The Neuroprotective Effects of Benzylideneacetophenone Derivatives on Excitotoxicity and Inflammation via Phosphorylated Janus Tyrosine Kinase 2/Phosphorylated Signal Transducer and Activator of Transcription 3 and Mitogen-Activated Protein K Pathways

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ABSTRACT

To search for new neuroprotective compounds, novel benzylideneacetophenone compounds (JC1, (3E)-4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one; JC2, (1E)-1-(4-hydroxy-3-methoxyphenyl)hept-1-en-3-one; JC3, (2E)-3-(4-hydroxy-3-methoxyphenyl)phenylpro-2-en-1-one; JC4, (1E)-1-(4-hydroxy-3-methoxyphenyl)-5-phenylpent-1-en-3-one; JC5, (1E)-3-(4-hydroxy-3-methoxyphenyl)-6-phenylhex-1-en-3-one; JC6, (1E)-1-(4-hydroxy-3-methoxyphenyl)-7-phenylhept-1-en-3-one) were synthesized, and their potential to prevent neurotoxicities were evaluated. All compounds (JC1–JC6) showed considerable effect on free radical scavenging, the inhibition of glutamate-induced neurotoxicity in cortical cells, and the suppression of lipopolysaccharide (LPS)-induced nitric oxide (NO) generation in microglia. (2E)-3-(4-Hydroxy-3-methoxyphenyl)phenylpro-2-en-1-one (JC3) exhibited the most potent neuroprotective effect in ischemia model using organotypic hippocampal culture and middle cerebral artery occlusion (MCAO). Based on the above-mentioned results, the mechanisms underlying the biological activity of JC3, which exhibited potent antixcitotoxic and anti-inflammatory effects, were determined using cortical neuronal cells and microglia. Compound JC3 exerted a neuroprotective effect on oxygen-glucose deprivation- and hydrogen peroxide-induced cytotoxicity in cultured cortical cells. In addition, it suppressed the generation of NO, proinflammatory cytokines, and reactive oxygen species in LPS-treated microglial cells. It also suppressed the activation of phosphorylated Janus tyrosine kinase 2/phosphorylated signal transducer and activator of transcription 3 and mitogen-activated protein kinase (MAPK) in activated microglia and in cortex and striatum after 3 days of the MCAO in mice. These results demonstrated that JC3 might affect a set of intracellular signaling cascades, including the Janus tyrosine kinase/signal transducers and activators of transcription and MAPK pathways. This study suggests that benzylideneacetophenone derivative could be useful antineurototoxic agents.

The major pathogenic mechanisms of stroke include excitotoxicity, production of reactive free radicals, inflammation, and apoptosis (Mergenthaler et al., 2004). Activation of glutamate receptors, through the attendant failure of ion homeostasis and increase in intracellular Ca^{2+} concentration,
is a major factor involved in initiating ischemic cell death. The N-methyl-D-aspartate receptor controls an ion channel that is permeable to Ca\(^{2+}\), Na\(^{+}\), and K\(^{-}\). Antagonists at this receptor demonstrate robust neuroprotection when given before or at the time of occlusion of the middle cerebral artery (MCAO) in models of permanent or temporary ischemia (Dinagla et al., 1999).

The reaction product of superoxide and nitric oxide, which is generated after the activation of calcium-calmodulin-dependent types of nitric-oxide synthase, is the highly reactive peroxynitrite radical. The important role of oxygen free-radicals in cell damage associated with stroke is underscored by the fact that even delayed treatment with free-radical scavengers can be effective in experimental focal cerebral ischemia. The Ca\(^{2+}\)-related activation of intracellular second messenger systems, the increase in oxygen free radicals, as well as hypoxia itself, trigger the expression of several pro-inflammatory genes by inducing the synthesis of transcription factors, including nuclear factor-\(\kappa\)B, hypoxia-inducible factor 1, interferon regulatory factor 1, and STAT3. Thus, mediators of inflammation, such as platelet-activating factor, tumor necrosis factor (TNF)-\(\alpha\), and interleukin (IL)-1\(\beta\) are produced by injured brain cells (Dinagla et al., 1999). A major role in inflammation is ascribed to microglia. Microglial cells are the primary immunoeffector cells of the central nervous system. Especially in the penumbra, microglia would be activated, and the activated microglia are able to produce a multitude of proinflammatory cytokines, toxic metabolites (especially free oxygen radicals) and enzymes (cathepsin). Therefore, the inhibition of microglial activation would be an effective therapeutic approach to relieving the progression of neurodegenerative disorders. In addition to microglia, astrocytes have an important part in stroke-induced brain inflammation. They are able to produce both proinflammatory cytokines and neuroprotective factors such as erythropoietin, transforming growth factor-\(\beta\), and metallothionein-2. Because of the Janus-faced nature of microglia products (destructive, e.g., free radicals versus protective, e.g., growth factors), the overall role of microglia in cerebral ischemia is not clear at present. It is very likely that microglia at different time points play different roles, with protective or regenerative activities occurring days or even weeks after the onset of ischemia (Mergenthaler et al., 2004).

Apoptosis is characterized by a biochemical cascade that leads to the activation of certain kinds of protease: the caspases. Caspases catalyze the destruction of the cell and caspase-3 has a central part in the apoptotic signaling cascade, not only in cerebral ischemia. The genetical disruption as well as the pharmacological blockade of caspase-3 shows a robust neuroprotective effect in stroke models. The substructures metabolized by caspase-3 are DNA-repair enzymes such as poly(ADP-ribose) polymerase and DNA-dependent protein kinases (Mergenthaler et al., 2004).

Based on the findings of epidemiologic and laboratory studies, it has become increasingly evident that a variety of dietary or medicinal phytochemicals have substantial chemopreventive properties (Dragsted et al., 1993; Surh, 1999; Chun et al., 2002). *Alpinia oxyphylla*, which belongs to the ginger family (Zingiberaceae), has been used in oriental herbal medicine (Chun et al., 2002). Yakuchinone B (1-\([4']\)-hydroxy-3'-methoxyphenyl)-7-phenylhept-1-en-3-one) is a component of the seeds of *A. oxyphylla*. The conjugated 1,4-enones containing a phenyl ring belongs to an important class of natural chalcones that have shown a wide range of biological activities (Yamazaki et al., 2000). It has been reported that yakuchinone B showed several significant biological activities, such as anti-inflammatory (Srimal and Dhanwan, 1973), antitumor (Samaha et al., 1997), antiviral (Ninomiya et al., 1990), and neuroprotective effects (Oh et al., 2006). Because of their interesting structural features and characteristic therapeutic effects, we decided to design and develop novel compounds such as JC1 to JC5.

