

Original Article

A novel GSK-3 β inhibitor YQ138 prevents neuronal injury induced by glutamate and brain ischemia through activation of the Nrf2 signaling pathway

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Aim: To discover neuroprotective compounds and to characterize the discovered active compound YQ138 as a novel GSK-3 β inhibitor.

Methods: Primary rat cerebellar granule cells (CGCs) were treated with glutamate, and cell viability was analyzed with MTT assay, which was used as *in vitro* model for screening neuroprotective compounds. Active compound was further tested in OGD- or serum deprivation-induced neuronal injury models. The expression levels of GSK-3 β downstream proteins (Nrf2, HO-1, NQO1, Tau and β -catenin) were detected with Western blotting. For evaluating the neuroprotective effects *in vivo*, adult male rats were subjected to transient middle cerebral artery occlusion (tMCAO), then treated with YQ138 (10 mg/kg, iv) at 2, 4 and 6 h after ischemia onset.

Results: From a compound library consisting of about 2000 potential kinase inhibitors, YQ138 was found to exert neuroprotective effects: pretreatment with YQ138 (0.1–40 μ mol/L) dose-dependently inhibited glutamate-induced neuronal death. Furthermore, pretreatment with YQ138 (10 μ mol/L) significantly inhibited OGD- or serum deprivation-induced neuronal death. Among a panel of seven kinases tested, YQ138 selectively inhibited the activity of GSK-3 β (IC₅₀=0.52 nmol/L). Furthermore, YQ138 dose-dependently increased the expression of β -catenin, and decreased the phosphorylation of Tau in CGCs. Moreover, YQ138 significantly increased the expression of GSK-3 β downstream antioxidative proteins Nrf2, HO-1, NQO1, GSH and SOD in CGCs. In rats with tMCAO, administration of YQ138 significantly decreased infarct volume, improved the neurological deficit, and increased the expression of Nrf2 and HO-1 and the activities of SOD and GSH in the cerebral cortex.

Conclusion: A novel GSK-3 β inhibitor YQ138 effectively suppresses brain ischemic injury *in vitro* and *in vivo*.

Keywords: GSK-3 β inhibitor; YQ138; cerebellar granule cells; glutamate excitotoxicity; OGD; middle cerebral artery occlusion; brain ischemia; neuroprotection; Nrf2

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Introduction

Glutamate plays important roles as the predominant excitatory neurotransmitter in the mammalian brain^[1]. However, excessive release of glutamate results in excitotoxicity and is a major factor in neuronal injury associated with many acute and chronic brain disorders, including brain ischemia^[1–3]. Ischemic stroke is one of the major causes of mortality and

disability in adults, and it has become a burden worldwide^[4]. Currently, there are no pharmacological treatments to ameliorate glutamate excitotoxicity and provide neuroprotection for brain ischemic stroke^[5]. This indicates an urgent need to search for novel compounds with neuroprotective effects for the treatment of brain ischemic stroke.

Several lines of evidence have suggested that glycogen synthase kinase-3 β (GSK-3 β) is involved in ischemic brain injury^[6], and glutamate incubation in neurons increases GSK-3 β activation^[6]. GSK-3 β is a serine/threonine protein kinase, which is abundant in the central nervous system (CNS), particularly in neurons^[7]. GSK-3 β phosphorylates and regulates many important metabolic and signaling proteins, structural proteins

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and transcription factors, and as a result, it plays a key role in glycogen metabolism, embryogenesis, mitotic regulation, inflammation and neuroplasticity^[8]. One of the important substrates of GSK-3 β is the microtubule binding protein Tau, which plays a critical role in microtubule function in specific neurons^[9]. Tau phosphorylation is increased in transgenic mice conditionally overexpressing GSK-3 β ^[10]. Recent studies have shown that the inhibition of GSK-3 β attenuates apoptotic signals and prevents neuronal death^[11, 12]. GSK-3 β phosphorylation at tyrosine 216 residue is increased after middle cerebral artery occlusion (MCAO), indicating the involvement of active GSK-3 β ^[13]. GSK-3 β inhibition by lithium has been shown to be protective against cerebral ischemia and to prevent neurons from glutamate-induced apoptosis, suggesting that GSK-3 β inhibition improves brain neurons survival^[14–16].

The redox-sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), a member of the cap 'n' collar family of transcription, is the downstream substrate of GSK-3 β , which interacts with Kelch-like ECH-associated protein 1 (Keap1) under normal conditions^[17]. When cells are exposed to oxidative, nitrosative or electrophilic stress, the Keap1-Nrf2 complex is dissociated; Nrf2 accumulates in the nucleus and, together with small Maf proteins, binds to anti-oxidant response element (ARE) regions, thus promoting the generation of antioxidant proteins, such as heme oxygenase (HO-1)^[18]. HO-1 is a ubiquitous and redox-sensitive enzyme, which has some favorable effects, such as anti-inflammatory, antioxidant and anti-proliferative effects. It also participates in the conversion of heme into biliverdin^[19]. This antioxidant protein HO-1 may play an important role in suppressing neuronal damage or death.

In this study, using a well-characterized and reliable model, glutamate-induced neuronal injury in rat cerebellar granule cells (CGCs), we screened our in-house compound library consisting of 2000 small-molecule inhibitors against potential kinases and discovered the novel neuroprotectant small molecule YQ138. We determined the neuroprotective effects of YQ138 and the relevant underlying mechanisms. Our results indicated that the novel GSK-3 β inhibitor YQ138 shows neuroprotective effects through activation of the antioxidative Nrf2 signaling pathway, further increasing glutathione (GSH) and superoxide dismutase (SOD) levels and thereby suppressing ischemia-induced brain injury.

Materials and methods

Materials

Cell-culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3,5-triphenyltetrazolium chloride (TTC), trypsin, poly-L-lysine were purchased from Sigma-Aldrich (St Louis, MO, USA). Primary antibodies used for Western blotting analysis were mouse anti- β -actin (1:2000) and rabbit anti-Nrf2 (1:1000), purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-HO-1 (1:1000), purchased from Abcam (Cambridge, UK); and rabbit anti-NAD(P)H:quinone oxidoreductase 1 (NQO1, 1:1000),

rabbit anti-phospho-GSK-3 β ^{Ser9} (1:1000), rabbit anti-phospho-Tau (1:1000), and rabbit anti- β -catenin (1:1000), purchased from Cell Signaling Technology (Danvers, MA, USA). The GSH and SOD assay kits and RIPA buffer were purchased from Beyotime Institute of Biotechnology (Nanjing, China). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN, USA). All other reagents were obtained from Sigma-Aldrich.

Primary CGC cultures

Primary CGCs were isolated from 8-d old Sprague-Dawley rat pups, through previously described procedures^[20]. Cerebella were collected and placed into ice-cold Hanks' balanced salt solution (HBSS). The cerebella were dispersed into the same buffer containing 0.025% trypsin-EDTA after removal of the meninges and then digested for 15 min at 37°C. Trypsin digestion was stopped by the addition of two volumes of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) medium and 0.1 mg/mL DNase I. Digested tissues were centrifuged at 1500 \times r/min for 5 min at 4–10°C after gentle trituration. The tissue was resuspended in a complete Neurobasal culture medium supplemented with 2% B27 and 0.5 mmol/L GlutaMax and filtered through a 70- μ m nylon cell restrainer. The cells were diluted to 1 \times 10⁶ cells/mL in poly-L-lysine coated plates. Cultures were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Primary fetal cortical neuron cultures

Day E16 to E18 embryos were prepared for primary cultures of cortical neurons, as previously described^[21]. Pregnant Sprague-Dawley rats were sacrificed by cervical dislocation. To isolate the cortical neurons, the fetuses were removed from the abdomens of the E16 to E18 rats. Cerebral cortices were placed into ice-cold HBSS. After the scalp and skull were peeled away, the cortex was removed and placed into ice-cold HBSS. Next, the tissue was incubated with 0.05% trypsin-EDTA for 10 min at 37°C. The digestion was stopped by the addition of Neurobasal medium with 0.1 mg/mL DNase I. The following steps were performed in the same manner as the primary rat CGCs culture protocol.

