Neuroprotective Effects of Purpurin Against Ischemic Damage via Anti-inflammatory and MAPK Pathway

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Abstract

Purpurin has various effects, including anti-inflammatory effects, and can efficiently cross the blood-brain barrier. In the present study, we investigated the effects of purpurin on oxidative stress in HT22 cells and ischemic damage in the hippocampal CA1 region of gerbils. Oxidative stress induced by H_2O_2 was significantly ameliorated by treatment with purpurin, based on changes in cell death, DNA fragmentation, formation of reactive oxygen species, and apoptosis (Bcl-2)/antiapoptosis (Bax)-related protein levels. In addition, treatment with purpurin significantly reduced the phosphorylation of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK), and p38 signaling in HT22 cells. Transient forebrain ischemia in gerbils led to a significant increase in locomotor activity 1 day after ischemia and significant decrease in number of surviving cells in the CA1 region 4 days after ischemia. Administration of purpurin reduced the travel distance 1 day after ischemia and increased the number of NeuN-immunoreactive neurons in the hippocampal CA1 region of the dentate gyrus 4 days after ischemia. Purpurin treatment significantly decreased microglial activation in the hippocampal CA1 region 4 days after ischemia and ameliorated the ischemia-induced increases in interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α 6 h after ischemia. In addition, purpurin significantly alleviated the ischemia-induced phosphorylation of JNK, ERK, and p38 in the hippocampus 1 day after ischemia. These results suggest that purpurin has neuroprotective potential to reduce inflammatory processes and the phosphorylation of JNK, ERK, and p38 in the hippocampus.

Introduction

Ischemic stroke is a life-threatening disease that affects approximately 15 million people worldwide annually [1]. Interruption of the blood flow into the brain causes a reduction in the supply of oxygen and glucose into the brain, resulting in damage to affected areas, including the hippocampus [2, 3]. Reperfusion of interrupted vessels into the brain enormously increases the blood supply to the brain, but glucose metabolism is impaired via the pyruvate dehydrogenase pathway in neurons and pyruvate carboxylase pathway in astrocytes [4]. Normally, oxygen radicals are generated from 0.2–2% of oxygen by the electron transport chain [5] and scavenged by antioxidants in the body [6, 7]. However, ischemia/reperfusion significantly increases the formation of oxygen radicals, exceeding the scavenging capacity of antioxidant enzymes in neurons, and finally causing oxidative damage and propagating inflammatory damage in neurons after ischemia [8, 9].

Many attempts have been made to prevent and reduce brain damage after ischemic damage using herbal extracts because of their high phenolic and flavonoid contents [10, 11]. Anthraquinones have a 9,10-dioxoanthracene core substituted with phenolic hydroxyl and aliphatic groups in the two benzene rings. Anthraquinones are less highlighted, although they have various biological effects that inhibit the progression of diseases [12]. Purpurin, an anthraquinone, exhibits antioxidant, anti-inflammatory, and antifungal effects in *in vitro* assays [13, 14] and anti-angiogenic effects in a zebrafish model [15]. In addition, purpurin inhibits monoamine oxidase and shows potential for drug development in depression

[16, 17]. Purpurin is able to cross the blood-brain barrier (BBB) assessed in human brain-like endothelial cells [18], which mimic the in vivo BBB [19].

However, no studies have examined the effects of purpurin against oxidative damage in HT22 cells and ischemic damage in the gerbil hippocampus. In the present study, we elucidated the effects of purpurin and its mechanisms based on H₂O₂-induced oxidative stress in HT22 cells and ischemia-induced neuronal damage in the gerbil hippocampal CA1 region.

