Hydroxysaflror Yellow A Together with Blood–Brain Barrier Regulator Lexiscan for Cerebral Ischemia Reperfusion Injury Treatment

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ABSTRACT: Pharmacodynamic and biodistribution effects are two important factors in drug research. As a clinical drug, the neuroprotective effects and mechanisms of hydroxysafflor yellow A (HSYA) have been widely reported but have still not been described in enough detail. In this study, we first aimed to improve the pharmacology of HSYA in nerve injury treatments. The down-regulative expression of cytokines, including NLRP3, ASC, Caspase-1, GSDMD, IL-1β, IL-18, LDH, NF-xB, and p-p56, suggested that HSYA could both suppress pyroptosis and apoptosis pathway activation during the nerve injury. Additionally, HSYA improved the cellular viability in an oxidative stress damage cell model. Second, to further improve the therapeutic effect of the HSYA, we tried to enhance the concentration of HSYA in a lesion. The FDA-approved adenosine receptor agonist Lexiscan (Lex) could inhibit the expression of P-glycoprotein on the endothelial cell surface to transiently increase the permeability of the blood–brain barrier (BBB) without any sustained damage, which was used to assist HSYA in passing through the BBB to increase the accumulation in the brain. Furthermore, living image and distribution detection in vivo showed that the accumulation of HSYA in the brain could be significantly increased with the addition of Lex. Lastly, HSYA together with Lex (Lex-HSYA) could significantly reduce the volume of cerebral infarction, improve the histopathological morphology, and recruit brain-derived neurotrophic factors to alleviate the cerebral ischemia reperfusion injury. In conclusion, the pyroptosis pathway could act as a novel therapeutic target of HSYA in nerve injury treatment, and Lex-HSYA could be a promising candidate for nerve injury treatments.

INTRODUCTION

As a serious morbidity worldwide, ischemic stroke causes a high number of deaths annually. The clinical principle of the treatment for this disease is to restore the blood supply to the ischemic area in time, which can reduce the mortality risk of the patient. However, many pathological features during blood recovery, such as the production of radical oxygen species (ROS), calcium overload, energy failure, cell apoptosis, and an inflammatory reaction, precipitate the permanent deterioration of the central nervous system (CNS), which causes long-term disability.1–3 Therefore, protection and repair of the CNS after blood supply recovery are important for alleviating cerebral ischemia-reperfusion (CIR) injury.

Recently, an increasing number of reports have indicated that pyroptosis is a novel program cell death mechanism that could lead to inflammation and result in the aggravation of damage during CIR injury.9 Similar to apoptosis, pyroptosis is a form of programmed necrosis, which is also mainly mediated by Caspase-1.10 In ischemia cerebral injury, the necrotic cells in the ischemic core usually release various cell components to cause further inflammasome formation and Caspase-1 activation in the adjacent cell, which leads to pyroptosis occurring. Then, relative inflammatory factors, such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), are released, resulting in the secondary injury. This cascade
amplifies the inflammatory reactions, aggravating the injury.\textsuperscript{11} Therefore, pyroptosis not only participates in the initiation of inflammatory reactions but also plays a critical role in spreading inflammatory signals and amplifying inflammatory reactions. To date, although some mechanisms of HSYA on CIR therapy have been reported,\textsuperscript{12} the effect on the pyroptosis pathway is not clear. Previous studies on the anti-inflammatory activity of HSYA considered the inhibition of caspase-dependent activity for the inhibition of cell apoptosis.\textsuperscript{4} However, it may actually inhibit the occurrence of pyroptosis by inhibiting the expression of Caspase-1. A detailed investigation of the pharmacological mechanism of HSYA is important for its further application in therapeutic effect improvement for CIR injury therapy. Therefore, in this study, we explored whether pyroptosis is a novel mechanism of HSYA for alleviating initial cell damage.