Cell viability assay

CGCs were pretreated with compounds for 24 h and then were incubated with 200 μ mol/L glutamate or without nutrient B27 for an additional 24 h, or were subjected to OGD conditions. The primary fetal cortical neurons were treated with glutamate at concentrations of 100 μ mol/L for 10 min in an experimental buffer consisting of 120 mmol/L NaCl, 3.5 mmol/L KCl, 0.4 mmol/L KH₂PO₄, 5 mmol/L NaHCO₃, 20 mmol/L HEPES, 1.2 mmol/L Na₂SO₄ supplemented with 15 mmol/L glucose and 1.2 mmol/L CaCl₂ at pH 7.4. Cultures were rinsed with a 1.2 mmol/L MgCl₂-supplemented experimental buffer and returned to preconditioned medium. The cells were then incubated with a serum-free medium containing MTT at a final concentration of 0.5 mg/mL in a humidified

atmosphere of 5% CO₂ and 95% air at 37°C. The medium was replaced with DMSO after 4 h, and the absorbance was measured at 570 nm with a BD plate-reader.

Oxygen and glucose deprivation (OGD)

To induce ischemia, cells were pretreated with YQ138 for 24 h, and the culture medium was replaced with pre-warmed HBSS (PH 7.4). Cell cultures were then placed in a hypoxic chamber containing a mixture of 95% N₂ and 5% CO₂ for 6 h. OGD was stopped by replacing HBSS with the Neurobasal medium, and cells were then incubated for an additional 24 h under normal conditions. Control cells without OGD were maintained under normal conditions.

Western blotting analyses

After treatment, cells were lysed in Tris-Glycine SDS lysis buffer, and the cell lysis was boiled for 10 min. Whole protein samples were separated on SDS-PAGE gels. After separation on SDS-PAGE gels, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes at 100 V for 3 h. The membranes were blocked for 2 h with 5% bovine serum albumin (BSA) in Tris-buffered saline plus Tween 20 (TBST, PH 7.4) and incubated with primary antibodies diluted (1:1000) in blocking buffer with BSA. After several washes with TBST, the membranes were incubated with the secondary antibodies for 1 h at room temperature. The membranes were washed again, and the transferred proteins were visualized through chemiluminescence with a Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA, USA).

GSH and SOD measurement

The cellular GSH and SOD levels were measured by using the GSH and SOD assay kit according to the manufacturer's instructions after treatment. For the brain tissues, the rats were sacrificed, and the brains were collected at 24 h after MCAO. Next, brain cortices were homogenized, and the GSH and SOD concentrations were also measured by using the GSH and SOD assay kit.

Kinase assay

The human kinome, consisting of 518 kinases, is classified into CMGC, AGC, TK, TKL, CAMK, STE and 7 other subfamilies, on the basis of DNA sequences and evolution. GSK-3 β belongs to CMGC family. Here, other family members were used to evaluate the selectivity of GSK-3 β inhibitors, including PKC ϵ (AGC family), JAK2 (TK family), BRAF (TKL family), DRAK2 (CAMK family) and IKK β (other family). The recombinant GST-GSK-3 β protein was expressed in *Escherichia coli* strain BL21-Codon Plus (DE3), purified by GSTrap affinity chromatography, and cleaved by thrombin. The GSK-3 β kinase assay was performed with a Z'-LYTE™ kinase assay kit Ser/Thr 9 Peptide substrate (Invitrogen, Grand, NY, USA) in a 10- μ L reaction volume containing 50 nmol/L enzyme, 30 μ mol/L ATP and 2 μ mol/L substrate peptide. DRAK2 proteins were provided by Professor Jiang-ping Wu (Laboratory

of Immunology, Research Centre, CHUM, Notre Dame Hospital, Pavillon DeSève). The DRAK2 kinase reaction was performed in a final assay volume of 3.4 μ L using the ADP-GLO kinase assay kit (Promega, Madison, WI, USA), according the ADP-GLO protocol and was read on an EnVision plate reader (Perkin Elmer, Wellesley, MA, USA). The recombinant PKC ϵ , IKK β , and JAK2 with N-terminal His-tag were expressed using the baculovirus expression system and purified with Ni-Beads. BRAF protein was obtained from Carna Biosciences, Inc (Kobe Port Island, Japan). Related kinase reactions were performed in a final assay volume of 10 μ L using the related HTRF assay kit (Cisbio, Codolet, France). Reactions were performed according the HTRF protocol and were read on an EnVision plate reader. The CDK2/CycA2 protein was obtained from Carna Biosciences, Inc. The CDK2/CycA2 kinase assay was performed with a Invitrogen Z'-LYTE™ kinase assay kit (Ser/Thr 12 peptide substrate), with a final enzyme concentration of 2 nmol/L. All reactions were performed in triplicate. IC₅₀ values (concentration at which a 50% of enzyme inhibition is shown) were derived from a nonlinear regression model (curve fit) based on sigmoidal dose response curve (variable slope) and computed using Graphpad Prism version 5.02, Graphpad Software (San Diego, CA, USA). Data are expressed as the mean \pm SD.

Preparation of compound YQ138

The synthetic approach of compound YQ138 is outlined in Figure 1. Indole **1** was reacted with oxalyl chloride in Et₂O, followed by sodium methoxide to obtain **2**. N-alkylation of **2** with 1-(2-chloroethyl)-1H-imidazole resulted in the key intermediate **3**. Condensation of glyoxilic esters **3** with 2-(benzo[d]isoxazol-3-yl)acetamide **4** in the presence of *t*-BuOK in tetrahydrofuran yielded the target compound YQ138.

Animals

Adult male Sprague-Dawley (SD) rats (260–280 g, Grade II) were purchased from Zhejiang Laboratory Animals Center (Hangzhou, China) and maintained under standard housing conditions at temperatures between 20°C and 23°C with a 12-h light/dark cycle and a relative humidity of 50%. All procedures were performed according to the US National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals published by the US National Academy of Sciences (<http://oacu.od.nih.gov/regs/index.htm>). All animal tests and experimental procedures were approved by the Administration Committee of Experimental Animals in Jiangsu Province and the Ethics Committee of China Pharmaceutical University. The animal experiments were performed according to the National Research Council's guidelines.

Drug treatment

Animals were randomly divided into four groups ($n=8-12$ per group): sham, vehicle, YQ138 (10 mg/kg), and edaravone (3 mg/kg). Rats were intravenously treated with YQ138 and edaravone at 2 h, 4 h and 6 h after ischemia onset.