In our previous research, benzylideneacetophenone analogs (JC1–JC5) were designed and synthesized based on a structural modification of yakuchinone B (JC6) in an effort to develop neuroprotective agents with beneficial effects in neurodegenerative diseases. The biological activities of benzylideneacetophenone derivatives were evaluated for their free-radical scavenging activities, suppression of LPS-induced NO generation, and antieuxitoxic effects in vitro. Favorable scavenging ratios, in the 50% range at a concentration of approximately 50 \(\mu\)M, were found in all synthesized compounds (JC1–JC6) in the previous results (Oh et al., 2006).

The mechanisms associated with the inhibitory actions of neuroexcitotoxic and inflammatory effects were determined in both in vitro and in vivo models of ischemia. The results of this study suggest that synthesized benzylideneacetophenone derivatives could be useful neuroprotective agents in the treatment of ischemia and neurodegenerative diseases.

### Materials and Methods

**Reagents.** L-Glutamate and LPS (*Escherichia coli*, O111:B4) were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). All other reagents were obtained from Sigma-Aldrich.

**Compound Synthesis.** The synthesis of benzylideneacetophenone derivatives (JC1–JC6) was initiated as described previously (Oh et al., 2006). The 4-hydroxy-3-methoxy cinnamaldehyde was protected with tert-butyldimethylsilyl trifluoromethanesulfonate in the presence of 2,6-lutidine or 2-(trimethylsilyl)ethoxymethyl chloride (SEM–Cl)/\(N,N\)-disopropylethylamine to form aldehydes in 95 and 97% yields, respectively. Six benzylideneacetophenones (JC1–JC6) were fully identified by infrared and NMR spectroscopies, including high-resolution mass spectroscopy (Oh et al., 2006).

**Mixed Cortical Culture and Measurement of Cell Cytotoxicity and Viability.** The procedures were approved by the Animal Care and Use Committee of the School of Medicine, Ewha Womans University in Seoul, Korea. All efforts were made to minimize animal suffering and the number of animals used. After CO\(_2\) anesthesia, cerebral cortices were removed from the brains of 15.5-day-old ICR fetal mice. The neocortices were triturated and plated on 24-well plates (with approximately 1 \(\times\) 10\(^6\) cells/well), which were precoated with 100 \(\mu\)g/ml poly-d-lysine and 4 \(\mu\)g/ml laminin in modified Eagle’s medium (MEM) and supplemented with 5% horse serum, 5% fetal bovine serum (FBS), 2 mM glutamate, and 20 mM glucose. After 6 days in vitro (DIV), the cultures were shifted to the plating media containing 10 \(\mu\)M cytosine arabinoside without FBS. The cultures were then fed twice per week. After 12 to 13 days, more than 90% of neurons were microtubule-associated protein 2-positive to immunocytochemical staining and sat on the top of a confluent monolayer of astrocytes. Mixed cortical cell cultures containing neurons and glia (DIV 12–14) were exposed to the excitatory amino acid L-glutamate in MEM without 10% horse serum for 24 h to measure the condition of the cells.

Cell death was assessed by measuring the activity of lactate dehydrogenase (LDH) released in the culture medium according to the method described by Koh and Choi (1987). Culture medium collected during DIV 12–14 was used as a control for LDH activity. Growth hormone (GHR) and growth hormone releasing factor (GHRF) were used as an additional factor to stimulate the survival of the neurons in mixed cortical cultures.
after 18 to 24 h drug treatment was used unless otherwise indicated. An aliquot of 25 µl of culture medium was transferred to a microplate, and 100 µl of NADH solution (0.3 mg/ml NADH and 0.1 M potassium phosphate, pH 7.4) was added to the medium. After 2 min, 25 µl of pyruvate solution (22.7 mM pyruvate and 0.1 M potassium phosphate, pH 7.4) was added. After adding the pyruvate solution, the decrease in absorbance at 340 nm, indicating the conversion of NADH to NAD⁺, was measured using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). LDH activity was normalized on the basis of the reference scale such that the sham-treated culture and culture showing complete cell death were taken as 0 and 100%, respectively, and normalized LDH activity was regarded as an indicator of cell death.

Cell numbers and viability were assessed using the WST-1 reagent (Roche Diagnostics, Indianapolis, IN). This colorimetric assay measures the metabolic activity of viable cells based on cleavage of the tetrazolium salt WST-1 substrate 4-[3-(4-iodophenyl)-2-(-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] into formazan by phenyl-2H-tetrazolium salt WST-1 substrate 4-[3-(4-iodophenyl)-2-(-nitro-

Microglia Cell Culture and Nitrite Assay. The murine BV2 cell line (a generous gift from W. Kim, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea), which is immortalized after infection with a v-raf/v-myc recombinant retrovirus, exhibits the phenotypic and functional properties of reactive microglial cells (Bocchini et al., 1992). BV2 cells were maintained at 37°C at 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin. BV2 cells were grown in 24-well plates at a concentration of 1 × 10⁵ cells/well followed by proper treatment.

Primary microglia were prepared from 1-day-old Sprague-Dawley rats (Daehan Biolink, Eumsung, Korea) as described previously (Frei et al., 1987), with some modifications. In brief, the cortices were triturated into single cell in MEM containing 10% FBS and then plated into a 75-cm² T-flask for 10 to 14 days. To prepare the conditioned media at 37°C for 2 h. After thorough shaking, the formazan produced by the metabolically active cells in each sample was measured at a wavelength of 450 nm, whereas the reference wavelength was 650 nm. Absorbance readings were normalized against control wells with the medium alone.

Fig. 1. Structures of benzylideneacetophenone derivatives.

