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(*S*)-ZJM-289, a nitric oxide-releasing derivative of 3-*n*-butylphthalide, protects against ischemic neuronal injury by attenuating mitochondrial dysfunction and associated cell death

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ABSTRACT

Pharmacological compounds that release nitric oxide (NO) have been recognized as the potential therapeutic agents for acute stroke. (*S*)-ZJM-289 is a novel NO-releasing derivative of 3-*n*-butylphthalide (NBP) with enhanced anti-platelet and anti-thrombotic actions. The present study was performed to investigate the neuroprotective effects and related mechanisms of (*S*)-ZJM-289 on ischemic neuronal injury *in vitro* and *in vivo*. Primary cortical neuronal cultures were exposured to oxygen–glucose deprivation followed by recovery (OGD/R), a model of ischemia-like injury, and treated with (*S*)-ZJM-289 before OGD. *In vitro* results showed that (*S*)-ZJM-289 attenuated OGD/R-induced neuronal injury, which was associated with the maintenance of mitochondrial integrity and function by alleviating intracellular calcium overload and reactive oxygen species (ROS) accumulation, preventing mitochondrial membrane depolarization and preserving respiratory chain complexes activities. Moreover, (*S*)-ZJM-289 treatment suppressed mitochondrial release of cytochrome *c* (cyt *c*) and nuclear translocation of apoptosis-inducing factor (AIF), thereby blocking mitochondria-mediated cell death, which may be partially mediated by up-regulation of Hsp70. The neuroprotection by (*S*)-ZJM-289 was also studied using a model of middle cerebral artery occlusion (MCAO). Oral administration of (*S*)-ZJM-289 at the onset of reperfusion for 3 d significantly reduced the brain infarct size, improved neurological deficit and prevented neuronal loss and apoptosis. In current study, (*S*)-ZJM-289 appears to be more potent in ischemic neuroprotection than NBP, in particular at the lower doses, which may be due to the synergistic action of NBP and NO. These findings point to that (*S*)-ZJM-289 could be an attractive alternative to NBP in preventing the process of ischemia/reperfusion (I/R) injury.

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1. Introduction

Stroke is the second most common cause of death and a major cause of disability with 15,000,000 new cases per year worldwide. The incidence is predicted to grow with the increasing age of the population (Donnan et al., 2008). More than 80% of all strokes are caused by cerebral ischemia. Multiple mechanisms have been implicated in the pathophysiology of ischemia–reperfusion (I/R) injury including excitotoxicity, oxidative stress, intracellular calcium overload and eventual cell death, which are associated with mitochondrial dysfunction (Janardhan and Qureshi, 2004; Crack and Taylor, 2005; Duchen et al., 2008). Accumulating evidence suggests that mitochondria are major targets of I/R injury (Galluzzi et al., 2010; Niizuma et al., 2010).

Nitric oxide (NO) plays a pivotal role in vascular protection during cerebral ischemia (Walford and Loscalzo, 2003) by promoting vasodilation, inhibiting platelet aggregation and inhibiting leukocyte adherence, suggesting that application of NO donors to improve post-ischemic cerebral blood flow is a promising therapeutic strategy. However, owing to the dual role of NO in cerebral ischemia (Moro et al., 2004), the use of NO donors as neuroprotectants is limited. More recently, the development of hybrid NO donor drugs have increased exponentially. Notably, these drugs could retain the actions of their parent compounds, accompanied

Abbreviations: AIF, apoptosis-inducing factor; cyt *c*, cytochrome *c*; Hsp70, heat shock protein 70; I/R, ischemia/reperfusion; MCAO, middle cerebral artery occlusion; NBP, 3-*n*-butylphthalide; NO, nitric oxide; OGD, Oxygen–glucose deprivation; ROS, reactive oxygen species; (*S*)-ZJM-289, (*S*)-[2-(1-diethyla-minoacetoxy) pentyl] benzoic acid-{2-methoxy-4-[2-(4-nitrooxybutoxycarbonyl)-vinyl]}phenyl ester hydrochloride.

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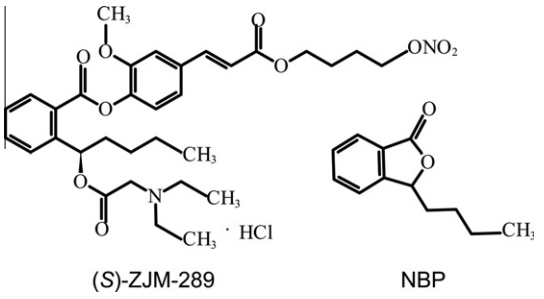

Fig. 1. Chemical structures of (*S*)-[2-(1-diethyla-minoacetoxy) pentyl] benzoic acid-{2-methoxy-4-[2-(4-nitrooxybutoxycarbonyl)-vinyl]} phenyl ester hydrochloride ((*S*)-ZJM-289) and 3-*n*-butylphthalide (NBP).

with additional beneficial actions of NO, which may have better clinical prospects.