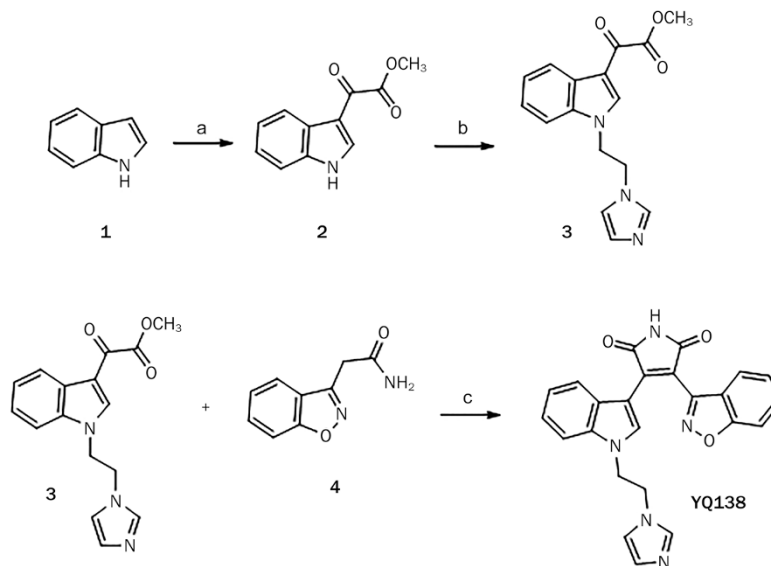


Figure 1. Synthetic route to YQ138. Reagents and conditions: (a) i (COCl)₂, Et₂O; ii CH₃ONa, CH₃OH; (b) NaH, DMF, 1-(2-chloroethyl)-1H-imidazole; (c) i t-BuOK, THF; ii concentrated HCl.

Animal surgery

Transient middle cerebral artery occlusion (tMCAO) was performed as previously described^[22]. The right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) of individual rats were exposed. The right MCA was occluded by inserting a monofilament nylon suture (diameter of approximately 0.26 mm) with a round tip into the internal carotid artery, and it was advanced further until it closed the origin of the MCA. After 2 h of MCAO, the filament was withdrawn to restore blood flow (reperfusion). Sham-operated control mice received the same surgical procedure without insertion of a filament. The body temperature was kept at 37°C with a temperature control system. All animals had free access to food and water.

Measurement of neurological deficit, infarct size and brain-water content

The neurological deficits of individual rats were measured with Longa's method with minor modifications in a blinded manner at 24 h after MCAO^[23]. The neurological deficits were scored on a five-point scale: 0, normal function; 1, flexion of the torso and contralateral forelimb after lifting the animal by the tail; 2, circling to the contralateral side but normal posture at rest; 3, recline to the contralateral side at rest; and 4, absence of spontaneous motor activity. After assessment of neurological deficit, the rats were euthanized at 24 h after tMCAO, and the brains were collected and dissected on ice and sectioned into 2 mm coronal sections. Sections were soaked in 2% TTC phosphate buffer for 20 min at 37°C in the dark. Whereas infarct tissues stained white, the normal brain tissues stained red. The ratio percentages of the infarct areas to the total brain areas were calculated by morphometric analysis (image-pro plus). With regard to the brain-water content, some brain

samples were weighed immediately to obtain the wet weight, subsequently dried for 8 h in an oven at 120°C, and then reweighed to obtain the dry weight. The brain-water content was calculated with the following formula: [(wet weight-dry weight)/(wet weight)]×100.

Statistical analysis

All values are expressed as the mean±SD from at least three independent experiments. The results were analyzed using one-way ANOVA followed by Bonferroni's *post hoc* test. *P*<0.05 was considered statistically significant.

Results

Discovery of YQ138 using a glutamate-induced injury model in cerebellar granule neurons (CGCs)

Using our established glutamate-induced neuronal cell damage model to randomly screen an in-house compound library consisting of approximately 2000 potential kinase inhibitors, we found the small-molecule YQ138, which exhibited a potential neuroprotective effect by preventing CGCs from glutamate-induced injury (Figure 2). YQ138 pretreatment at 24 h before glutamate incubation inhibited glutamate-induced cell death in a dose-dependent manner in CGCs (Figure 2A). Pretreatment of YQ138 at 2 h before glutamate addition also significantly prevented neuronal injury induced by glutamate, but YQ138 incubation concurrently with or after glutamate incubation did not prevent glutamate-induced neuronal death (Figure 2B).

Neuroprotection of YQ138 in OGD- or B27 deprivation-induced neuronal injury model in CGCs

OGD insult, followed by reoxygenation and nutrient recovery, is thought to mimic the process of ischemia/reperfusion.