Fig. 2. Neuroprotective effect of benzylideneacetophenone derivatives on l-glutamate toxicity in cultured cortical cells and the suppression of NO generation in LPS-treated BV2 microglial cells. A, l-glutamate (60 µM) only or l-glutamate plus JC1-JC6 was applied for 24 h. JC3 potentially inhibited the glutamate-induced cell death. B, cells were treated with 100 ng/ml LPS or with LPS plus JC1 to JC6 for 24 h. At the end of incubation, 50 µl of the medium was collected to measure nitrite production. The amount of NO in the supernatant fractions was measured by using the Griess reagent. All values are expressed as mean ± S.E.M. from three independent experiments. #, p < 0.01 indicates significant difference between the sham group and l-glutamic acid- or LPS-treated group, respectively. *, p < 0.05 and **, p < 0.01 indicate significant differences between the glutamate or LPS group and compound-treated group, respectively. The vehicle (0.1% dimethyl sulfoxide) containing JC1-JC6 and the tested compound itself did not affect the viability or NO level of cells.
microglia, cells were removed from the T-flasks by mild shaking. Detached microglial cells were plated on 24-well plates. After 1-h incubation at 37°C, the medium containing suspended cells was discarded, and adherent cells were incubated until the experiments were performed. The homogeneity of cells was determined by immunostaining for OX-42 and generally found to be higher than 95%.

NO production from activated microglial cells was determined by measuring the amount of nitrite, a relatively stable oxidation product of NO, as described previously (Green et al., 1982). Cells were incubated with or without LPS in the presence or absence of various concentrations of compounds for 24 h. The nitrite accumulation in the supernatant was assessed by the Griess reaction. In brief, an aliquot of the conditioned medium was mixed with an equal volume of 1% sulfanilamide in water and 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid. The absorbance was determined at 540 nm in an automated microplate reader.

Organotypic Hippocampal Slice Culture. Preparation of OHC was carried out according to the procedures described by Stoppini et al. (1991) with modifications as described below. Cerebella from the brains of 6-day-old male and female Sprague-Dawley rat pups (Dae-han Biolink) were aseptically removed. The hippocampus was dissected and transversely sliced into 350-μm-thick sections on a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Slices were transferred to Millicell membranes (Mil-

Fig. 3. Neuroprotective effect of JC3 on OGD-induced neurotoxicity in OHC and on MCAO-induced cerebral ischemic injury in mice. A, hippocampal tissue slices were pretreated with JC3 for 30 min and then subjected to OGD for 2 h. After reoxygenation for 48 h, slices were examined by fluorescence microscope with PI staining. JC3 significantly inhibited the OGD-induced cellular damage. In addition, positive control was shown by MK-801. The result was representative of four separate experiments. The vehicle (0.1% dimethyl sulfoxide) in which compounds JC1 to 6 were dissolved did not affect cell death in hippocampal slices. Results are representative of three independent experiments. Magnification, 200×. B, compound JC3 (30 mg/kg) was administered intraperitoneally at 30 min before MCAO. The triphenyltetrazolium chloride-stained infarction area was measured 3 days after MCAO in coronal brain sections. The infarct volume was greatly decreased by JC3 in cortex and striatum. Scale bar, 50 mm. All values are expressed as the mean ± S.E.M. *p < 0.05 and **p < 0.01 indicate a significant difference between the MCAO group and compound-treated group, respectively (for each group, n = 6). The vehicle (2% dimethyl sulfoxide and 10% Cremophor EL) containing JC1 to JC8 and the tested compound itself did not affect the infarction or behavior in animals.
lipore, Saint-Quentin-en-Yvelines, France). Cultures were maintained at 37°C in 1 ml of the serum-based medium containing 50% MEM, 25% HBSS, 25% horse serum, 20 mM HEPES, 6 g/liter glucose, 1 mM L-glutamine, and 50 mg/ml penicillin/streptomycin, pH 7.2, for 2 days. After 10 to 12 DIV, cultures were maintained in serum-free medium, and organotypic hippocampal slices were then selected with propidium iodide (PI; 2.5 μg/ml) before the experiment.

**Oxygen-Glucose Deprivation Treatment.** In cortical culture, OGD was achieved by introducing cells into an anaerobic chamber with an atmosphere of 5% O2 and 95% N2. The culture medium was replaced three times with a glucose-free balanced salt solution (116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 1 mM NaH2PO4, and 0.01 mM glycine, pH 7.2) as described previously (Goldberg and Choi, 1993) with some modifications, that had been incubated in an anaerobic chamber for 2 h before use. In vitro ischemic incubation was initiated by placing the cultures in a humidified 37 °C incubator located in an anaerobic chamber. After 1 h of incubation, the culture wells were removed from the anaerobic chamber, and the media was changed to the glucose-free balanced salt solution mentioned above but containing glucose.

After 12 days in culture, hippocampal slices were rinsed twice with serum-free media (75% MEM, 25% HBSS, 25% horse serum, 20 mM HEPES, 1 mM glutamine, and 5 mg/ml glucose). For the stimulated ischemic injury, slices were placed in a humidified oxygen-free chamber with deoxygenated glucose-free media for 2 h. OGD was terminated by returning the slices to a normoxic incubator, and the media were switched to prewarmed normal growth media, where slices were reperfused with the normal serum-free media. In experiments conducted to evaluate the temporal development of neuronal death, PI (2.5 μg/ml) was added during the last 2 h of reperfusion, and slices were examined at 48 h after OGD. PI was included for the entire reperfusion period for the experiments involving the testing of compounds. PI fluorescent signals were viewed under a fluorescent microscope (Carl Zeiss, Jena, Germany) with a rhodamine filter set, and the images were captured and subsequently analyzed using an imaging software package (Axiovision and KS300; Carl Zeiss, Germany). Control cultures were maintained for the same period under a normoxic atmosphere in glucose-containing medium.

**Reverse Transcription-Polymerase Chain Reaction.** Rat primary microglia were stimulated with LPS in the absence or presence of JC3 for 24 h. Total RNA was isolated from microglial cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. For cDNA synthesis, 2 μg of total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). cDNA was amplified by polymerase chain reaction (PCR) using primers for IL-1β, TNF-α, iNOS, and IL-6; PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The primer sequences of rat iNOS, TNF-α, IL-1β, IL-6, and β-actin were described previously (Bhat and Fan, 2002; Kim et al., 2002; Jang et al., 2007).

