Therefore, we next investigated the effects of **5d** on MAPK and PI3K/Akt signaling pathways. Results showed that Akt inhibition by a PI3K inhibitor, LY294002, or a Akt-specific siRNA, blocked **5d**-induced translocation of Nrf2 to the nuclear. These findings indicate that **5d** promotes the nuclear translocation of Nrf2 through a PI3K/Akt signaling pathway, thereby protects brains against nervous injury induced by I/R or OGD/R. Interestingly, it was found that **5d** down-regulated the

expression of p-p38 and p-JNK compared with the Model group. It is well known that the activated MAPKs could play a significant role in neural cell fate via the mediation of early gene induction, which is responsible for cell survival/death following ischemic injury (Nozaki et al., 2001). These kinases are activated via the phosphorylation of specific threonine and/or tyrosine residues and subsequently phosphorylate distinct but sometimes overlapping downstream targets (Pearson et al., 2001). Thus, these results indicated that in addition to the activation of PI3K/AKT/Nrf2 signaling pathway, the inhibition of p38 and JNK signaling pathway is also involved in the neuroprotection of **5d** against cerebral ischemia.

NADPH oxidase is a major complex that produces reactive oxygen species (ROS) during the ischemic period and aggravates brain damage and cell death after ischemic injury. Although many approaches have been tested for preventing production of ROS by NADPH oxidase in ischemic brain injury, the regulatory mechanisms of NADPH oxidase activity after cerebral ischemia are still unclear. In this study, we demonstrate for the first time the role of Nrf2 as a novel negative regulator of NADPH oxidase activity using a Nrf2 siRNA. Results showed that **5d** administration inhibited the upregulation of NOX4 caused by I/R. Furthermore, siRNA transfection against Nrf2 mRNA in cortical primary neurons enhanced the expression of NOX4 elicited by **5d** treatment. These data indicate that the activation of Nrf2 results in NADPH oxidase inhibition, which is implicated in the neuropeotection of **5d** against I/R or OGD/R injury. However, the specific mechanism underlying the decreased NADPH oxidase activity induced by Nrf2 activation after I/R has not been fully elucidated. It has been

reported that Nrf2 regulated hyperoxia-induced NOX4 transcription via AREs in lung endothelium (Pendyala et al., 2011). Thus, we hypothesize that AREs might be the gene product induced by Nrf2 that is able to inhibit NADPH oxidase ensemble or activity. To verify this hypothesis is the focus of our following study.

In the light of current knowledge, **5d** induced a neuroprotection against I/R *in vivo* and protected cultured cortical neurons from OGD/R-induced injury *in vitro*. The impairment of ROS generation and the improvement of neuronal cell viability by **5d** treatment is due to the decrease of NADPH oxidase activity by promoting Nrf2 nuclear localization through PI3K/Akt signaling pathway. This current work offers an experimental basis for the clinical usage of **5d**, and also provides promising approaches or drug combinations for ischemic stroke therapy.

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Conflict of interest: The authors declare no competing financial interests.

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Figure Legends

Fig.1 **5d** protects primary cultured cortical neurons against OGD/R-induced cytotoxicity. (A) Effects of **5d** on neuronal cell viability by a MTT reduction assay. (B) Effects of **5d** on the neuronal cell viability after OGD/R injury. (C) Effects of **5d** on the neuronal cell death by LDH assessment. Data are expressed as means ± SD (n = 6). $^{*}P < 0.05$ *vs* Control group, $^{\#}P < 0.05$ *vs* OGD/R group, $^{\&}P<0.05$ *vs* NBP group at the corresponding concentration.

Fig.2 **5d** protects primary cultured cortical neurons against OGD/R-induced oxidative damage. (A) Effects of **5d** on intracellular ROS production after OGD/R injury. (B) and (C) The content of MDA and 8-OH-dG in the cerebral cortex after I/R. Data are expressed as means ± SD (n = 8). $^{*}P < 0.05$ *vs* Control or Sham group, $^{\#}P < 0.05$ *vs* OGD/R or Model group, $^{\&}P<0.05$ *vs* NBP group at the corresponding concentration.