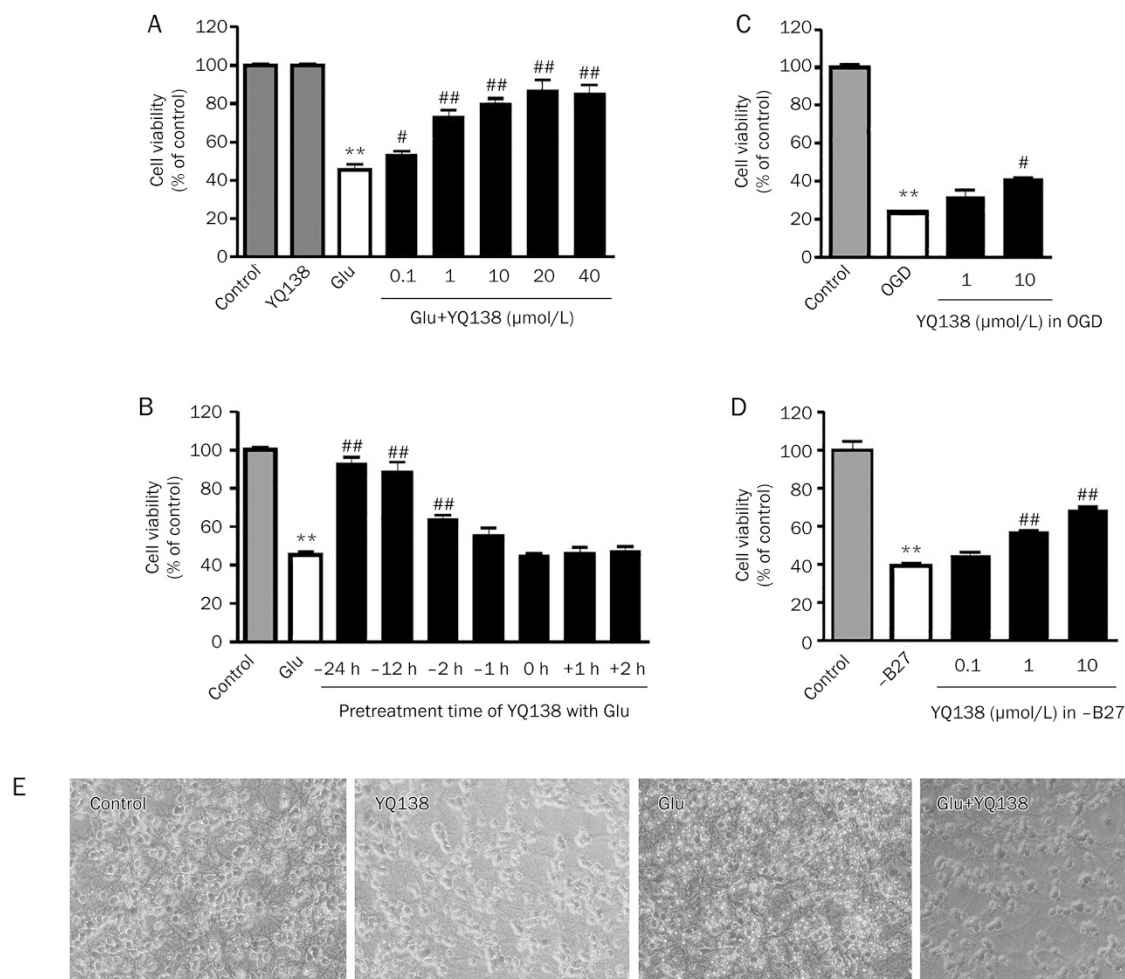


Figure 2. Neuroprotective effects of YQ138 on glutamate-induced neuronal injury. (A) Cerebellar granule neuronal cells (CGCs) were preincubated with various concentrations of YQ138 for 24 h and then treated with 200 μmol/L glutamate (Glu) for an additional 24 h. (B) CGCs were treated with YQ138 (10 μmol/L) at different time points before, during, or after 24-h Glu incubation. (C) CGCs were pretreated with YQ138 (10 μmol/L) for 24 h, and then exposed to oxygen-glucose deprivation (OGD) for 6 h, followed by 24 h of reoxygenation, or were maintained under normal conditions. (D) CGCs were preincubated with YQ138 for 24 h, followed by B27 deprivation (-B27) for an additional 24 h. (E) Morphology of CGCs pretreated with YQ138 (10 μmol/L) for 24 h followed by Glu incubation for an additional 24 h. Photomicrographs were obtained using an inverted light microscope. Cell viability was measured by using the MTT assay. The results are expressed as the mean±SD of at least three independent experiments. ***P*<0.01 vs control; #*P*<0.05, ##*P*<0.01 vs Glu, OGD, or -B27.

YQ138 significantly inhibited CGCs injury induced by OGD conditions (Figure 2C). If the nutrient B27, a serum substitute, is removed, the majority of CGCs die via a cell apoptotic process^[24]. As shown in Figure 2D, Y138 also significantly inhibited CGC injury caused by serum deprivation (B27 deprivation, -B27).

YQ138 induces GSH and SOD in CGCs

Glutamate-induced cell apoptosis usually occurs via reactive oxygen species (ROS) production^[25, 26]. GSH and SOD play important roles in the antioxidant system^[27]. GSH, an endogenously synthesized tripeptide thiol, is involved in scavenging free radicals and protecting against oxidative stress^[28]. SOD, an endogenous mitochondrial anti-oxidant enzyme, exhibits the effect of scavenging free radical and prevents the

accumulation of superoxide^[29]. We investigated the content of GSH and SOD after incubation with YQ138 and glutamate in CGCs and found that YQ138 reversed glutamate-caused decreases in GSH and SOD content in CGCs, thus protecting neurons against cellular injury caused by glutamate (Figure 3). However, YQ138 alone did not increase the GSH and SOD production in CGCs (Figure 3).

YQ138 induces Nrf2, HO-1 and NQO1 expression in CGCs

Nrf2 is a member of the cap 'n' collar family, and plays a crucial role in regulating cellular antioxidant systems^[30]. Exposure to glutamate resulted in a decrease in the level of Nrf2 in a time-dependent manner in CGCs, whereas YQ138 (10 μmol/L) completely prevented this effect (Figure 4A). Moreover, YQ138 increased Nrf2 expression in glutamate-incubated

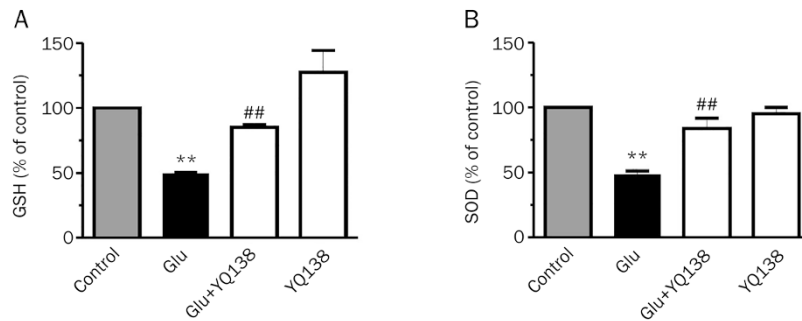


Figure 3. Effects of YQ138 on GSH concentration and SOD activity in CGCs. CGCs were preincubated with YQ138 for 24 h and then exposed to 200 $\mu\text{mol/L}$ glutamate (Glu) for an additional 24 h to determine GSH concentration (A) and SOD activity (B). The results are expressed as the mean \pm SD of at least three independent experiments. ** P <0.01 vs control; ## P <0.01 vs Glu.