**Immunoblot Analysis.** Primary microglial cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in ice-cold modified lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 0.01% Triton X-100, and protease inhibitors, pH 8.0), and cellular debris was cleared by centrifugation. Samples were assayed for protein concentration using bicinchoninic acid reagents ( Pierce Chemical, Rockford, IL). The supernatants were aliquoted and stored at −70°C until use. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 solution. The blots were incubated with the phospho- or total forms of JAK2, STAT3, extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, c-Jun NH2-terminal kinase (JNK) (Cell Signaling Technology Inc., Danvers, MA), β-Actin (Santa Cruz Biotechnology Inc., Danvers, MA); and the images were captured and subsequently analyzed using an imaging software package (Axiovision and KS300; Carl Zeiss, Germany). Control cultures were maintained for the same period under a normoxic atmosphere in glucose-containing medium.

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Fig. 4. Antioxidant effect of JC3 in OGD and H2O2-treated cortical cells. A, cells were subjected to OGD for 2 h and then reoxygenation for 48 h in the absence or presence of JC3. JC3 elevated the cell viability against OGD. B, cells were treated with 100 μM H2O2 for 30 min in the absence or presence of JC3. JC3 inhibited the H2O2-induced cell death in the WST-1 assay. All values are expressed as mean ± S.E.M. from three independent experiments. *, p < 0.05 indicates significant difference between the sham group and OGD- or H2O2-treated group, respectively. †, p < 0.01 indicate significant difference between the sham group and OGD- or H2O2-treated group, respectively. ‡, p < 0.01 indicate significant difference between OGD and H2O2 group and compound-treated group, respectively.
with a mixture of N2O and O2 (70:30) containing 2.5% isoflurane, and the following brief modifications. The animals were anesthetized with Naloxone filament method, as described previously (Kilic et al., 2001), with the exception that pentobarbital (50 mg/kg) was administered instead of barbiturate. Adult male ICR mice (male; 24–32 g) were used for the study. An MCAO was carried out according to the intraluminal filament technique, as described previously (Kilic et al., 2001), with the following modifications. The animals were anesthetized with pentobarbital (50 mg/kg) and perfused with heparinized saline from the heart. Animals were subjected to perfusion fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and decapitated. Excised brains were immersed in 4% paraformaldehyde for 24 h, then transferred to 30% sucrose. After 2 days, brains were sectioned at 30 μm, and every 5th section was immunostained with antibodies to TNF-α, IL-1β, IL-6, and β-actin. Representative sections were photographed with a microscope camera. The images were analyzed using ImageJ software (NIH, USA). The area of the ipsilateral hemisphere was determined using a computerized image analysis system (TDI Scope Eye 3.0 for Windows; Olympus, Tokyo, Japan).

**Measurement of Intracellular ROS**

Intracellular accumulation of ROS was measured with dichlorodihydrofluorescein diacetate (H2DCF-DA). The nonfluorescent compound H2DCF-DA accumulates within cells upon deacetylation and then reacts with ROS to form a fluorescent dichlorofluorescein (Qin et al., 2005). To test the effects of JC3 on the LPS-induced generation of ROS, cells were incubated with 30 μM H2DCF-DA in HBSS buffer for 1 h at 37 °C and washed twice with HBSS buffer. Compound JC3 was added 30 min before treatment with LPS for 8 h. Dichlorofluorescein fluorescence intensity was measured at an excitation of 485 nm, and an emission of 535 nm on a fluorescence plate reader (Molecular Devices).

**MCAO and 2,3,5-Triphenyl Tetrazolium Chloride Staining.**

ICR mice (male; 30–35 g) were obtained from Daehan Biolink and maintained under standard conditions (24 ± 1°C) under a 12-h light/dark cycle (light on at 8:00 AM) with free access to food and water. All procedures were performed according to the guidelines issued by the Animal Care and Use Committee of the School of Medicine, Ewha Womans University. Adult male ICR mice (male; 24–32 g) were used for the study. An MCAO was carried out according to the intraluminal filament method, as described previously (Kilic et al., 2001), with the following modifications. The animals were anesthetized with a mixture of N2O and O2 (70:30) containing 2.5% isoflurane, and they were maintained by the inhalation of 1.5% isoflurane during the operation. A midline incision was made on the ventral surface of the neck, and the right common carotid artery and external carotid artery were isolated and ligated with an 8.0 silk suture. A polyamide monofilament (Ethicon; Johnson & Johnson International, Brussels, Belgium) coated to a round tip with silicone resin (Xantopren; Beyer Dental, Leverkusen, Germany) (thread thickness) was introduced into the intracranial internal carotid artery through an incision in the common carotid artery. The filament was carefully advanced approximately 10 mm distal to the carotid bifurcation, which was beyond the origin of the middle cerebral artery. After 90 min of occlusion, recirculation was initiated by pulling out the thread. In the sham-operated rats, an incision was made over the MCA, but the artery was not occluded. The rectal temperature was maintained at 37 ± 0.5°C throughout the surgery using a heating pad (Biomed S.L., Madrid, Spain). The mice subjected to MCAO were randomly assigned to receive either drug or the vehicle treatment (n = 8 in each group). After 3 days of reperfusion, the mice were decapitated, and the brain was then immediately removed.

Removed brain was readily sectioned and the 2-mm-thick coronal sections were immediately stained by immersion in 1% triphenyltetrazolium chloride at 37°C for 15 min. Sections were incubated in 4% paraformaldehyde solution for preservation. To assess the extent of cerebral infarct volume, the area of ischemic lesion was measured in each section by subtracting the area in the ipsilateral hemisphere from that of the contralateral hemisphere. The area of the infarction (square millimeters) in each section was determined using a computerized image analysis system (TDI Scope Eye 3.0 for Windows; Olympus, Tokyo, Japan).