Fig.3 Effects of **5d** on the cellular locations of Nrf2 and Keap-1 after I/R. (A) The cytosolic extracts and nuclear extracts were prepared from the cortex of ischemia hemisphere of brain and the expressions of Nrf2 and Keap1 were examined by western blot. β-actin and Lamin B were used as loading controls for cytosol and nucleus, respectively. (B) Quantitation of the western blot analysis for Nrf2 (nucleus) compared with Lamin B. (C) Quantitation of the western blot analysis for Keap-1 (cytosol) compared withβ-actin. Values are expressed as a percentage of the corresponding Sham value. Data are expressed as means ± SD (n = 4). $^{*}p<0.05$ *vs*

Sham group, $^{\#}p<0.05$ *vs* Model group.

Fig.4 Effects of **5d** on the nuclear translocation of Nrf2 after OGD/R. (A) The expression of nuclear Nrf2 in neurons after OGD/R. (B) Statistical results from the densitometric measurements after normalization against Lamin B was calculated as the mean ± SD (n = 4). Values are expressed as a percentage of the corresponding Control value. (C) Neurons were transfected with Negative siRNA or Nrf2 siRNA for 24 h, and then cells were harvested to detect Nrf2 protein levels by Western blot. (D) Neurons were transfected with siNrf2 or Negative siRNA for 24 h. The transfected cells were treated with **5d** for 24 h before OGD/R treatment. Cell viability was measured by MTT assay. Data are expressed as means ± SD (n = 6). $^{*}p<0.05$ *vs* Control group, $^{\#}p<0.05$ *vs* OGD/R group.

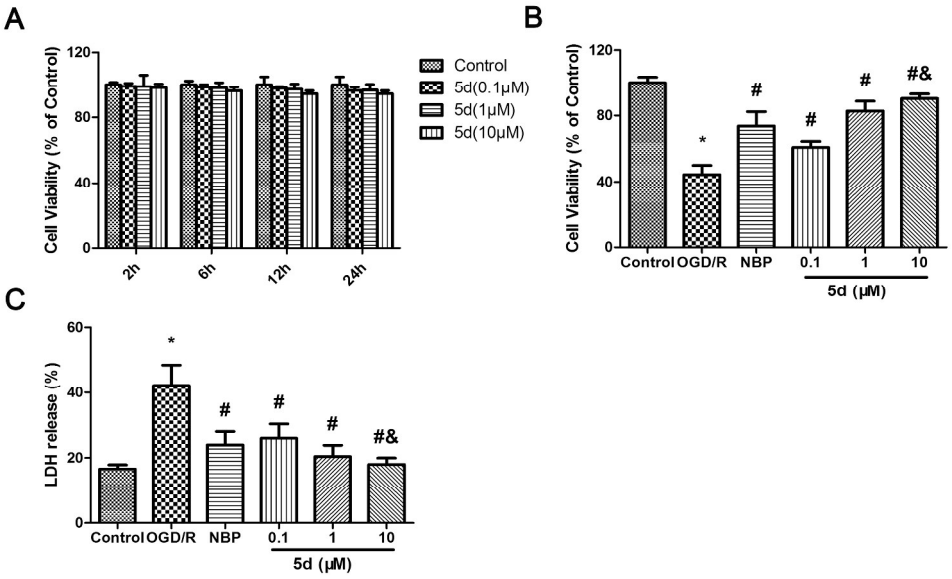
Fig.5 Effects of **5d** on MAPK and PI3K/Akt signaling pathways in the brains of rats after I/R. (A) Representative Western blots of ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK, Akt and phospho-Akt in the brains of rats after I/R. (B) Relative band densities of phospho-p38 to p38. (C) Relative band densities of phospho-Akt to Akt. (D) Relative band densities of phospho-ERK to ERK. (E) Relative band densities of phospho-JNK to JNK. Data are expressed as means ± SD (n = 4). $^{*}P < 0.05$ *vs* Sham group, $^{\#}P < 0.05$ *vs* Model group.

Fig.6 Activation of Nrf2 by **5d** via PI3K/Akt signaling pathway in cortical neurons

after OGD/R. (A) After treatment with LY294002, the expression of nuclear Nrf2 was detected by Western blot. (B) Statistical results from the densitometric measurements after normalization against Lamin B were calculated as the mean ± SD (n = 4). Values are expressed as a percentage of the corresponding OGD/R value. (C) Neurons were transfected with Negative siRNA or Akt siRNA for 24 h, and then cells were harvested to detect Akt protein levels by Western blot. (D) Neurons were transfected with siAkt for 24 h. The transfected cells were treated with **5d** for 24 h before OGD/R and the expression of nuclear Nrf2 protein was examined by Western blot. (E) Statistical results from the densitometric measurements after normalization against Lamin B were calculated as the mean ± SD (n = 4). Values are expressed as a percentage of the corresponding OGD/R value. $^{*}P < 0.05$; ns, nonsignificant.