Materials And Methods

Cell preparation and determination of cellular toxicity in HT22 cells

Murine hippocampal HT22 cells were obtained from ATCC (Manassas, VA, USA) and cultured in *Dulbecco's modified Eagle's medium as described in previous studies [20, 21]. Purpurin was dissolved in 200-mM dimethyl sulfoxide (DMSO) and various concentrations of purpurin (1–200 μ M) were added to HT22 cells for 60 min. The cells were then harvested to observe the cellular toxicity of purpurin in HT22 cells. Cellular toxicity was assessed by measuring the fluorescence of formazan produced using the WST-1 assay kit (Sigma, St. Louis, MO, USA) and a Fluoroskan ELISA plate reader (Labsystems Multiskan MCC/340, Helsinki, Finland) as described in previous studies [20, 21].*

Measurements of reactive oxygen species, DNA fragmentation, and cell viability in HT22 cells

Cells were exposed to 25- μ M purpurin or DMSO immediately after treatment with 1-mM H₂O₂ to induce oxidative stress. For reactive oxygen species (ROS) formation, 20- μ M 2',7'-dichlorofluorescein diacetate (DCF-DA) *was added to HT22 cells 10 min after H₂O₂ treatment to induce the formation of DCF, which has strong fluorescence. Cells were harvested 30 min after DCF-DA treatment. DNA fragmentation was validated using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining as described in previous studies [20, 21]. Briefly, cells were harvested 3 h after H₂O₂ treatment, and DNA fragmentation was visualized using a TUNEL staining kit (Sigma). Microphotographs from DCF-DA and TUNEL staining were taken using a confocal fluorescence microscope (LSM 510 META NLO; Zeiss GmbH, Jena, Germany), and the fluorescence intensity was measured using a Fluoroskan ELISA plate reader (Labsystems Multiskan MCC/340). Cell death was assessed using a WST-1 assay 5 h after H₂O₂ treatment, and formazan fluorescence was measured using a Fluoroskan ELISA plate reader.*

Western blot analysis in HT22 cells

To elucidate the possible mechanisms of purpurin's effects against oxidative stress, cells were harvested 6 h after H₂O₂ treatment. Thereafter, cells were lysed with ice-cold radioimmunoprecipitation assay buffer (Thermo Scientific, IL, USA), and western blotting for mitogen-activated protein kinases (MAPKs) was performed as described in a previous study [22]. Briefly, the following primary antibodies were used: rabbit anti-c-Jun N-terminal kinase (JNK), p-JNK, anti-extracellular signal-regulated kinase 1/2 (ERK), anti-

p-ERK, anti-p38, and anti-p-p38), Bax, Bcl-2, and β -actin (1:2,000; Abcam). All antibodies except β -actin were purchased from Cell Signaling (Danvers, MA, USA) and used at the same dilution (1:1000).

Experimental animals

Mongolian gerbils (male, 3 months old) were obtained from Japan SLC Inc. (Shizuoka, Japan), and the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU-200313-2). Ischemic surgery was conducted as described in previous studies [20-22]. Obstruction of both common carotid arteries was confirmed by observing the retina artery using an ophthalmoscope (Heine Optotechnik, Herrsching, Germany). Immediately after ischemia/reperfusion, vehicle (saline containing 0.5% sodium carboxymethyl cellulose), 1, 3, or 6 mg/kg 1,2,4-trihydroxyanthraquinone (purpurin, Sigma, St. Louis, MO) was orally administered to gerbils. The dosage was chosen based on the antidepressant-like effects of 6 mg/kg purpurin treatment in mice [17]. In addition, we did not use DMSO as a vehicle for in vivo studies because it shows neuroprotective effects against ischemic damage [23].

Spontaneous motor activity

Motor activity was monitored 1 day after ischemia for 60 min because hyperactivity was induced due to cellular damage in the hippocampal CA1 region [24]. Traveling activity was recorded using a digital camera system (Basler 106200, Ahrensburg, Germany), and the travel distance and duration of immobile/mobile phases were analyzed using Ethovision XT14 (Wageningen, Netherlands).