On the other hand, the targeting efficiency of the drug also determines the therapeutic effect.\textsuperscript{13} As a hydrophilic drug, the dissatisfactory accumulated efficiency in the brain has limited the wide application of HSYA in clinics, although it can be used for direct injection.\textsuperscript{14} Therefore, how to increase the concentration of HSYA in the lesion is another research focus in this study, after clarifying the mechanism of drug efficacy. As the defense structure of the brain, which is constructed by endothelial cells and junction molecules, the blood–brain barrier (BBB) protects the brain from damage in a normal state and hinders drug delivery to some CNS disease therapies.\textsuperscript{15} Although some have reported that the BBB was temporarily opened in the acute phase of CIR injury, it was subsequently closed.\textsuperscript{16,17} Therefore, BBB is still an important factor that prevents drugs from accumulating in the brain. A number of attempts, including physical disruption, drug modification, nano-drug delivery systems, and intrathecal injection, have been used for the improvement of drug enrichment in the brain.\textsuperscript{18–20} However, because of the shortcomings of these methods, such as the lack of large-scale production processes, side effects, and other problems, no products have been used in clinical applications.\textsuperscript{21,22} Therefore, a simple, safe, and effective method of overcoming the drug delivery barrier can indeed improve the therapeutic effect of CIR injury.

P-glycoprotein (P-gp) is one of the most widely known and investigated drug efflux transporters and is highly expressed on brain endothelial cells to inhibit drug delivery to the brain via the paracellular pathway.\textsuperscript{23,24} Previous research has indicated that regulating the expression of P-gp would improve the permeability of BBB.\textsuperscript{25} The \(\alpha_2\)A adenosine receptor (\(\alpha_2\)A AR) is distributed in glial cells and endothelial cells to regulate the immune responses.\textsuperscript{26} Moreover, the activation of \(\alpha_2\)A AR could induce the degradation of P-gp to change the permeability of BBB.\textsuperscript{27} The FDA-approved-specific \(\alpha_2\)A AR agonist \(2\{-4\{[(methylamino)carbonyl]-1H-pyrazol-1-yl}\}\) adenosine (Lexiscan, Lex) has been reported, which could increase the accumulation of drugs in the brain in a time- and dose-dependent manner, without side-effects.\textsuperscript{28} Therefore, in this study, Lex was employed in an attempt to increase the concentration of HSYA in the brain.

In this study, as described in Scheme 1, we first studied the pharmacodynamic effects of HSYA in vitro and clarified the effect of HSYA on the pyroptosis pathway. Then, the targeting efficiency of the Lex-HSYA was investigated both in vitro and in vivo. Evans blue (EB) staining, living images, and high-performance liquid chromatography (HPLC) were used to monitor the distribution of HSYA. The changes of the BBB structure during the administration were observed by transmission electron microscopy (TEM). Finally, the therapeutic effect of Lex-HSYA was evaluated on the middle cerebral artery occlusion (MCAO) models. All results supported that Lex could improve the ameliorating effect of HSYA in CIR treatments.

# RESULTS AND DISCUSSION

**Cytotoxicity of HSYA In Vitro.** Among the multiple pathogenic factors that participated in the neuronal death and
Figure 1. Effects of HSYA on pyroptosis and apoptosis induced by oxidative stress. (A) Protective effect of HSYA with different dosages in an oxidative stress damage cell model. (B) Fluorescence image of PC12 oxidative stress injured cells stained with Calcein AM/PI. Viable cells exhibited green fluorescence while dead cells appeared red. (C) Dead−live cell analysis of the PC12 cells in an oxidative stress damage cell model. (D) Mechanism of the protective effect of HSYA on the oxidative stress injured cell. mRNA detection of NLRP3 (E), ACS (F), Caspase-1 (G), GSDMD (H), IL-18 (I), LDH (J), and IL-1β (K) at 6 and 24 h after administration, respectively. (L) Expression of NLRP3, Caspase-1, and IL-1β in different groups. (M) Quantitative data on NLRP3, Caspase-1, and IL-1β protein content. The expression (N) and the quantitative data (O) of ASC and GSDMD in each group. The concentration of LDH (P) and IL-18 (Q) in each group. The expression (R) and the quantitative data (S) of NF-κB and p-p65 in each group. Statistical difference from each group (*p < 0.05). Values are the mean ± SD.
neurological dysfunction, the production of ROS was the major factor that results in inflammation or cell apoptosis. Therefore, in this study, we first opted the PC12 cell injury induced by hydrogen peroxide models to evaluate the pharmacodynamic effects of HSYA in vitro.4 As shown in Figure 1A, the cell viability was improved as the dose of HSYA increased, until 10 μM. The cell viability decreased with further increasing of the dosage. Therefore, 10 μM was the optimal concentration of HSYA for the following study in this paper. By laser scanning confocal microscopy, after Calcein-AM/PI staining, viable cells exhibited green fluorescence while dead cells appeared red (Figure 1B). Within 24 h after HSYA administration, the dead cell rate of PC12 cells damaged by H2O2 was decreased (Figure 1C). These results suggested a dose-dependent effect of HSYA on the protection of nerve cells from oxidative damage.