(*S*)-ZJM-289, a novel NO-releasing derivative of 3-*n*-butylphthalide (NBP) (Fig. 1), requires enzymatic hydrolysis or non-enzymatic pathway to durably liberate low level of NO at a constant rate. As the parent compound, NBP was approved by the State Food and Drug Administration (SFDA) of China as a new drug mainly for the treatment of ischemic stroke (Zhang et al., 2006). However, the clinical application of NBP has been limited due to its poor aqueous solubility and its therapeutic efficacy would be improved only when administered by combination with other anti-platelet drugs. Interestingly, it has been recently reported that (*S*)-ZJM-289 displayed improved aqueous solubility and enhanced anti-platelet aggregation and anti-thrombotic potency by synergistic action of NBP and NO (Wang et al., 2011). Increased focus has therefore been put on the therapeutic potential of (*S*)-ZJM-289 for cerebral ischemia.

Therefore, the aims of the present study were to investigate whether (*S*)-ZJM-289 would improve the protective effects on ischemic neuronal injury *in vitro* and *in vivo* and to demonstrate the possible mechanisms underlying the neuroprotective effects of (*S*)-ZJM-289 associated with mitochondria.

2. Materials and methods

2.1. Materials

(*S*)-ZJM-289 and NBP were kindly provided by Center of Drug Discovery, China Pharmaceutical University. The purity of (*S*)-ZJM-289 and NBP are higher than 95% (Wang et al., 2011).

2.2. Cortical neurons culture

Primary cortical neurons were prepared from E17 Sprague–Dawley rats. Primary cultured cortical neurons were isolated and cultured as described previously (Brewer, 1995) with some modifications. 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 collected by centrifugation and resuspended in DMEM/F12 (1:1) with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin. 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. The neurons were maintained for 7–10 d in primary cultures until used for the following experiments.

2.3. 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 °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 recovery period. (*S*)-ZJM-289 and NBP were applied to cortical cell cultures 2 h before and during OGD/R. The vehicle-treated cultures received 0.1% DMSO.

2.4. Cell viability and apoptosis 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 an LDH kit (JianCheng Bioengineering Institute, Nanjing, China).

For detection of cell apoptosis, cells were stained with 1 μg/mL Hoechst 33258 for 10 min at 37 °C. Then, the cells were photographed under a fluorescence microscope (OLYMPUS CX41, Tokyo, Japan). The apoptotic cells were determined as condensed or fragmented nuclei with strong bright Hoechst 33258 staining.

2.5. Mitochondrial membrane potential ($\Delta\psi m$) measurement

To measure $\Delta\psi m$, the fluorescent probes JC-1 and tetramethylrhodamine ethyl ester (TMRE) were used. JC-1 selectively incorporates into mitochondria, where it forms monomers (fluorescence in green, 529 nm) or aggregates, at high transmembrane potentials (fluorescence in red, 590 nm). Mitochondria depolarization is specifically indicated by a decrease in the red-to-green fluorescence intensity ratio. At 24 h after OGD exposure, cells were incubated with 10 μg/mL JC-1 for 20 min at 37 °C. The fluorescence was then immediately examined using a confocal laser scanning microscope (OLYMPUS YMPUSFV1000, Tokyo, Japan) and a flow cytometry (BD FACSCanto, NJ, USA).

Neuronal cultures in black 96-well plates were loaded in the dark with TMRE (0.5 μM) at 37 °C in a 5% CO_2 incubator. After 20 min loading, the cells were rinsed three times with PBS. Experiments were performed in PBS containing 1 mg/mL glucose at 37 °C. TMRE fluorescence was measured with a Gemini EM microplate reader (λ_{ex} = 510 nm and λ_{em} = 590 nm). Data were expressed as a percentage of the intensity of the untreated control culture as follows: $\% \ \Delta\psi m_{sample} = (\text{TMRE-fluorescence}_{sample} - \text{TMRE-fluorescence}_{background}) \times 100/(\text{TMRE-fluoresence}_{control} - \text{TMRE-fluorescence}_{background})$.

2.6. Intracellular calcium and reactive oxygen species (ROS) measurements

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was monitored using the fluorescent Ca^{2+} indicator Fluo-3/AM. Cells were loaded with 5 μM Fluo-3/AM for 45 min at 37 °C and washed twice with PBS to remove any extracellular dye. After 30 min OGD, the stained cells were analyzed with a confocal laser scanning microscope and a flow cytometry. Intracellular ROS production was estimated using a membrane-permeable fluorescent probe DCFH-DA.

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