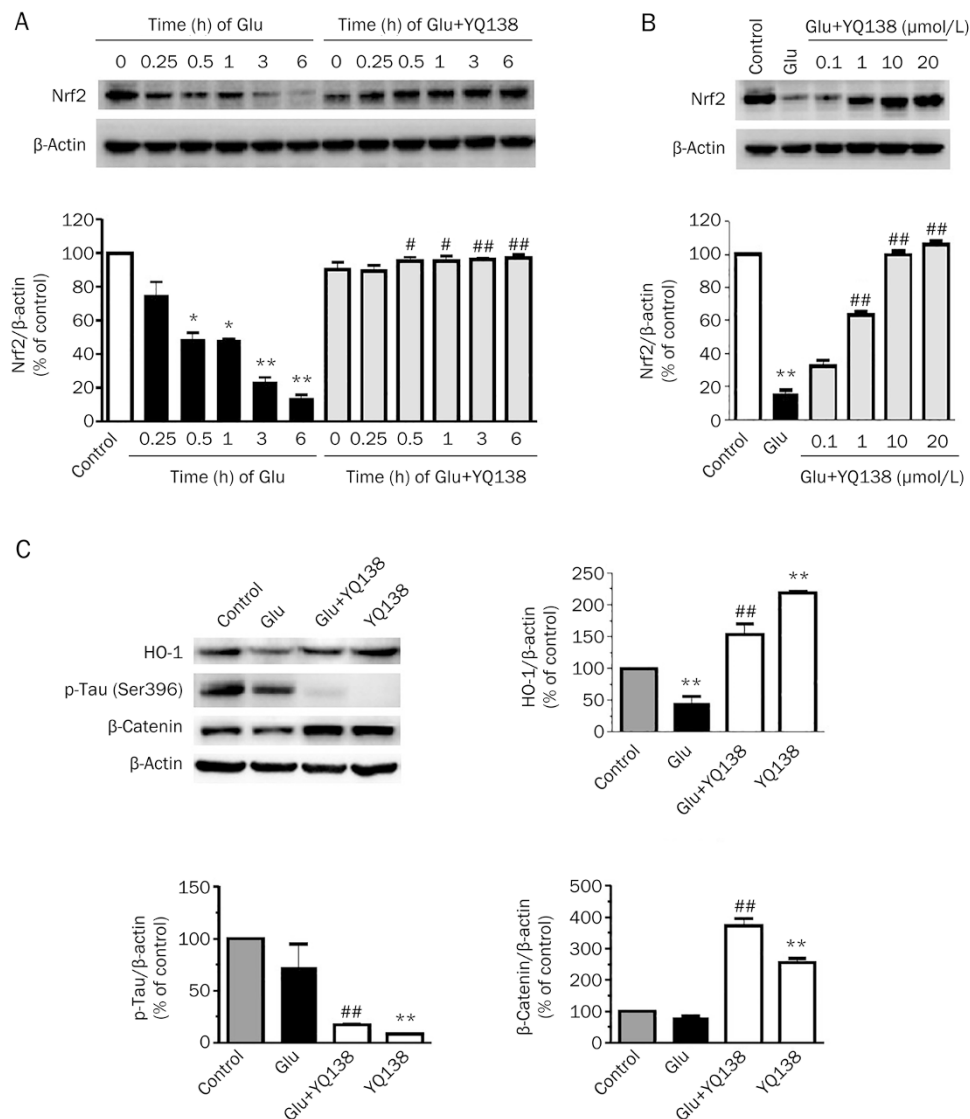


Figure 4. YQ138 induces Nrf2 protein expression in CGCs. (A) CGCs pretreated with YQ138 (10 $\mu\text{mol/L}$) were treated with 200 $\mu\text{mol/L}$ glutamate (Glu) for different times before preparation of protein extracts. (B) Dose-response of YQ138 on the induction of Nrf2 protein levels in Glu-incubated CGCs for 24 h. (C) YQ138 treatment reversed the Glu-induced decrease of Nrf2 downstream target HO-1 protein levels via inhibition of cellular GSK-3 β activity indicated by a decrease in Tau phosphorylation (Ser396) and increase in β -catenin expression in CGCs. Protein expression was measured using Western blotting analysis. β -Actin was used as an internal control. One representative blot is shown. The results are expressed as the mean \pm SD of at least three independent experiments. * P <0.05, ** P <0.01 vs control; # P <0.05, ## P <0.01 vs Glu.

CGCs in a dose-dependent manner (Figure 4B). YQ138 also enhanced the protein expression levels of HO-1 and β -catenin and decreased the GSK-3 β substrate Tau phosphorylation levels in CGCs (Figure 4C).

Under stress, Nrf2 translocates into nuclei and binds AREs of phase II and antioxidant defense enzymes, such as HO-1 and NQO1, thus inhibiting oxidative stress^[18]. HO-1 is a ubiquitous and redox-sensitive inducible stress protein, and it degrades heme to CO, iron, and biliverdin. NQO1, a detoxifying enzyme, reduces reactive quinones and quinone imines to less reactive and less toxic hydroquinones. We investigated the expression of Nrf2, HO-1 and NQO1 after incubation with YQ138 in CGCs. YQ138 increased the expression of Nrf2 and its downstream substrate, such as HO-1 and NQO1, in CGCs, in a dose-dependent manner (Figure 5A). We also found that YQ138 promoted the accumulation of Nrf2 in the nucleus (Figure 5B).

YQ138 inhibits GSK-3 β activity with high selectivity

Given that GSK-3 β down-regulated the transcriptional factor Nrf2 and antioxidant cell defense^[31, 32], we speculated that YQ138 may interact with a GSK-3 β protein and inhibit its activity to manifest its neuroprotective effects. The reported GSK-3 β inhibitors lithium chloride (5 mmol/L)^[16] and AR-A014418 (10 μ mol/L)^[33] exhibited similar neuroprotective effects to those of YQ138 (Figure 6A). YQ138 directly inhibited GSK-3 β activity at a molecular level with an IC₅₀ value of 0.52 nmol/L (Figure 6B). In addition, YQ138 displayed high selectivity for GSK-3 β over other tested kinases (Table 1).

Table 1. Selectivity of tested kinases of YQ138.

Kinase assay	Inhibition (%) at 2 μ mol/L YQ138
GSK-3 β	99.07 \pm 4.30
CDK2	5.68 \pm 0.18
JAK2	2.39 \pm 0.18
PKC ϵ	21.34 \pm 2.38
IKK β	6.55 \pm 0.05
BRAF	0.47 \pm 0.56
DRAK2	6.6 \pm 1.39

Effects of YQ138 on downstream substrates of GSK-3 β in primary neurons

Tau protein is a microtubule-associated protein (MAP) and is abundantly expressed in CNS. Previous reports have demonstrated that hyperphosphorylated Tau causes cognitive impairment^[34]. Phosphorylation modification of Tau is directly regulated by protein kinases and the protein phosphatase system. GSK-3 β is the most important kinase involving Tau phosphorylation^[9, 10]. As a GSK-3 β inhibitor, YQ138 decreased the phosphorylation of Tau protein dose-dependently in CGCs (Figure 6C). In addition, inactivating GSK-3 β activity causes the stabilization and activation of β -catenin^[35]. YQ138 increased the stability and expression of β -catenin by inactivating GSK-3 β (Figure 6C).

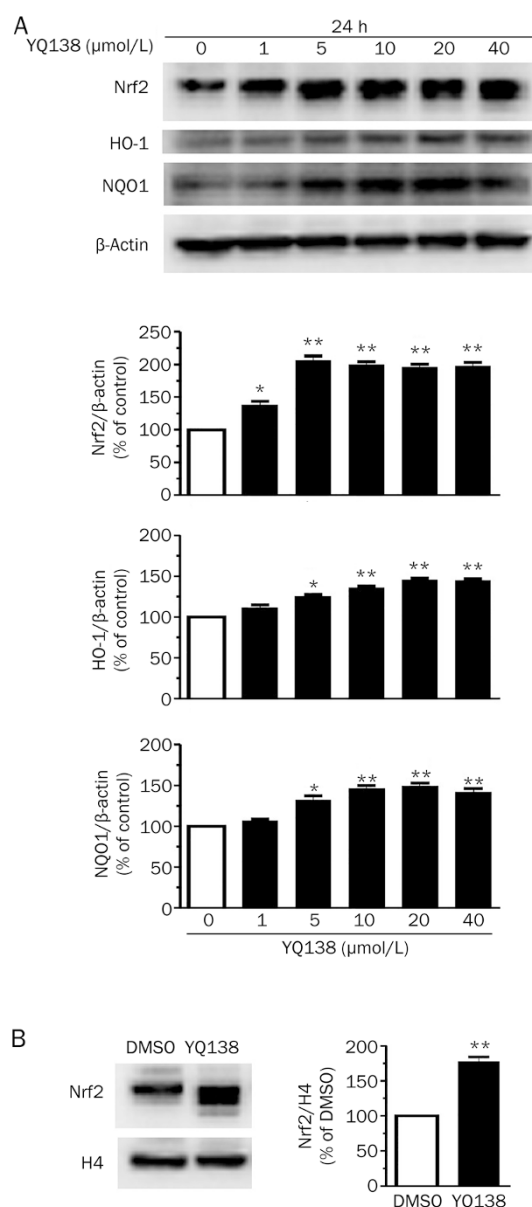


Figure 5. YQ138 induces Nrf2, HO-1 and NQO1 expression in CGCs. (A) CGCs were treated with various concentrations of YQ138 for 24 h to detect the expression of Nrf2, HO-1 and NQO1 using Western blotting analysis. (B) CGCs were treated with YQ138 (10 μ mol/L) for 24 h and nuclear extracts were prepared. The nuclear protein levels of Nrf2 were determined using Western blotting analysis. H4 and β -actin were used as internal controls for nuclear extracts and whole-cell lysates, respectively. The results are expressed as the mean \pm SD of at least three independent experiments. One representative blot is shown. * P <0.05, ** P <0.01 vs control.