Inhibition of the Pyroptosis Pathway. The pyroptosis pathway is a novel cellular injured mechanism found in recent years.29,30 As shown in Figure 1D compared to the traditional cell injury pathway, the pyroptosis pathway not only participates in the initiation of inflammatory reactions but also spreads inflammatory signals to amplify inflammatory reactions. However, similar to traditional apoptosis, pyroptosis is also mainly mediated by Caspase-1. However, HSYA could regulate the pyroptosis pathway to act as protection, which was then explored. The quantitative real-time PCR (qPCR) analysis was used to investigate the mRNA transcriptional expression of the relative cytokine. As shown in Figure 1E−J, the mRNA transcriptional expression of NACHT, LRR, and PYD domains-containing protein 3 (NLRP3), apoptosis—associated speck—like protein containing (ASC), cysteinyll aspartate specific protease-1 (Caspase-1), Gasdermin D (GSDMD), IL-1β, IL-18, and lactic dehydrogenase (LDH)

Figure 2. Investigation of the effect on pyroptosis in vivo. The protein expression (A) and the quantitative data (B) of NLRP3, ASC, Caspase-1, and GSDMD in brain tissues. The statistical difference from each group (**p < 0.01). Values are the mean ± SEM, and there were at least five samples in each group. Effect on BBB permeability change in vitro.

Figure 3. Investigation of the effect of Lex on the changing of the BBB. (A) BBB model in vitro was constructed by the b.End3 and C6 cell coculture system. The activation of the paracellular pathway resulted in the down-regulation of P-pg and F-actin, and the permeability of model was reflected by the intensity of the fluorescence probe HRP in the sublayer. (B) P Glycoprotein 1 protein immunofluorescence staining. (C) Quantitative analysis of P Glycoprotein 1 protein expression. (D) F-actin protein is exhibited by the red fluorescence, and the blue fluorescence is DAPI Staining Solution (DAPI). (E) Fluorescence intensity detection of HRP to evaluate the permeability change of the BBB in vitro. The statistical difference from each group (**p < 0.01). Values are the mean ± SEM, and there were at least five samples in each group.
were increased significantly in the model group, which indicated that the pyroptosis pathway could be activated by oxidative stress. Surprisingly, these phenomena could be inhibited by the addition of HSYA. In addition, because the inflammation and pyroptosis might be activated within hours, we have determined the mRNA level at 6 and 24 h after stimulation. Compared to the model group, the expression of NLRP3, Caspase-1, GSDMD, ASC, IL-18, LDH, and IL-1β was dramatically decreased in HSYA groups, which had the consistent trend in 6 and 24 h. Moreover, different from the other factors, the expression of IL-1β in 24 h was lower than that in 6 h but there was no significant difference. These results suggested that HSYA could inhibit the activity of pyroptosis pathway in the acute phase. Moreover, the results of the Western blot (WB), as shown in Figure 1K–N, and the ELISA, as shown in Figure 1OP, indicated that the expression of NLRP3, ASC, Caspase-1, GSDMD, IL-1β, IL-18, and LDH was significantly increased during injury and decreased after administration. Besides, we further evaluated the regulatory effect of HSYA on the apoptosis dependent on the nuclear factor kappa-B (NF-κB) signaling activation. As shown in Figure 1QR, the expression of NF-κB and the phosphorylation of p65 in the oxidative stress PC12 cells could be down-regulated by the HSYA. These results indicated that HSYA could decrease the activity of NF-κB signaling pathway. In a conclusion, these remarkable phenomena suggested that HSYA was likely to alleviate inflammation after injury by both inhibiting the activation of the pyroptosis pathway and apoptosis signaling pathway.