Fig.7 Nrf2 is a novel negative modulator of NADPH oxidase. (A) The expression of NOX4 in the brains of rats after I/R determined by Western blot. (B) Statistical results from the densitometric measurements after normalization against β-actin were calculated as the mean ± SD (n = 4). Values are expressed as a percentage of the corresponding Sham value. $^{*}p<0.05$ *vs* Sham group or Control group, $^{\#}p<0.05$ *vs* model group or OGD/R group, $^{\&}p<0.05$ *vs* NBP group. (C) The NOX4 protein expression in whole cell lysates from Nrf2 siRNA-transfected primary cortical neurons subjected to OGD determined by Western blot. (D) Statistical results from the densitometric measurements after normalization against β-actin were calculated as the mean ± SD (n = 4). Values are expressed as a percentage of the corresponding

OGD/R value. $^{*}P < 0.05$; ns, nonsignificant.



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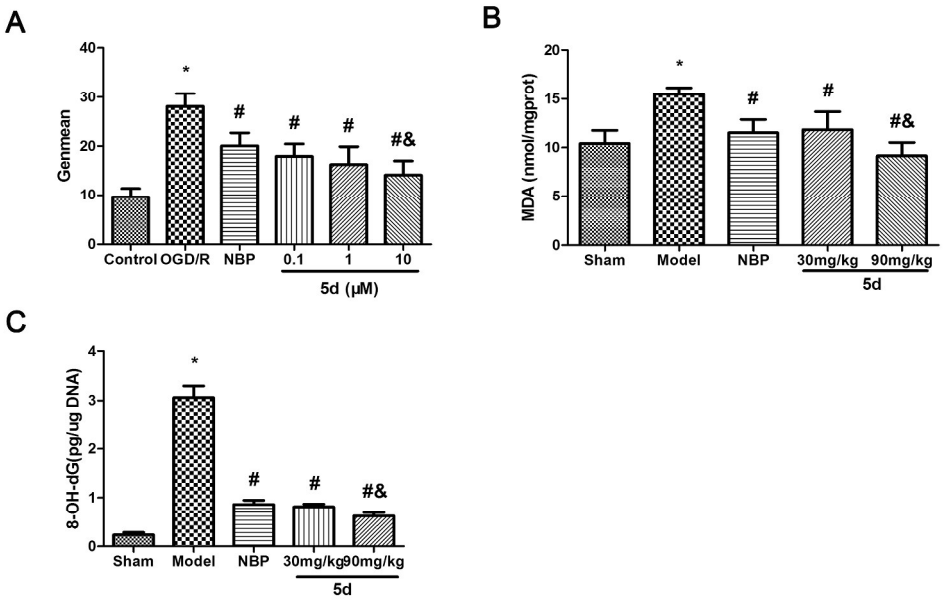

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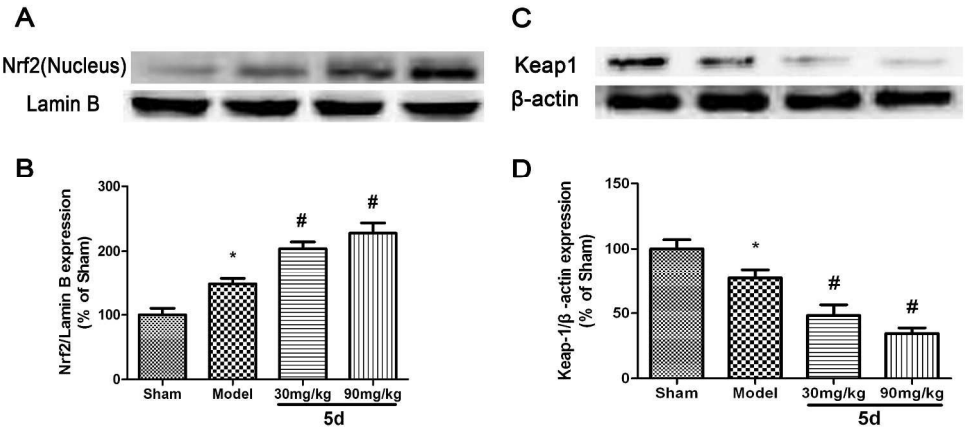