Molecular modeling study

The binding mode between GSK-3 β (1Q3D) and YQ138 was proposed using the Discovery studio 2.5/LigandFit, as shown in Figure 7. Compound YQ138 tightly occupies the ATP binding site of GSK-3 β , resulting in the docking result, which is consistent with its potent GSK-3 β inhibitory activity. The NH and carbonyl group in a maleimide ring of YQ138 can

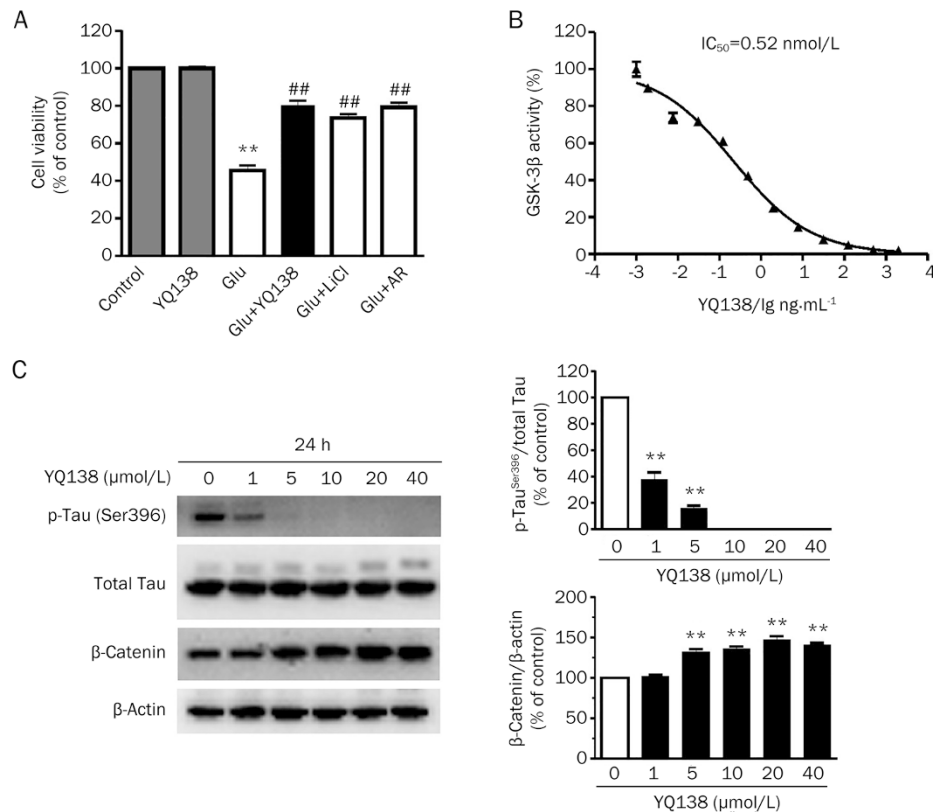


Figure 6. YQ138 inhibits GSK-3 β activity. (A) Neuroprotective effects of GSK-3 β inhibitors, lithium chloride (LiCl) and AR-A014418 (AR), on glutamate (Glu)-induced neuronal injury. CGCs were preincubated with LiCl (5 mmol/L), AR (10 μ mol/L) or YQ138 (10 μ mol/L) for 24 h and then treated with 200 μ mol/L Glu for an additional 24 h. Cell viability was measured by using the MTT assay. (B) IC₅₀ curve of YQ138 against GSK-3 β activity. (C) YQ138 increases GSK-3 β substrate β -catenin expression but decreases Tau phosphorylation (pTau) in CGCs. CGCs were treated with various concentrations of YQ138 for 24 h to measure the expression of pTau and β -catenin using Western blotting analysis. β -Actin was used as an internal control. One representative blot is shown here. The results are expressed as the mean \pm SD of at least three independent experiments. ** P <0.01 vs control, ## P <0.01 vs Glu.

strongly interact with Asp133 and Val135 in the hinge domain of GSK-3 β via two hydrogen bonds, respectively. In addition, the 3-position nitrogen atom of the imidazole ring of YQ138 can form another hydrogen bond with Asp200.

Neuroprotection of YQ138 in the OGD-induced neuronal injury model in primary rat cortical neurons

OGD conditions induce neuronal damage in the cortex and striatum during ischemic stroke. OGD insult, followed by reoxygenation, is thought to mimic the pathological conditions of ischemia. YQ138 significantly inhibited cortical neuronal injury induced by OGD (Figure 8A). In addition, OGD conditions decreased the protein expression levels of Nrf2 and β -catenin, but YQ138 treatment reversed the OGD-induced decrease of Nrf2 expression in primary rat cortical neurons (Figure 8B).

YQ138 decreases infarct volume and improves neurological deficit in tMCAO rats

Rats were intravenously treated with YQ138 (10 mg/kg) and Ederavone (3 mg/kg) at 2 h, 4 h and 6 h after ischemia onset. These results showed that YQ138, compared with vehicle,

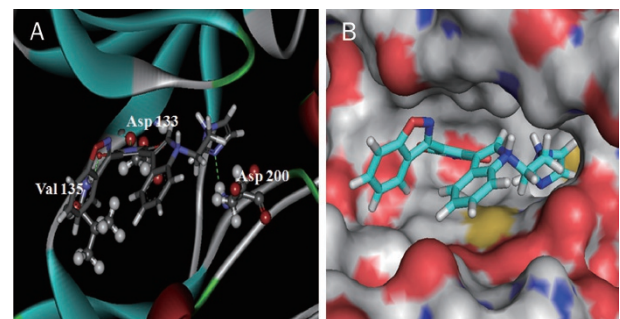


Figure 7. Molecular docking of YQ138 into the GSK-3 β crystal structure. (A) The ribbon shows the compound YQ138 bound to GSK-3 β . (B) The surface shows the compound YQ138 docking into ATP-binding pocket of GSK-3 β .

decreased infarct volume and water content (Figure 9A–9C). Moreover, YQ138 administration improved the neurological deficit scores in cerebral ischemic rats (Figure 9D). These findings indirectly suggested that YQ138 may protect the blood-brain barrier and exhibit neuroprotective effects.

YQ138 increased the levels of Nrf2, HO-1, GSH and SOD in tMCAO rats

We investigated the antioxidative proteins Nrf2, HO-1, GSH and SOD levels in the cerebral cortex of rats with tMCAO. A significant decrease in the levels of GSH and SOD was revealed in rats 24 h after MCAO when compared with the sham treatment, whereas YQ138 treatment significantly increased the levels of GSH and SOD (Figure 10A, 10B). In addition, YQ138 treatment significantly enhanced the MCAO-caused decrease in the protein expression levels of Nrf2, HO-1, β -catenin and phosphorylated GSK-3 β (Ser9) in the cerebral cortex of ischemic rats (Figure 10C).

Discussion

In this study, a novel GSK-3 β inhibitor YQ138 was found to exhibit neuroprotective effects in primary neuronal injury models or cerebral ischemic rat models. The underlying mechanisms for YQ138's neuroprotective role involved the inhibition of GSK-3 β activity at the molecular and cellular levels, as well as the enhancement of GSK-3 β downstream of the Nrf2 signaling pathway and related antioxidative systems.