Furthermore, the antipyroptosis effect of HSYA was investigated in the MCAO model. As shown in Figure 2AB, the expressions of the NLRP3, ACS, Caspase-1, and GSDMD were significantly up-regulated during the CIR injury. Continuously, the expressions of the relative proteins exhibited the decrease trend by the addition of HSYA. However, there was no significant difference between the model group and the HSYA (5 mg/kg) group. With the increase dosage of HSYA to 10 and 20 mg/kg, the inhibition of the protein expression became more and more obvious. These phenomenal suggested that the activation of the pyroptosis pathway could be inhibited by sufficient HSYA.

Effect on BBB Permeability Change In Vitro. The BBB protects brain tissues from damage by foreign substances but also hinders the therapeutic effect of drugs on the brain. Therefore, the regulation of BBB permeability is important to accumulate drugs in the brain. It has been reported that Lex, as a kind of A2A AR agonist, could down-regulate the expression of P-gp to change the permeability of BBB, which could assist drugs attempting to cross the BBB by the paracellular pathway. Therefore, we constructed an extracorporeal BBB model for validation of the Lex effect (Figure 3A). In this experiment, we selected immortalized mouse brain microvascular endothelial (b.End3) cells and mouse astrocyte (C6) cells to construct an in vitro BBB model by noncontact coculture, which referred to the method of Hurst and Fritz. This classic noncontact coculture had a good correlation with the in vivo results and was frequently used in the study of material permeability. In this model, the interaction between cells through the secreted substances could truly reproduce the interaction between various cells under physiological or pathological conditions. Specifically, similar to the structure in vivo, C6 cells could induce b.End3 cells to form the barrier characteristics of BBB. More importantly, the cell culture was convenient, and the immortal cell line coculture model had good stability and repeatability, and the culture was relatively simple. As shown in Figure 3B,C, the expression of P-gp was down-regulated by Lex, which was consistent with a previous report. F-actin is composed of two intracellular filamentous structures, including microfilaments and filaments. It is an endothelial cytoskeletal protein in BBB that is involved in cell attachment and cell morphology retention and establishment. The distribution of microfilaments in the cells can be clearly displayed by staining with the fluorescently labeled phalloidin. Thus, the expression of F-actin could reflect the
change of BBB permeability in vitro. As shown in Figure 3D, the red fluorescence of F-actin in the control group was a network distribution with a large number of distributions. However, in the Lex group, the red fluorescence was sparse. A comparison of the two groups revealed that Lex could reduce the expression of F-actin and increase the permeability of the BBB in vitro. Moreover, the permeability changes were further analyzed, which are exhibited in Figure 3E. The fluorescence intensity of horseradish peroxidase (HRP) transmission in the Lex group was significantly stronger than in the other two groups. These results illustrated that Lex had an effect on promoting BBB permeability in vitro.

**Permeability Evaluation of BBB In Vivo.** Next, to explore whether Lex could increase the permeability of the BBB in vivo, EB dye was used as the tracer for qualitative detection. As shown in Figure 4A, after the administration in normal rats, the blue part of the EB-Lex group could be observed more obviously than that in the EB group, which indicated that Lex could increase BBB permeability in normal rats. Then, MCAO model rats were established to examine this role of Lex. EB was injected after CIR injury, and the blue part in the cerebral cortex reached the highest level at 1 d and then gradually decreased within the next 5 days. This phenomenon suggested that the tight junction of the BBB partially recovered on the third day and completely recovered on the fifth day. The significant difference between EB-IR and EB-IR-Lex indicated that Lex could further extend the retention time of EB in the brain by enhancing the BBB permeability, which
might be beneficial to the enrichment of drugs in the brain. In addition, we quantified EB extravasation (Figure 4B), and the results were consistent with those that are shown in Figure 4A.