**Immunohistochemistry.** After transient focal ischemia, mice were anesthetized with pentobarbital (50 mg/kg) and perfused with heparinized saline from the heart. Animals were subjected to perfusion fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and decapitated. Excised brains were immersed in the same fixative for 7 days and then transferred to 30% sucrose. After 2 days, brains were sectioned at 30 μm, and every 5th section was immunostained with antibodies to TNF-α, IL-1β, IL-6, and β-actin. Representative sections were photographed with a microscope camera. The images were analyzed using ImageJ software (NIH, USA). The area of the ipsilateral hemisphere was determined using a computerized image analysis system (TDI Scope Eye 3.0 for Windows; Olympus, Tokyo, Japan).

**Fig. 5.** Effects of JC3 on NO generation and iNOS mRNA level in LPS-treated microglia. Primary microglial cells were pretreated with JC3 for 30 min and then treated with 1 μg/ml LPS for 24 h. A, JC3 decreased LPS-induced NO generation in activated microglia. All values are expressed as mean ± S.E.M. from three independent experiments; #, p < 0.01 indicates significant difference between the sham group and LPS-treated group, respectively. ***, p < 0.01 indicates a significant difference between the LPS group and compound-treated group, respectively. B, total RNA was extracted after 24 h of LPS-treatment. iNOS mRNA levels were measured using RT-PCR. JC3 repressed the LPS-induced expression of iNOS mRNA in activated microglia. Results are representative of three independent experiments.

**Fig. 6.** Reduction of LPS-induced proinflammatory cytokine mRNA expression and ROS generation by JC3 in microglia. A, primary microglial cells were treated with JC3 for 30 min and then with LPS for 24 h. mRNA was extracted for reverse transcription-PCR analysis. The expression levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 were decreased by JC3 at a concentration of 20 μg/ml in activated microglia. The result was representative of three separate experiments. B, cells were incubated with JC3 for 1 h and then treated with LPS at the indicated dose for 8 h. The elevated ROS level in LPS-treated microglia was decreased by JC3 in a dose-dependent manner. All values are expressed as mean ± S.E.M. from three independent experiments; #, p < 0.01 indicates significant difference between the sham group and LPS-treated group, respectively. ***, p < 0.01 indicates a significant difference between the LPS group and compound-treated group, respectively.
Effect of JC3 on the LPS-induced activation of JAK/STAT and MAPK activation in microglia. A, primary microglial cells were pretreated with JC3 for 30 min and stimulated with 1 µg/ml LPS for 24 h. Phosphorylation of JAK2 and STAT3 was measured by immunoblot analysis. JC3 suppressed the phosphorylation of JAK2 and STAT3 in activated microglia at 10 min and 3 h, respectively. β-Actin was used as an internal control. B, cell extracts were collected from cultured microglia after activation by LPS with pretreatment of JC3 and immunoblot analysis was performed using phospho- or total JNK, p38, and ERK antibodies. JC3 inhibited the activation of MAPK at different times, JNK and p38 at 10 min and ERK at 3 h. β-Actin was used as an internal control. All values are expressed as mean ± S.E.M. from three independent experiments. #, p < 0.01 indicate significant difference between the sham group and LPS-treated group, respectively. *, p < 0.05 and **, p < 0.01 indicate a significant difference between the LPS group and compound-treated group, respectively. Results are representative of three independent experiments.
fixative solution for overnight at 4°C and then transferred to 30% sucrose in PBS, pH 7.4, where they were kept for 48 h at 4°C. Frozen sections (20 µm) were prepared in the coronal plane using a cryostat.

Free-floating sections were incubated 24 h in PBS containing monoclonal rat anti-CD11b antibody (OX-42, 1:1000 dilution; Serotec, Oxford, UK), polyclonal rabbit anti-pJNK, anti-p-p38 antibodies (1:1000 dilution; Cell Signaling Technology Inc.), or monoclonal mouse anti-pERK (1:1000 dilution; Santa Cruz Biotechnology, Inc.), 0.3% Triton X-100, 0.5 mg/ml bovine serum albumin, and normal serum within ATP-binding cassette kit (Vector Laboratories, Burlingame, CA). The sections were then incubated for 90 min with biotinylated anti-rat, anti-rabbit, and anti-mouse IgG (1:200 dilution; Vector Laboratories) as secondary antibody, respectively, treated with avidin-biotin-peroxidase complex (1:100 dilution; Vector Laboratories) for 1 h at room temperature, and reacted with 0.02% 3,3′-diaminobenzidine and 0.01% hydrogen peroxide (H₂O₂) for approximately 3 min. After each incubation step, sections were washed three times with PBS. Finally, they were mounted on gelatin-coated slides, dehydrated in an ascending alcohol series, and cleared in xylene. Cell counts were measured using a computerized image analysis system (Leica, Wetzlar, Germany). Cells were counted in the cortex and striatum areas in six slices of four mice per group by one person blinded to treatment group identities.

Statistical Analysis. All values were expressed as mean ± S.E.M., and comparisons between groups were performed using analysis of variance followed by one-way analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons. The results are representative of three independent experiments done in duplicate. Differences with *, p < 0.01, **, p < 0.05, and ***, p < 0.01 were considered as statistically significant.

Results

Benzylideneacetophenone Derivatives Inhibited the L-Glutamate-Induced Neurotoxicity in Cortical Cells and Suppressed the LPS-Induced Generation of NO in Microglia. The chemical structures of benzylideneacetophenone derivatives are shown in Fig. 1. Analogs of alkyl, phenyl, and alkylyphenol group were introduced on the yakuchinone B backbone. The neuroprotective effect of benzylideneacetophenone derivatives on glutamate-induced cytotoxicity was investigated. Cortical cells were treated with L-glutamate (60 µM) in the presence or absence of benzylideneacetophenone derivatives for 24 h, and cell death was then assayed using LDH assay. The result showed that JC3, JC4, and JC5 exerted cytotoxic effects on L-glutamate-induced excitotoxicity in cultured cortical cells (Fig. 2A).

To investigate the anti-inflammatory effect of benzylideneacetophenone derivatives JC1-JC6, the LPS-induced production of NO in the presence or absence of benzylideneacetophenone derivatives in BV2 microglial cells. Microglial cells were pretreated with benzylideneacetophenone derivatives for 30 min and then stimulated with LPS for 24 h. Most of these compounds suppressed the generation of NO in activated microglia in a dose-dependent manner (Fig. 2B). These findings suggest that benzylideneacetophenone derivatives may suppress the LPS-induced inflammatory response through inhibition of NO generation. Therefore, these results showed that JC3 and JC5 had neuroprotective effects on excitotoxicity and inflammatory response.