Neuronal survival and microglial activation

Neuronal survival and microglial activation were assessed using immunohistochemical staining for neuronal nuclei (NeuN) and ionized calcium-binding adapter molecule 1 (Iba-1), respectively, as described previously [21]. Briefly, the animals were sacrificed with a mixture of alfaxalone (Alfaxan, 75 mg/kg; Careside, Seongnam, South Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, South Korea) 4 days after ischemia and perfused transcardially with saline and 4% paraformaldehyde. Coronal serial sections (30- μ m thickness) were made based on brain atlas between 2.0 and 2.7 mm caudal to the bregma [25]; five sections (90 μ m apart from each other) were incubated with mouse anti-NeuN antibody (1:1000; EMD Millipore, Temecula, CA, USA) and rabbit anti-Iba-1 antibody (1:500; Wako, Osaka, Japan). Sections were reacted with 3,3'-diaminobenzidine tetrachloride (Sigma) to visualize immunoreactive signals. The number of NeuN-immunoreactive neurons and Iba-1 immunoreactivity was calculated using OPTIMAS software (version 6.5; CyberMetrics[®] Corporation, Phoenix, AZ, USA) and ImageJ software version 1.53 (National Institutes of Health, Bethesda, MD, USA), respectively.

Measurements of pro-inflammatory cytokines

To elucidate the mechanisms of purpurin's effects against ischemic damage, animals (n = 5 in each group) were euthanized with 75 mg/kg alfaxalone and 10 mg/kg xylazine 6 h after ischemia/reperfusion,

when pro-inflammatory cytokine levels were significantly increased [26, 27]. In brief, interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α levels were measured based on comparisons with linear calibration curves generated using IL-1 β , IL-6, and TNF- α standard solutions.

Western blot analysis in gerbil hippocampus

To elucidate the MAPK pathway in gerbil hippocampus after ischemia, animals were sacrificed 24 h after ischemia. Hippocampi were obtained from the brain and homogenized. Thereafter, cells were lysed with ice-cold radioimmunoprecipitation assay buffer (Thermo Scientific, IL, USA), and western blotting for mitogen-activated protein kinases (MAPKs) was performed described above.

Statistical analysis

Data are presented as mean with the standard deviation, and differences in means were compared and statistically analyzed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Neuroprotective effects of purpurin against oxidative stress in HT2 cells

First, we validated the toxicity of purpurin in HT22 cells to determine the effective, but non-toxic, concentration of purpurin. Purpurin treatment for 60 min showed no toxic effects at a concentration 25 μ M, and higher concentrations of purpurin decreased cell viability in a concentration-dependent manner (Fig. 1A).

ROS formation was visualized by the formation of DCF fluorescence after H₂O₂ treatment of HT22 cells. In the control group, DCF fluorescence was faintly detected, but in the DMSO-treated group, some cells showed strong DCF fluorescence, although no statistically significant difference in DCF fluorescence was detected between the control and DMSO-treated groups. In the DMSO and H₂O₂-treated (H₂O₂+DMSO) group, numerous DCF fluorescent cells were found, and the fluorescence intensity was significantly higher (511.3%) than that in the control group. In the purpurin and H₂O₂-treated (H₂O₂+Purpurin) group, a few DCF fluorescent cells were found, and fluorescence intensity was significantly lower than that in the H₂O₂+DMSO group (Fig. 1B).

DNA fragmentation was observed using TUNEL staining after H₂O₂ treatment of HT22 cells. In the control and DMSO groups, few TUNEL-positive cells were detectable among the HT22 cells and the TUNEL fluorescence intensity was low. In the H₂O₂+DMSO group, many TUNEL-positive cells were observed among HT22 cells, and the fluorescence intensity was significantly increased to 490.3% of that in the control group. In the H₂O₂+Purpurin group, few TUNEL-positive cells were found, and the fluorescence intensity was significantly lower than that in the H₂O₂+DMSO group at 201.5% of the intensity in the control group (Fig. 1C).

Cell viability was measured using formazan fluorescence from tetrazolium salts after H₂O₂ treatment in HT22 cells. In the DMSO group, the cell viability was similar to that of the control group, but cell viability in the H₂O₂+DMSO group was significantly lower after H₂O₂ treatment at 56.6% of that of the control group. In the H₂O₂+Purpurin group, cell viability was significantly increased compared to that in the H₂O₂+DMSO group, and cell viability in this group was at 78.3% of that of the control group (Fig. 1D).