On the other hand, to further clarify the mechanism of Lex in BBB permeability in vivo, the ultrastructure of rat brain tissue was structurally identified by TEM. As shown in Figure 4C, the nucleus of the oval cerebral neurons, as abundant and structurally intact cytoplasmic organelles, could be observed in the sham group. However, in the Lex group, the whole microstructure could not be found; the brain tissue was dissolved, the tight junction between the endothelial cells was almost invisible, and vacuolization occurred everywhere. The same result appeared in the model group. These results indicated that Lex could modify the structure of the BBB to change the permeability. Moreover, to our surprise, a normal histomorphology, such as the tightly connected endothelial cells, capillaries with mild edema, and an intact structure, could be observed 5 days after the HSYA administration. These results illustrated that Lex could change the permeability of the BBB to enhance the permeability and finally to increase the concentration of drugs in the brain.

**Biodistribution of HSYA.** In order to study the effect of Lex on the distribution of HSYA in vivo, the hydrophilic fluorescent probe Cyanine 7 NHS ester near-infrared fluorescent dye (Cy7) with a similar molecular weight was used to replace HSYA for the detection. On the one hand, the Cy7 dye could simulate the entry of drug molecules into the body. On the other hand, the fluorescence emitted by Cy7 at a specific wavelength makes it easier for us to monitor the changes of the drug in the brain. Near-infrared fluorescence imaging technology was used to observe the real-time fluorescence distribution in mice. The fluorescence signal could not be detected in the blank group and the Lex group (Figure 5A). The fluorescence in the Cy7 group and Cy7-Lex group increased gradually from 2 to 8 h after administration, and the fluorescence intensity reached the peak at 8 h and weakened within 24 h (Figure 5B). Subsequently, the rats were sacrificed to obtain the tissue for further investigation (Figure 5C). The Cy7-Lex group had a stronger fluorescence intensity than the Cy7 group, which indicated that Lex could increase the concentration and residence time of drugs in the brain. From another perspective, we also found that the drug concentration began to diminish at 24 h, so a daily dose was chosen for the dosing regimen.

To further verify the above results, the HPLC was used to detect changes in the content of HSYA in vivo, especially in the brain. The HSYA concentrations in the brain tissue and serum of different groups after administration were detected. The HPLC curves are shown in Figure 5D,E, and the quantitative analysis is shown in Figure 5F,G. It was easily found that HSYA could be detected after administration. Additionally, the concentration of the Lex-HSYA group was remarkably higher than the HSYA group (*p < 0.05). On the other hand, for the concentration of HSYA in serum, no significant difference could be found between the HSYA group and Lex-HSYA group. It could be summarized that Lex could only increase the concentration of HSYA in the brain but not in the serum. Therefore, it could be considered that Lex...
Figure 7. HE stains of brain tissue slices in different groups.

Figure 8. (A) Nissl staining images. (B) Quantitative analysis of Nissl-positive cells in the ischemic penumbra (*p < 0.05). The statistical difference between the HSYA (20 mg/kg) group and Lex-HSYA (20 mg/kg) group is significant (*p < 0.05). Values are the mean ± SD, and there were six samples in each group.
Each group.

were 0% (sham group), 25.6 ± 0.06% (Lex group), 21.0 ± 0.48% [Lex-HSYA (10 mg/kg) group], and 19.83 ± 0.052% [Lex-HSYA (20 mg/kg) group], respectively. Compared with the model group, the levels of cerebral infarction were significantly lower in HSYA (20 mg/kg), Lex-HSYA (10 mg/kg), and Lex-HSYA (20 mg/kg) groups. It is gratifying to note that the brain tissue structure was almost complete and clear in the Lex-HSYA (20 mg/kg) group.

Moreover, little therapeutic effect could be found on the individual treatment groups was significantly lower in HSYA (20 mg/kg), Lex-HSYA (10 mg/kg), and Lex-HSYA (20 mg/kg) groups (*p < 0.05), which suggested that HSYA could alleviate CIR injury. Furthermore, the cerebral infarction volumes in the HSYA (20 mg/kg) and Lex-HSYA (10 mg/kg) groups were similar while the Lex-HSYA (20 mg/kg) group had a lower infarct volume than the HSYA (20 mg/kg) group (*p < 0.05). This could be attributed to the fact that Lex-assisted HSYA accumulates in the brain to enhance the therapeutic effect. Furthermore, we detected the neurological function scores of each group, and Figure 6C illustrated that the injured groups displayed the behavior that was characteristic of neuron damage with the high neurological deficit scores. These damaged animals were ameliorated to a great extent after administration. With the prolong of the treatment, the recovery of the neurological function in the treatment groups was significant. Compared to the individual HSYA group, the Lex-HSYA groups exhibited more remarkable neurological functional improvement. In addition, compared with the model group, the water content of brain tissue was reduced in the treatment group (Figure 6D), which was consistent with the results of the infarct volume detection. Moreover, little therapeutic effect of Lex could be found on the MCAO model. In conclusion, HSYA, together with Lex, could improve the therapeutic effect on CIR treatments.