Glutamate, one of the most important excitatory amino acids in the nervous system, plays a crucial role in many functions of the mammalian brain^[1]. Excitotoxicity caused by glutamate receptor overactivation has been shown to result in neuronal injury and infarct growth after acute ischemic stroke^[36]. The cerebellar granule cells in culture are more vulnerable to glutamate-induced neuronal excitotoxicity than are cultured cortical neurons. The *in vitro* model of glutamate excitotoxicity using primary culture of cerebellar granule cells and an *in vivo* model of cerebral ischemia have been used to determine potential neuroprotective compounds in previous studies^[6, 15]. Thus, we chose to study cerebellar granule cells *in vitro*, which is a well-characterized and reliable model to analyze mechanisms and excitotoxic neuronal damage. We found that YQ138 functioned as a GSK-3 β inhibitor, and it suppressed the excitotoxicity induced by glutamate in rat primary cerebellar granule neuronal cells (Figure 2). Stroke is the neurological condition that develops when a portion of

the brain is deprived of oxygen and glucose. Therefore, we further investigated the role of YQ138 in ischemic neuronal injury using primary cerebellar granule neuronal cells and primary cortical neurons exposed to OGD. Our data showed that YQ138 prevented neuronal death induced by OGD insult (Figure 2 and 8). The differentiated neurons in CNS require nutrients, such as serum, to survive against apoptosis and to exert their functions^[23]. In our study, we found that pretreatment with YQ138 partially blocked B27 deprivation-induced neuronal cell death (Figure 2).

GSH is involved in numerous basic cellular processes, including maintaining redox status, conjugation/detoxification reactions, and scavenging free radicals and electrophilic intermediates^[26, 27]. SOD is a free radical scavenger that converts superoxide radicals formed by cerebral ischemia into the less reactive hydrogen peroxide form^[28]. In this study, we found increases in the levels of GSH and SOD upon YQ138 treatment after cells were exposed to glutamate (Figure 3). In addition, we also investigated the levels of GSH and SOD in the cerebral cortex of rats with tMCAO, and we observed that YQ138 significantly increased the activity of GSH and SOD (Figure 10) compared with the vehicle group.

Previous research has established that Nrf2 activation is an effective strategy for the prevention of oxidative stress-induced cellular damage, and it delays the progression of inflammation^[29-32]. We found that YQ138 increased the level of Nrf2 in a time-dependent and dose-dependent manner against the excitotoxicity caused by glutamate (Figure 4). We also found that nuclear Nrf2 accumulation was significantly enhanced after incubation with YQ138 (Figure 5).

Previous reports have shown that HO-1 may provide protection against excitotoxicity and cerebral ischemia^[19]. Moreover, NQO1 is induced by electrophilic metabolites and oxidative stress, and it is transcriptionally regulated by ARE^[30]. In this study, we found that HO-1, NQO1 and Nrf2 protein levels were increased after incubation with YQ138 in a dose-dependent manner (Figure 5).

The biological activity of Tau is regulated by its degree of phosphorylation. However, hyperphosphorylation of Tau

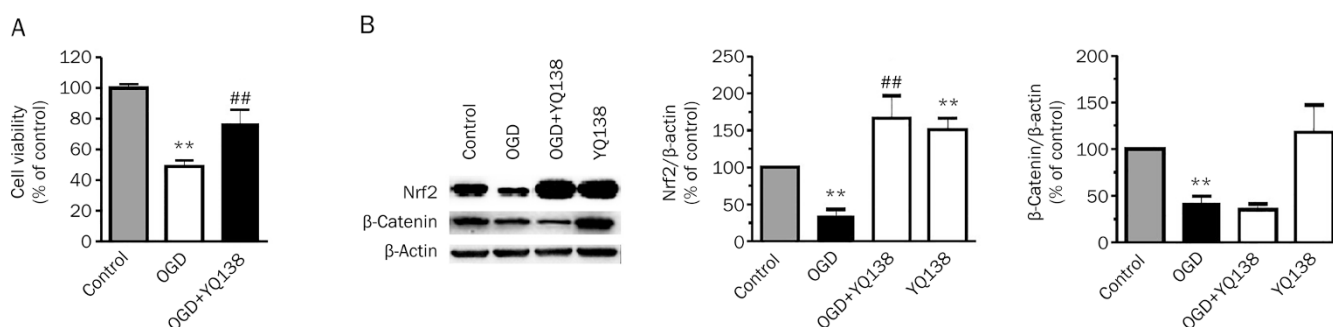


Figure 8. YQ138 protects cortical neurons from OGD-induced cell death. Primary rat cortical neurons were treated with YQ138 (10 μ mol/L) for 24 h and then exposed to OGD for 6 h followed by 24 h of reoxygenation or maintained under normal conditions (normoxia). (A) Cell viability was measured by using the MTT assay. (B) YQ138 treatment reversed the OGD-induced decrease of Nrf2 expression levels in cortical neurons. Nrf2 protein expression was measured using Western blotting analysis. β -Actin was used as an internal control. The results are expressed as the mean \pm SD of at least three independent experiments. One representative blot is shown. ** P <0.01 vs control; ## P <0.01 vs OGD.

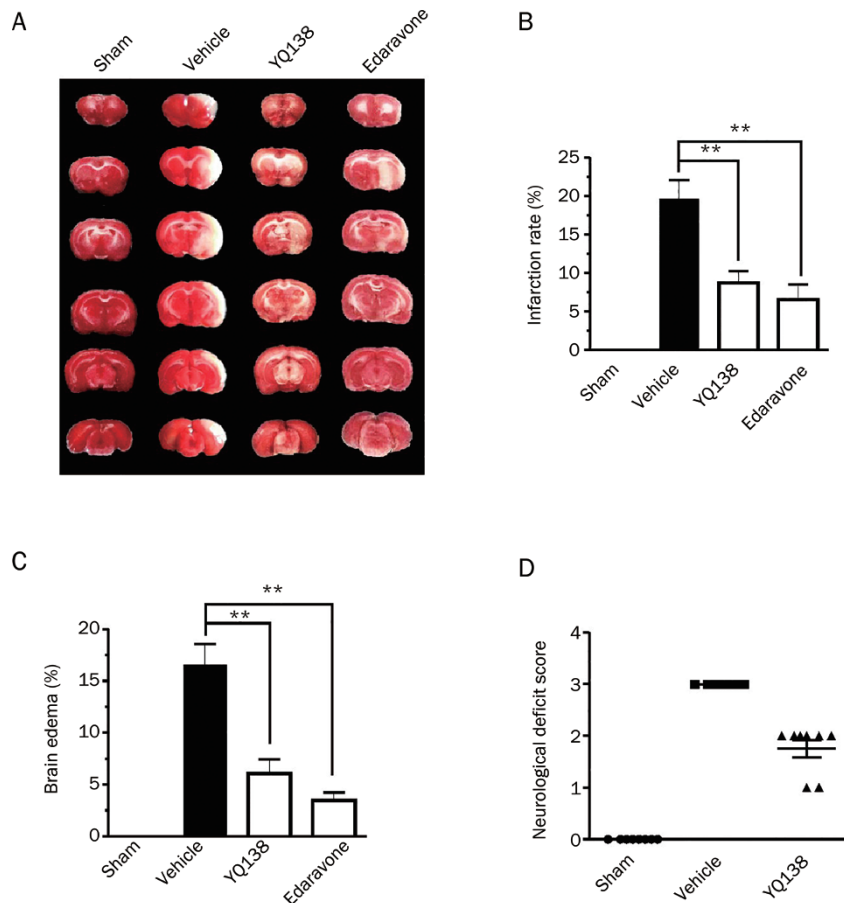


Figure 9. The effect of YQ138 on acute cerebral ischemia in rats at 24 h after transient middle cerebral artery occlusion (tMCAO). YQ138 and Edaravone decreased ischemia-induced infarct volume (A, B) and brain edema (C). YQ138 administration also improved the neurological deficit score in cerebral ischemic rats (D). Data are expressed as the mean \pm SD of individual groups of rats ($n=8-12$). ** $P<0.01$ vs vehicle (tMCAO group).

not only converts it into a cytotoxic protein, which sequesters MAPs but also damages its biological activity^[37]. Overexpression of GSK-3 β in transgenic mice results in the hyperphosphorylation of Tau^[10]. Moreover, β -catenin, which regulates the coordination of cell-cell adhesion and gene transcription, is also regulated by GSK-3 β ^[35]. Thus, we investigated the effects of YQ138 on the expression of Tau and β -catenin. Our data indicated that YQ138 inhibited cellular GSK-3 β activity, as indicated by the enhanced protein levels of Tau phosphorylation and β -catenin in neurons (Figure 6).