Neuroprotective mechanisms of purpurin against oxidative stress in HT2 cells

Bax and Bcl-2 protein levels were measured using western blotting after H₂O₂ treatment of HT22 cells. In the DMSO group, Bax and Bcl-2 protein levels did not show any significant changes relative to those in the control group. However, in the H₂O₂+DMSO group, Bax protein levels were significantly higher at 469.7% of those in the control group, while Bcl-2 levels were dramatically lower at 24.2% of those in the control group. In the H₂O₂+Purpurin group, changes in Bax and Bcl-2 levels were ameliorated compared to those in the H₂O₂+DMSO group, respectively, and they were 336.5% and 55.9% of those in the control group, respectively (Fig. 2A).

JNK, ERK, p38 proteins and their phosphorylated forms (p-JNK, p-ERK1/2, and p-p38) were assessed using western blotting after H₂O₂ treatment of HT22 cells, and the ratio of phosphorylated and naïve forms were analyzed. In the DMSO group, the p-JNK/JNK, p-ERK/ERK, and p-p38/p38 ratios were similar to those in the control group. In the H₂O₂+DMSO group, the p-JNK/JNK, p-ERK/ERK, and p-p38/p38 ratios were significantly higher at 381.8%, 472.3%, and 176.4% of those in the control group, respectively. In the H₂O₂+Purpurin group, the ratio of p-JNK/JNK, p-ERK/ERK, and p-p38/p38 was significantly lower than those in the H₂O₂+DMSO group at 222.2%, 283.5%, and 109.7% of the ratios in the control group, respectively (Fig. 2B).

Neuroprotective effects of purpurin against ischemic damage in gerbils

The neuroprotective effects of purpurin were validated using locomotor behavior 1 day after ischemia. In the vehicle-treated ischemic group, the time in the mobile and immobile phases was significantly changed to 115.6% and 57.3% of those in the control group, respectively. The traveled distance in the vehicle-treated ischemic group was significantly longer than that in the control group (292.9% of that in the control group). In the 1 or 3 mg/kg purpurin-treated ischemic groups, the time spent in the mobile and non-mobile phases was similar to those in the vehicle-treated group, but in the 6 mg/kg purpurin-treated group, they did not show significant differences compared to those in the vehicle-treated group control or vehicle-treated ischemic group. Similarly, the traveled distance was significantly longer in 1 or 3 mg/kg purpurin-treated ischemic groups than in the control group. However, in the 6 mg/kg purpurin-treated group, the traveled distance was significantly less than that in the vehicle- or 1 mg/kg purpurin-treated ischemic groups (181.9% of that in the control group) (Fig. 3A).

The neuroprotective effects of purpurin were confirmed using immunohistochemical staining for NeuN in the hippocampus 4 days after ischemia. In the control group, abundant NeuN-immunoreactive cells were

found in the hippocampus. In the vehicle-treated ischemic group, a few NeuN-immunoreactive cells were detected in the hippocampal CA1 region (5.1% of control), whereas in other regions, NeuN-immunoreactive cells were similar levels were seen as in the control group. In the 1 or 3 mg/kg purpurin-treated groups, NeuN-immunoreactive neurons were similarly observed in the hippocampal CA1 region compared to vehicle-treated group (7.5% and 9.9% of control). In the 6 mg/kg purpurin-treated ischemic group, many NeuN-immunoreactive cells were found in the CA1 region, and the number of NeuN-immunoreactive neurons was significantly higher (60.2% of control) than that in the vehicle-treated ischemic group (Fig. 3B).

Neuroprotective mechanisms of purpurin's effects against ischemic damage in gerbils

The neuroprotective mechanisms of 6 mg/kg purpurin were evaluated in terms of anti-inflammatory responses in the hippocampus using an ELISA assay for IL-1 β , IL-6, and TNF- α 6 h after ischemia. In the vehicle-treated ischemic group, IL-1 β , IL-6, and TNF- α levels were significantly higher at 529.6%, 312.4%, and 1255.0% of those in the control group, respectively. In the purpurin-treated ischemic group, IL-1 β , IL-6, and TNF- α levels were significantly lower than those in vehicle-treated ischemic group and were 203.2%, 178.2%, and 626.1% of those in the control group (Fig. 4A).