Histopathological Study. To further evaluate the protective effect of Lex-HSYA on CIR, the histopathology was studied with H&E staining, Nissl staining, and TUNEL staining. First, as shown in Figure 7, the normal neuron cells with the clear membranes, and ordinarily shaped and centered nuclei were histologically displayed in the brain tissue in the sham group. In the model group, the ischemic areas were visible in the cerebral cortex, in addition to vascular congestion, nuclear rupture, and the disintegration of some neurons. The situation of the Lex group was similar to that of the model group. Fortunately, these histological changes were decreased, and an improvement in histological appearance was observed in HSYA (20 mg/kg), Lex-HSYA (10 mg/kg), and Lex-HSYA (20 mg/kg) groups. It is gratifying to note that the brain tissue structure was almost complete and clear in the Lex-HSYA (20 mg/kg) group.

Second, the Nissl staining was applied to examine the magnitude of neuronal injury. As shown in Figure 8A,B, the model group was similar to the Lex group. The number of Nissl corpuscle neuronal cells in HSYA groups was much higher than that of the model group, and the Lex-HSYA (20 mg/kg) group exhibited the greatest improvement (*p < 0.05). These results revealed that Lex-HSYA has a prominent effect on terms of protecting brains from CIR.

Finally, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was used to investigate the neuronal apoptosis in each group. Compared to the sham group, more apoptotic cells could be found in the ischemic penumbra in injured groups. Simultaneously, the data clearly showed that less-apoptotic cells presented in the HSYA-treated group compared to the model group. Among them, the Lex-HSYA group had fewer apoptotic cells than the HSYA group (*p < 0.05, Figure 9). Histopathological studies indicated that under the synergy of Lex, HSYA inhibits neuron apoptosis and promotes the tissue repair of the CIR injured brain. Additionally, large doses of HSYA show better effects.

Expression of the Neurotrophic Factor In Vivo. For such CNS injury, the neurotrophic factor plays an important role in promoting the functional recovery and mainte-
nance. It has been reported that HSYA could increase the content of the BDNF protein and exert neuroprotective effects on cerebral ischemic injury. As shown in Figure 10, CIR resulted in the down-regulation of BDNF expression. To our surprise, with the intervention of HSYA, the content of BDNF in the injured area increased. This indicates that HSYA can significantly up-regulate the expression of BDNF in a dose-dependent manner. Moreover, the expression of BDNF in the Lex-HSYA group was further up-regulated, which might be attributed to the concentration increase of HSYA in the brain, together with Lex.

**CONCLUSIONS**

In this study, the FDA-approved A2AR agonist Lex was used to promote the accumulation of HSYA in the brain by the transitory enhancement of BBB permeability. Lex could down-regulate the expression of P-gp on the surface of endothelial cells to assist HSYA attempting to pass through the BBB by the cell-bypass pathway. This treatment for anti-CIR injury suggested that HSYA with Lex maintained a better protective performance than the single HSYA, which was attributed to improvement of the drug biodistribution in vivo. On the other hand, the therapeutic mechanisms of HSYA for CIR injury were further polished. The pyroptosis pathway would be a novel target of HSYA in the treatment of CIR injury. In conclusion, the improvement of targeting and the further clarification of the effective mechanism would provide a brilliant prospect for Lex-HSYA in the clinical application of CIR treatment.

**MATERIALS AND METHODS**

HSYA was supplied by Pusi Biotechnology Co. Ltd. (Chengdu, China), and the Lex was supplied by Yuanye Biotechnology Co. Ltd. (Shanghai, China). Cyanine 7 NHS ester near-infrared fluorescent dye (Cy7) (Shanghai, China), the Live & Dead Viability Kit (kai GENE bio TCH, Nanjing), Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Saint Louis, MO, USA) (DMEM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) (MTT) were used without any further purification. DAPI staining solution (DAPI) and all the HPLC-grade reagents, acetonitrile, methyl alcohol, and ethyl alcohol were purchased from Sigma-Aldrich. TTC was obtained from Sigma-Aldrich.