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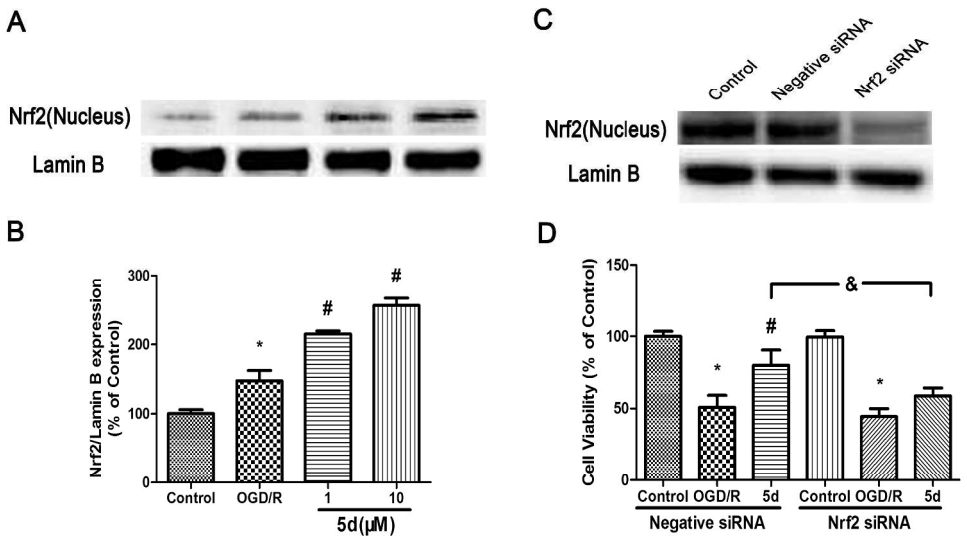

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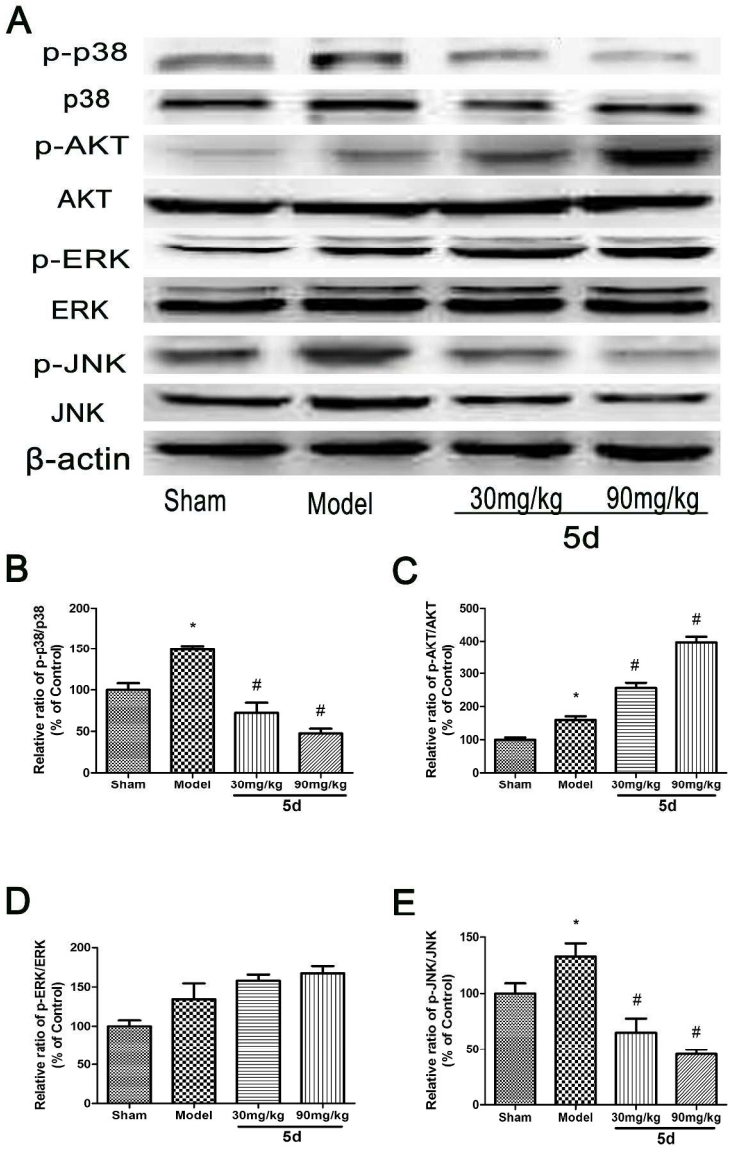