Microglia were visualized using immunohistochemical staining for Iba-1 4 days after ischemia. In the control group, Iba-1 immunoreactive microglia had a small cell body and thin processes. In the vehicle-treated ischemic group, Iba-1 immunoreactive microglia in the stratum pyramidale had a round cell body, but they had a hypertrophied cell body and thick processes in the stratum oriens and radiatum. In this group, Iba-1 immunoreactivity was significantly increased to 711.7% of that in the control group. In the purpurin-treated ischemic group, Iba-1 immunoreactive microglia had a large cell body and less-developed processes compared to those in the vehicle-treated ischemic group. In this group, Iba-1 immunoreactivity was significantly than that in the vehicle-treated ischemic group and was 459.9% of that in the control group (Fig. 4B).

MAPKs and their phosphorylated forms were validated using western blotting 1 day after ischemia in gerbil hippocampus and the ratio of phosphorylated and naïve forms were analyzed. In the vehicle-treated ischemic group, the ratios of p-JNK/JNK, p-ERK/ERK, and p-p38/p38 were significantly increased to 221.8%, 692.4%, and 223.9% of control group, respectively although naïve forms of MAPKs showed similar levels compared to respective control group. In the purpurin-treated ischemic group, the ratios of p-JNK/JNK, p-ERK/ERK, and p-p38/p38 were significantly lowered to 129.9%, 406.0%, and 124.2% of those in the control group compared to respective vehicle-treated ischemic group (Fig. 5).

Discussion

Purpurin, an alizarin-type anthraquinone, has free radical scavenging activity [14, 28-30] and antioxidant effects against Trp-P-2 carcinogen by reducing DNA adducts in the liver [31]. In the present study, we investigated the role of purpurin against oxidative stress induced by H₂O₂ in HT22 cells and against

ischemic damage in gerbils. First, we screened the toxicity of purpurin in HT22 cells to determine the optimal concentration without toxicity in HT22 cells. We observed that 25- μ M purpurin was the optimal concentration with minimal toxicity in HT22 cells. The optimal concentration may differ depending on the cell type. In 3T3-L1 adipose cells, 50- and 100- μ M purpurin had positive effects [30].

Oxidative stress was induced by treatment with H_2O_2 , which increases ROS formation and decreases cell viability in a concentration-dependent manner in HT22 cells [32]. Treatment with H_2O_2 significantly increased ROS formation and DNA fragmentation in HT22 cells, whereas it decreased cell viability. Purpurin treatment significantly ameliorated H_2O_2 -induced ROS formation, DNA fragmentation, and decreased cell viability in HT22 cells. This result was supported by previous studies showing that purpurin has H_2O_2 scavenging activity and reduces ROS levels in activated RAW 264.7 murine macrophages [14]. In addition, purpurin reduces *h*Tau accumulation in an in vitro culture system [18].

Next, we examined the protein levels of Bax and Bcl-2, which are the main components of the apoptosis and anti-apoptosis pathways, respectively, because high levels of ROS lead to mitochondrial membrane damage and release of pro-apoptotic proteins such as Bax [33]. Treatment with H_2O_2 significantly increased Bax levels and decreased Bcl-2 levels in HT22 cells, consistent with previous studies [32, 34]. Incubation with purpurin significantly ameliorated the changes in Bax and Bcl-2 induced by H_2O_2 treatment in HT22 cells. We also observed the phosphorylation of MAPKs, including JNK, ERK, and p38, because MAPKs play important roles in ROS-induced cell death and H_2O_2 significantly increased the expression of p-ERK 1/2, p-JNK, and p-p38 in HT22 cells [32]. Treatment with H_2O_2 significantly increased the p-JNK/JNK, p-ERK/ERK, and p-p38/p38 ratios in HT22 cells, and incubation with purpurin significantly mitigated the increase in the ratio.