Compound JC3 Prevented Both OGD-Induced Neurotoxicity in OHC and the MCAO-Induced Cerebral Ischemic Injury. OHC combined with OGD have great advantages over the other in vitro systems used to study ischemia-induced injuries. This system closely mimics an in vivo system because the cultured slices maintained their cellular architecture and interneuronal connections, which may make it useful in the test of therapeutic agents (Cho et al., 2004). To examine the protective effect of benzylideneacetophenone derivatives, ischemia was induced by OGD in an OHC, and the neurotoxicity was examined using PI staining. In brief, ischemic injury (2 h) followed by reperfusion under normoxic conditions caused significant neuronal death in the CA1 region, as measured by PI uptake. As shown in Fig. 3, OGD strongly induced neuronal cell death in the CA1 region of the hippocampus, whereas pretreatment with JC3 (10 µM) significantly decreased the amount of cell death in this region (Fig. 3A). This result suggested that JC3 exerts a potent neuroprotective effect against OGD-induced cell death. However, the other tested compounds did not affect the considerable neuroprotective effect in OGD-induced cell death (data not shown). Similar to JC3, blockade of N-methyl-d-aspartate receptors with MK-801 also exhibited a strong neuroprotective effect in this experimental system.

In addition, the neuroprotective effect of JC3 in cerebral ischemia was confirmed in animal models of ischemia. Compound JC3 was administered (30 mg/kg i.p.) at 30 min before MCAO, and the size of the infarct volume was assessed after 3 days of reperfusion. Pretreatment with JC3 (30 mg/kg) reduced the infarct volume in comparison with the saline-treated mice (Fig. 3B). This result showed that JC3 exerted a neuroprotective effect in an animal model of ischemia. Other tested compounds did not exhibit significant neuroprotective effects in MCAO model (data not shown). Therefore, JC3 was selected for further study; the biological pathway of JC3 was evaluated, and another experiment was carried out to determine the mechanisms underlying the potent neuroprotective effect of JC3.

Compound JC3 Blocked the OGD- or H₂O₂-Induced Cytotoxicity in Cultured Cortical Cells. The protective effects of JC3 were investigated under certain oxidative stress paradigms, such as OGD in cortical cells. Cell viability was reduced when cortical cells were exposed to OGD for 2 h and then supplied with oxygen and glucose for 48 h. However, JC3 inhibited OGD-induced cell death at concentrations of 1, 5, and 10 µM (Fig. 4A).

Furthermore, the protective effects of JC3 were examined under the other oxidative stress paradigm, H₂O₂ toxicity, which has become a well established model for oxidative stress on cortical cells (Lee et al., 2005). Application of JC3 at a concentration of 10 µM markedly protected neurons from H₂O₂-induced toxicity, whereas treatment with 100 µM H₂O₂ alone induced severe cytotoxicity (Fig. 4B). Therefore, these results indicated that JC3 exerted protective effects under different stress conditions, such as OGD and H₂O₂-induced toxicity, in cultured cortical cells.

Compound JC3 Suppressed the LPS-Induced Generation of NO and iNOS Expression. As shown in Fig. 2B, JC3 had an inhibitory effect on LPS-induced NO generation in BV2 microglial cells. Compound JC3 was applied to primary microglia for 30 min before treatment with 1 µg/ml LPS for up to 24 h. LPS treatment considerably increased NO generation, whereas JC3 significantly suppressed NO generation in primary microglia (Fig. 5A). In addition, iNOS mRNA expression was highly induced by LPS, and this expression was inhibited by JC3 (Fig. 5B). These results implied that suppression of NO generation by JC3 might be due to the inhibition of iNOS mRNA expression.
Compound JC3 Reduced the LPS-Induced Expression of Proinflammatory Cytokine mRNA and ROS Generation. JC3 exerted an anti-inflammatory effect on LPS-induced responses accompanied by the induction of proinflammatory cytokines and ROS generation. Primary microglial cells were pretreated with JC3 for 30 min and then stimulated with LPS for 24 h. The mRNA expression levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were reduced by treatment with JC3 at a concentration of 20 μM (Fig. 6A). In addition, the ROS level was highly elevated after LPS treatment for 8 h, whereas JC3 significantly suppressed the generation of ROS in a dose-dependent manner (Fig. 6B). These results indicated that JC3 had an anti-inflammatory effect on the expression of LPS-induced proinflammatory cytokines and ROS generation in primary microglia.

The JAK/STAT and MAPK Pathways Mediated the Anti-Inflammatory Effects of JC3 in Activated Microglia. Compound JC3 exhibited anti-inflammatory effects against LPS-induced inflammation (Figs. 5 and 6). Therefore, the mechanisms underlying the anti-inflammatory activity of JC3 were investigated in the JAK/STAT-signaling cascade in primary microglia. As shown in Fig. 7A, JAK2 phosphorylation was strongly increased by LPS, whereas JC3 inhibited LPS-induced JAK2 phosphorylation. STAT3 phosphorylation is mediated by cytokines, followed by the recruitment of cytosolic pJAK2 and JC3 inhibited LPS-induced STAT3 phosphorylation (Fig. 7A). These results suggested that JC3 might suppress NO expression through inhibition of the JAK/STAT pathway.

Furthermore, glial cells were pretreated with JC3 for 30 min before LPS stimulation to investigate whether inhibition of iNOS expression by JC3 occurred through alteration of MAPK activities. Immunoblot analysis was then performed using the phospho- or total forms of antibodies against mitogen-activated protein kinases, such as p38, ERK1/2, and JNK. Compound JC3 significantly inhibited the LPS-stimulated phosphorylation of JNK and p38 at 10 min. In addition, ERK phosphorylation was reduced by JC3 at 3 h (Fig. 7B). JC3 may exert its anti-inflammatory effect by regulating the MAPK pathway to suppress NO generation.