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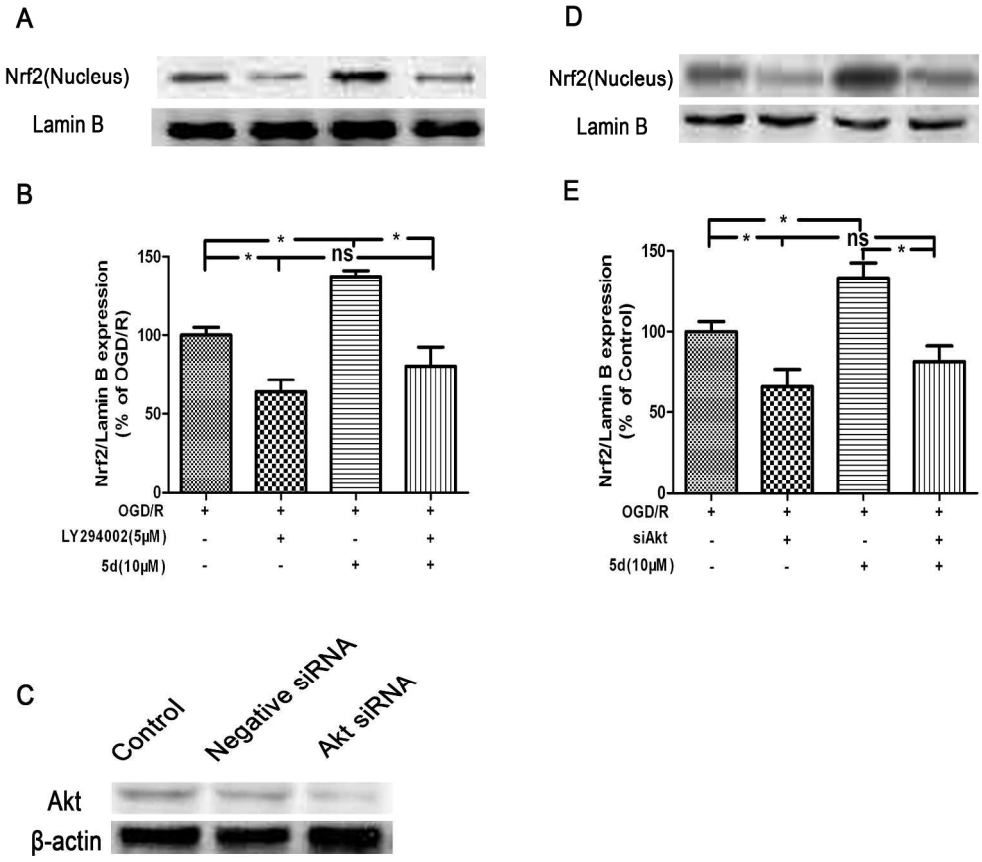

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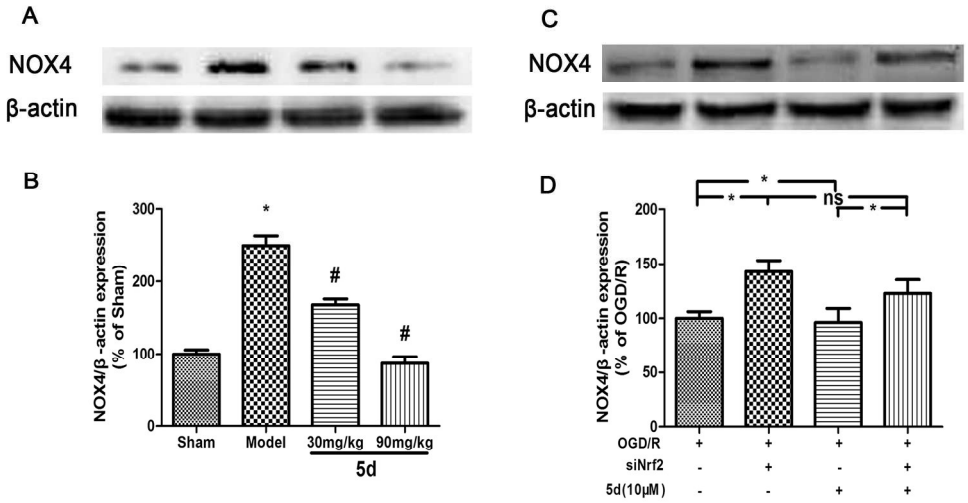

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