In the present study, we also investigated the effects of purpurin against ischemic damage following oral treatment with 6 mg/kg purpurin because purpurin is able to cross the blood brain barrier [18, 35]. In addition, purpurin caused no significant changes in physiological or blood chemistry variables in an acute oral toxicity study [36]. We observed the locomotor activity 1 day after ischemia because the locomotor test is a predictive measure for assessing neuronal damage in the hippocampus [37, 38]. Transient forebrain ischemia significantly increased the travel distance and time in the mobile phase, indicating hyperactivity in gerbils 1 day after ischemia. Purpurin treatment significantly reduced the travel distance and time in the mobile phase. In addition, we confirmed that 6 mg/kg, not 1 or 3 mg/kg, purpurin treatment ameliorated the ischemia-induced reduction in NeuN-immunoreactive neurons in the hippocampal CA1 region. This result suggests that purpurin has the potential to reduce neuronal death induced by ischemia.

To elucidate the possible role of 6 mg/kg purpurin against ischemia, we observed the morphology of microglia and pro-inflammatory cytokines in the hippocampus because a recent study showed the anti-inflammatory roles of purpurin in RAW 264.7 murine macrophage cells [14]. The animals were sacrificed 6 h after ischemia to measure IL-1 β , IL-6, and TNF- α levels in the hippocampus because these levels are significantly increased in the early period of ischemia [26, 27, 39]. In addition, the IL-1 receptor antagonist

showed neuroprotective effects against ischemic damage in rats [40]. In the vehicle-treated group, IL-1 β , IL-6, and TNF- α levels were significantly increased 6 h after ischemia/reperfusion compared to those in the control group. In the purpurin-treated ischemic group, IL-1 β , IL-6, and TNF- α levels were dramatically lower in the hippocampal homogenates. This result suggests that purpurin treatment significantly reduces the release of pro-inflammatory cytokines in the hippocampus 6 h after ischemia. In addition, we confirmed microglial activation based on microglial morphology in the hippocampus 4 days after ischemia. In the vehicle-treated group, Iba-1-immunoreactive microglia had hypertrophied cell body and thickened processes (activated microglia), and the phagocytic form (round cell body without processes) of microglia were also found in the stratum pyramidale of the CA1 region 4 days after ischemia/reperfusion. This result was consistent with previous studies showing that ischemia induced microglial activation and morphological changes in the hippocampus [41, 42]. Treatment with purpurin reduced the phagocytic form of microglia in the stratum pyramidale, and overall Iba-1 immunoreactivity was significantly decreased in the hippocampal CA1 region compared to that in the vehicle-treated group. A molecular docking study demonstrated that purpurin had a strong inhibitory effect on the nucleotide-binding domain leucine-rich repeat and pyrin domain containing receptor 3, which is one of the main contributors to neuroinflammation [43].

In the present study, we also observed the ischemia significantly increased the ratios of p-JNK/JNK, p-ERK/ERK, and p-p38/p38 in the gerbil hippocampus 1 day after ischemia result. This result is consistent with in vitro study in HT22 cells that oxidative stress induced by H₂O₂ treatment significantly increased the phosphorylation of MAPKs. In addition, several studies demonstrate the increases of MAPK phosphorylation in the hippocampus after ischemia [22, 44, 45] and treatment with JNK blocker ameliorates the neuronal death induced by ischemia [46]. In addition, the close relationship has been reported between the cytokine-related inflammation and MAPKs [47, 48]. In the present study, we observed the purpurin treatment significantly decreased the activation of MAPK pathway in the hippocampus after ischemia.

Conclusions

The current findings suggest that purpurin may be a strong neuroprotective agent to reduce oxidative damage in HT22 cells by reducing phosphorylation of MAPKs in HT22 cells and ameliorating neuronal damage in the hippocampus by decreasing the release of pro-inflammatory cytokines and phosphorylation of MAPKs in the hippocampus after ischemia.

Declarations

Ethical Approval

The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU-200313-2).