Fig. 8. Inhibition of microglial activation by JC3 in brain after MCAO. A, compound JC3 (30 mg/kg) was administered intraperitoneally at 30 min before MCAO. The OX-42-immunopositive cells were highly elevated in the cortex and striatum of MCAO group, whereas they were suppressed by the treatment with the compound JC3. Scale bar, 50 μm. B, histogram indicates the number of OX-42-immunopositive cells per field (square millimeter). The histograms represent mean ± S.D. of four mice per group. For each regional cell was counted in four 100× fields by evaluator blinded to study groups. #, p < 0.01 indicates significant difference between the sham group and MCAO group, respectively. **, p < 0.01 indicates a significant difference between the MCAO group and compound JC3-treated group, respectively (for each group, n = 4). The vehicle (2% dimethyl sulfoxide and 10% Cremophor EL) containing JC3 and JC3 itself did not affect the OX-42 expression.
The JAK/STAT and MAPK Pathways Mediated the Anti-Inflammatory Effects of JC3 in MCAO Model. Based on anti-inflammatory effect of JC3 in microglia cells, the effect of JC3 was examined on inflammatory process in ischemic brain of mice. Compound JC3 was administered (30 mg/kg i.p.) at 30 min before MCAO. The expression of OX-42, marker of activated microglia, was assessed after 3 days by immunohistochemistry. In the sham-operated animals, OX-42-immunopositive cells were highly elevated in the cortex and striatum of MCAO group, whereas they were suppressed by the treatment with the compound JC3. Scale bar, 50 μm. B, histogram indicates the number of pJAK2- and pSTAT3-immunopositive cells per field (square millimeter). The histograms represent mean ± S.D. of four mice per group. For each mouse cells were counted in four 100× fields by evaluator blinded to study groups. #, p < 0.01 indicates significant difference between the sham group and MCAO group, respectively. **, p < 0.01 indicates a significant difference between the MCAO group and compound JC3-treated group, respectively (for each group, n = 4). The vehicle (2% dimethyl sulfoxide and 10% Cremophor EL) containing JC3 and JC3 itself did not affect the pJAK2 or pSTAT3 expression.
Fig. 10. Anti-inflammatory mechanisms of JC3 on activation of MCAO-induced MAPK pathways. A, compound JC3 (30 mg/kg) was administered intraperitoneally at 30 min before MCAO. The MAPK (pJNK, p-p38, and pERK)-immunopositive cells were highly elevated in the cortex and striatum of MCAO group, whereas they were suppressed by the treatment with the compound JC3. Scale bar, 50 μm. B, histogram indicates the number of MAPK (pJNK, p-p38, and pERK)-immunopositive cells per field (square millimeter). The histograms represent mean ± S.D. of four mice per group. For each mouse, cells were counted in four 100× fields by an evaluator blinded to study groups. *, p < 0.01 indicates significant difference between the sham group and MCAO group, respectively. **, p < 0.01 indicates a significant difference between the MCAO group and compound JC3-treated group, respectively (for each group, n = 4). The vehicle (2% dimethyl sulfoxide and 10% Cremophor EL) containing JC3 and JC3 itself did not affect the MAPK expression.
tive cells were barely detected in the brain, and JC3 only did not affect the OX-42 expression (data not shown). At 3 days after MCAO and reperfusion, the activated microglia were significantly detected in the cortex and striatum of ischemic hemisphere. In contrast, the activation of microglia was suppressed in the brains of JC3-administered mice (Fig. 8). Therefore, the anti-inflammatory effect of JC3 was confirmed through the decreased of OX-42-immunopositive cells in an in vivo model.

We evaluated the anti-inflammatory mechanisms of JC3 in ischemic brain, because the anti-inflammatory effect of JC3 was demonstrated through pJAK2/pSTAT3 and MAPK in activated microglia by LPS as shown in Fig. 8. In immunohistochemical analysis, little pJAK2 and pSTAT3 staining was shown in the cortex and striatum of the sham-operated mice. After transient MCAO, pJAK2 and pSTAT3-immunopositive cell numbers were highly increased in the ipsilateral cortex and striatum on the ischemic brain. The treatment of compound JC3 before MCAO significantly inhibited the expression of pJAK2 and pSTAT3 in ischemic brain (Fig. 9). In addition, the MAPK (pJNK, p-p38, and pERK) immunoreactivity was faint in the cortex and striatum of the sham-operated mice. The ipsilateral cortex and striatum showed the elevation of pJNK, p-p38, and pERK-immunopositive cells in ischemic brain. Among these kinases, expression of p-p38 was remarkably increased in ipsilateral striatum after MCAO. The elevated MAPK-immunopositive cells on cortex and striatum were suppressed by the treatment with JC3 (Fig. 10). The vehicle (2% dimethyl sulfoxide and 10% Cremophor EL (polyethoxylated castor oil; BASF Wyandotte, Wyandotte, MI) or JC3 itself did not affect the pJAK2/pSTAT3 or MAPK expression in mice. Compound JC3 had shown anti-inflammatory effect might via suppression of activation of pJAK2/pSTAT3 and MAPK in transient MCAO model.

Discussion

Over the past few decades, many researchers have attempted to develop antineurotoxic agents that are capable of preventing the release of glutamate (Hurtado et al., 2005), activation of microglia (Kim et al., 2004), oxidative stress (Gazaryan et al., 2007), Ca²⁺ influx (Weng and Kriz, 2007), apoptosis (Miao et al., 2007), and cell cycle arrest (White et al., 2004). Although many reagents, such as glutamate receptor antagonists, Ca²⁺ channel blockers, anti-inflammatory agents, and nitric-oxide synthase inhibitors, have shown neuroprotective effects, their serious side effects have limited their clinical application. Therefore, researchers searched for novel compounds with better neuroprotective effects and fewer side effects. The greater potency of the novel compounds and their effectiveness against neurodegenerative diseases have generated a great deal of excitement among neuroscientists, medicinal chemists, and clinicians.