To determine whether administration of YQ138 would attenuate the injury of ischemia *in vivo*, we investigated the effect of YQ138 in MCAO rats. Previous studies have suggested that MCAO mimics clinical ischemia stroke in animal models^[22, 38]. Our data showed that YQ138 improved the infarct volume and brain edema, and it also improved the neurological deficit in rats after MCAO (Figure 9). Improvement of antioxidative systems, such as Nrf2, HO-1, GSH and SOD, may be responsible for the beneficial effects of YQ138 in cerebral ischemic rats (Figure 10). YQ138 potentially decreased water content in the infarct region via protection of the blood-brain barrier, which can be disrupted by cerebral ischemia, resulting in vasogenic

brain edema. Glutamate excitotoxicity plays an important role in the pathogenesis of several brain disorders, including ischemic stroke, but there is currently no effective treatment for brain ischemic stroke. Together, these observations suggest that YQ138, a novel GSK-3 β inhibitor, may be developed as a neuroprotective agent to protect against ischemic stroke via an up-regulation of the anti-oxidative system.

In conclusion, in the present study, we provide the first evidence that YQ138, a GSK-3 β inhibitor, functions as a novel neuroprotective agent for ischemia stroke by increasing Nrf2 and antioxidative signaling.

Abbreviations

ARE, antioxidant response element; BSA, bovine serum albumin; CCA, common carotid artery; CGCs, cerebellar granule cells; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; DNase I, deoxyribonuclease I; EBSS, Earle's balanced salt solution; ECA, external carotid artery; Eda, edaravone; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; GSK-3 β , glycogen synthase kinase 3 β ; HBSS, Hank's balanced saline solution; HO-1, heme oxygenase-1; ICA, internal carotid

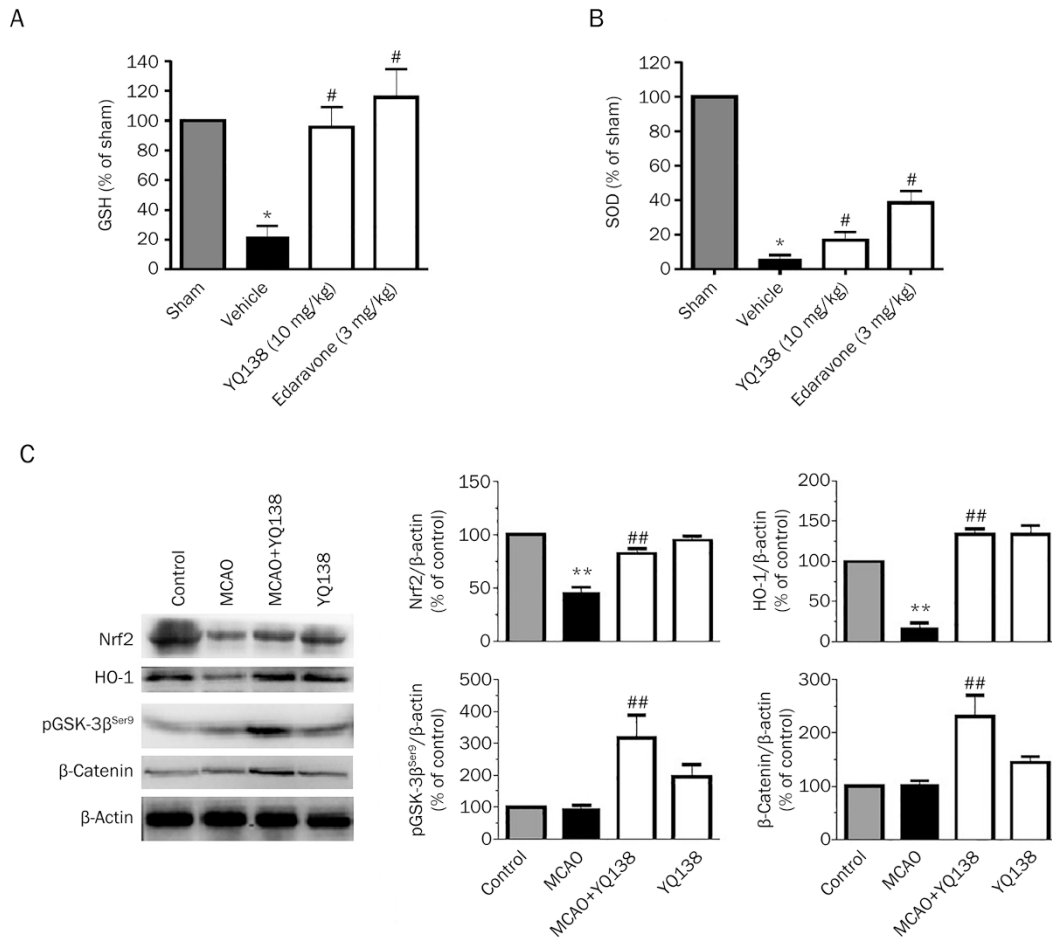


Figure 10. Effects of YQ138 on the antioxidative protein expression of Nrf2 and HO-1, GSH concentration and SOD activity in the cerebral cortex of rats with tMCAO. GSH concentration (A), SOD activity (B), the protein expression of Nrf2, HO-1, β -catenin, and phosphorylated GSK-3 β^{Ser9} (C), determined at 24 h after surgery. Data are expressed as the mean \pm SD of individual groups of rats ($n=3-5$). * $P<0.05$, ** $P<0.01$ vs sham group; # $P<0.05$, ## $P<0.01$ vs vehicle (MCAO group).

artery; Keap1, Kelch-like ECH-associated protein 1; MCAO, middle cerebral artery occlusion; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO1, uinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; OGD, oxygen and glucose deprivation; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SD, Sprague-Dawley; SOD, superoxide dismutase; TBST, Tris-buffered saline plus Tween20; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

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Author contribution

Tao PANG, Hong LIAO, Lu-yong ZHANG, Jian-rong GAO, Qing YE, and Jia LI designed the research; Tao PANG, Yun-jie WANG, Yuan-xue GAO, Yuan XU, Yu-bo ZHOU, and Lei XU performed the research; Qiu LI and Qing YE synthesized the compound YQ138; Tao PANG, Zhang-jian HUANG, Qing YE, and Jia LI analyzed the data; and Tao PANG, Qing YE, and Jia LI wrote the paper.

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