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Authors Contributions

W.K., H.J.K, H.Y.J., K.R.H., Y.S.Y., I.K.H, S.Y.C., and D.W.K. conceived the study. W.K., H.J.K., S.Y.C., and D.W.K. designed the study and wrote the manuscript. W.K., H.Y.J., and K.R.H. conducted the animal experiments. H.J.K., D.W.K., and S.Y.C. conducted biochemical experiments. Y.S.Y. and I.K.H participated in designing and discussing the animal study. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Competing Interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets and supporting materials generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Effects of purpurin against oxidative damage in HT22 cells. (A) Concentration-dependent WST-1 assay was performed in HT22 cells to determine the optimal concentration to show minimal neurotoxicity. (B) ROS formation, (C) DNA fragmentation, and (D) cell damage was assessed after H₂O₂-induced oxidative stress in HT22 cells using DCF staining, TUNEL staining, and WST-1 assay. Scale bar = 50 μm. DCF and TUNEL fluorescent intensities were observed using an enzyme-linked immunosorbent assay (ELISA)

reader. Data are expressed as mean value $\pm$ standard deviation and were analyzed using one-way ANOVA followed by Bonferroni's post hoc test (ap < 0.05, significantly different from the control group; bp < 0.05, significantly different from the DMSO group; cp < 0.05, significantly different from the H₂O₂+DMSO group).

Figure 2

Mechanisms of purpurin' effects against oxidative damage in HT22 cells. (A) Protein levels related to cell death and survival were measured after H₂O₂-induced oxidative stress in HT22 cells using western blot analysis for Bax and Bcl-2, respectively. Protein levels of Bax and Bcl-2 were calibrated to the β -actin level. (B) Cell signaling pathway related to MAPKs were validated using western blot analysis for JNK, ERK, p38, and their phosphorylated forms. Protein levels were converted into p-JNK/JNK, p-ERK/ERK, and p-p38/p38 ratios in each group. Data are expressed as mean value $\pm$ standard deviation and were analyzed using one-way ANOVA followed by Bonferroni's post hoc test (ap < 0.05, significantly different from the control group; bp < 0.05, significantly different from the DMSO group; cp < 0.05, significantly different from the H₂O₂+DMSO group).

Figure 3

Effect of purpurin against ischemic damage in gerbils. (A) Traveled distance and cumulative duration was measured in gerbils 1 day after ischemia in sham-operated (control), ischemia-induced vehicle-treated (vehicle), and ischemia-induced purpurin-treated (purpurin) groups (n = 10 per group). (B) Mature neurons are visualized to show the surviving neurons after ischemic damage in the control, vehicle, and purpurin groups using NeuN immunohistochemical staining. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μ m. The number of NeuN-immunoreactive neurons is shown as a

percentile value vs. control group (n = 10 per group). (A and B) Data are expressed as mean $\pm$ standard deviation and were analyzed using one-way ANOVA followed by Bonferroni's post hoc test (ap < 0.05, significantly different from the control group; bp < 0.05, significantly different from the vehicle group).

Figure 4

Anti-inflammatory mechanisms of purpurin against ischemic damage in gerbils. (A) Levels of pro-inflammatory cytokines were measured 6 h after ischemia in the gerbil hippocampus of control, vehicle,

and purpurin groups (n = 5 per group). (B) Microglia were visualized to show the morphological changes after ischemia in the CA1 region of the control, vehicle, and purpurin groups with Iba-1 immunohistochemical staining. Scale bar = 50 μ m. Optical density was measured and expressed as a percentage of the value vs. control group (n = 5 per group). Data are expressed as mean value $\pm$ standard deviation and were analyzed using one-way ANOVA followed by Bonferroni's post hoc test (ap < 0.05, significantly different from the control group; bp < 0.05, significantly different from the vehicle group).

Figure 5

Protein levels of JNK, ERK, p38, and their phosphorylated forms were validated 1 day after ischemia in the gerbil hippocampus of control, vehicle, and purpurin groups (n = 5 per group) using western blot analysis. Protein levels were converted into p-JNK/JNK, p-ERK/ERK, and p-p38/p38 ratios in each group. Data are expressed as mean value $\pm$ standard deviation and were analyzed using one-way ANOVA followed by Bonferroni's post hoc test (ap < 0.05, significantly different from the control group; bp < 0.05, significantly different from the vehicle group).