Yakuchinone B has been shown to have a variety of biological effects. Two major diarylheptanoids, yakuchinone A and yakuchinone B, which are found in A. oxyphylla, have been reported as strong inhibitors of prostaglandin biosynthesis in vitro (Kiuchi et al., 1992). Like curcumin, these substances have a diarylheptanoid moiety with carbonyl functional group and are thought to exhibit cancer chemopreventive activity. Because tumor promotion is closely linked to inflammation and oxidative stress, it is likely that compounds with strong anti-oxidative and anti-inflammatory activities can act as antitumor promoters (Surh, 1999). It has been reported that yakuchinone A and yakuchinone B down-regulate cyclooxygenase-2 and iNOS expression through suppression of nuclear factor-κB activation in the tumor promoter 12-O-tetradecanoylphorbol 13-acetate-treated mouse skin (Chun et al., 2002). Thus, for search for effective neuroprotective compounds, we designed and synthesized the novel compounds JC1 to JC5, which are based on the structure of JC6, and we evaluated their biological activity against ischemia in in vitro and in vivo models.

In this study, although almost all of the compounds (JC1–JC6) had considerable effects on the suppression of LPS-induced NO generation in microglia, JC3 to JC5 exerted antiexcitotoxic effects on glutamate induced neurotoxicity in cultured cortical cells. Interestingly, JC3 showed a significant anti-ischemic effect in OGD-induced neurotoxicity in organotypic hippocampal slices and in animal models of MCAO.

Compound JC3, which was selected on the basis of previous results, was the benzylideneacetophenone derivative to exert a potent neuroprotective effect in both in vitro and in vivo model. This presumably indicates that JC3 has a structurally stable form due to electron delocalization and that it engages in hydrogen bonding with its receptor binding site. These results implied that the elimination of radicals of benzylideneacetophenone derivatives could be accelerated to the coordination on the receptor binding sites. Especially, JC3 had the smallest highest occupied molecular orbital-lowest occupied molecular orbital gap, which signifies rapid electron and radical transfer between highest occupied molecular orbital and lowest occupied molecular orbital (data not shown). This might be one of the reasons that JC3 showed good free radical scavenging activity.

The antiexcitotoxic and anti-inflammatory effects of JC3 were investigated in cortical neuronal cells and microglia. JC3 inhibited the neurotoxicity that was induced under different oxidative stress paradigms, such as OGD and H₂O₂ application, in cortical cells. JC3 also suppressed the inflammatory responses to LPS-induced NO generation, iNOS gene expression, proinflammatory cytokine expression, and ROS generation in activated microglia. Finally, it was determined that these actions were initiated by a set of intracellular signaling cascades that includes the JAK2/STAT3 and MAPK pathways to regulate iNOS gene expression. Although the precise anti-inflammatory mechanism of compound JC3 was not identified in the present study, we partially demonstrated that the neuroprotective effect of JC3 prevented the JAK2 signaling pathway using AG490, a JAK-specific inhibitor (data not shown).

Transcription of the iNOS gene is induced by LPS or cytokines such as interferon-γ, IL-1β, or TNF-α. Cytokine signal transduction is thought to be mediated in the regulation of iNOS gene, predominantly through the JAK/STAT signaling pathway (Marrero et al., 1998; Gautron et al., 2002; Heinrich et al., 2003). STAT3, which is activated by several cytokines, recently emerged as a marker in neuronal cells. It is known that STAT3 activation is mediated by cytokines followed by the recruitment of cytokolic JAKs. Finally, phosphorylated STAT3, which forms hetero- or homodimers, is translocated into the nucleus; consequently gene expression is elevated by binding to STAT-responsive element (Singh et al., 2006; Jang et al., 2007). Recent study reported that transient MCAO in adult rats increased JAK2 and STAT3 phosphorylation in the ipsilateral cortex and striatum after 6 to 72 h of reperfusion (Satriotomo et al., 2006). In our experiment, pJAK2- and pSTAT3-stained cells were highly elevated after 3 days MCAO in mice, and its elevation was suppressed by JC3 treatment. Intracerebroventricular infusion of AG490 pre-
vented the postschematic JAK2 and STAT3 phosphorylation and significantly decreased the infarct volume, number of apoptotic cells, and neurological deficits compared with vehicle control in rats. Furthermore, intracerebral injection of small interfering RNA-specific STAT3 curtailed STAT3 mRNA expression and phosphorylation, decreased infarct volume, and improved neurological function following MCAO (Satriotomo et al., 2006). These results suggest that JAK2-STAT3 activation plays a role in postschematic brain damage and support our results that inhibition of ischemic-induced JAK2 activation and the downstream STAT3 phosphorylation might be one of the reasons in reduction of neurotoxicity.

Among the various pathways of inflammatory response, MAPK is extracellular to signals that the intracellular responses to an array of extracellular stimuli that include mitogens and other physical stressors (Gardiero et al., 2006). Two stress-activated kinases, JNK and p38 MAPK, are activated by H$_2$O$_2$, whereas the sensitivity of ERK, which normally mediates mitogenic and trophic factor signaling, to oxidative stress varies with the cell type and stimulus (Pawate et al., 2004). Other studies have shown that exposure of glia to H$_2$O$_2$ results in the activation of ERK, Akt, JNK, and p38 MAPK (Salsman et al., 2001). MAPKs and their upstream kinases finally activate several transcription factors and induce transcription from a variety of inflammatory genes in response to LPS and cytokines (Guha and Mackman, 2001).

In this study, we demonstrated that iNOS expression in response to LPS and cytokines (Guha and Mackman, 2001). MAPKs and their upstream kinases finally activate several transcription factors and induce transcription from a variety of inflammatory genes in response to LPS and cytokines (Guha and Mackman, 2001). In this study, we demonstrated that iNOS expression in activated microglia involves intracellular signaling via the MAPK pathway and elevated phosphorylated MAPK (pJNK, p-p38, and pERK) expression in the brain after MCAO was suppressed by the pretreatment with JCS in mice. These results suggest that the protective effect of benzylideneacetophenones against neurotoxicity is related to its antioxidant and anti-inflammatory activities. Thus, further studies are needed to identify the transcriptional levels and anti-oxidant mechanisms of JCS that are involved in the modulation of the JAK/STAT and MAPK pathways.

In conclusion, JCS can be used as a therapeutic means of suppressing cerebral ischemic injury, and it can be a useful lead molecule in the design and development of better therapeutic agents for neuroprotection.

**References**


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