For the WB assay, antibodies against P Glycoprotein 1, IL-1β, Caspase-1, nuclear factor kappa-B (NF-κB), phosphor-protein 65 (p-p65), the NLRP3, ASC, and Gasdermin D GSDMD antibody and HRP-conjugated secondary antibodies were obtained from Affinity Bioscience (Cincinnati, OH, USA). In addition, enzyme-linked immunosorbent assay (ELISA) kits for IL-18 and secreted LDH were purchased from Elabscience (Wuhan, China).

Mouse brain microvascular endothelial cells (b.End3) and the Pheochromocytoma cell line (PC12 cell) were grown in DMEM (Wako) supplemented with 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin–streptomycin. Cell culture was performed in a humidified atmosphere of 5% CO₂/95% air at 37°C in the incubator.

Male Sprague-Dawley (SD) rats and Balb/c male mice were purchased from Dashuo Bio-Technology Co. Ltd. (Chengdu, China). Male rats weighing between 280 and 300 g were housed under standard conditions with free access to food.
Table 1. Primers Used in This Study

| gene name | forward primer (5′−3′) | reverse primer (3′−5′) |
|-----------|------------------------|-----------------------|
| GAPDH     | CTGGAGAAGACCTGGCAGGCTG | GGTGGGAGAATGGGAGTGTG |
| NLRP3     | TCTTTGCGGCTATGCTAATCTC | TTCTAATAGGACCTCACTGT |
| Caspase-1  | CCGGGCAGCAGATGATTAT | AACCCTCGGTGCAAGGAATTG |
| ACS       | ATTTCAGGTCGCAAGTAGA | CTTTGTGACGGAAAGTGATG |
| GSDMD     | TGAGGTGCTGGTGGTCGAC | ATGGGGTGCTCTCCTCAAG |
| LDH       | ATCCAGACTCCTTGTCGGCATTCA | TGGCCTTGTAGTTGCTCTCAT |
| IL-1β     | TGACCTGTTCCTTGGAGGCTGAC | CATACCCACAGTACAGAG |
| IL-18     | AGTACGGCAGCGCCCTCAATCC | CACGACTGAGCGCAATGGT |

The protocol was authorized by the Institutional Animal Care and Use Committee of Chengdu Military General Hospital.

**Cell Viability.** The cell viability of Lex and HSYA was measured by an MTT assay on the PC12 cell line. In these tests, the cells were seeded in 96-well plates with a density of 8 × 10^3 cells per well for 24 h incubation. Lex and HSYA at different concentrations were added to the plates for another 4 h incubation. Then, 400 μM H_2O_2_ was added to the plates for another 4 h incubation. In the next step, 20 μL of MTT solution (5 mg/mL) was added to each well, and the solution was replaced with DMSO (160 μL per well) after 3 h. The absorbance was measured by a 680-model microplate reader from an infinite M200 microplate reader (Tecan, Durham, NC, USA).

The Cell Viability assay was used to directly observe living/dead cells. The cells were incubated with Calcein-AM/PI solution (2 μM Calcein-AM and 8 μM PI in phosphate buffered saline (PBS)) for 45 min and then washed three times in PBS before being imaged under the laser confocal microscope (A1si+/A1Rsi+, Nikon, Tokyo, Japan).

**RNA Extraction and QPCR.** The PC12 cells were pretreated with the indicated concentration of HSYA (10 μM) for 24 h before the addition of H_2O_2_. After the other coculture 24 h, the total mRNA was extracted from the cells of each group by TRIzol extraction. Both the amount and purity of the RNA preparation were confirmed by measuring the absorbance ratio at 260/280 nm. Total RNA (1 μg) was converted to cDNA using a PrimeScript RT reagent kit with gDNA Eraser and PCR amplification followed by an ABI Step One Plus instrument and software (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix. The RNA levels of the target genes were normalized by GAPDH according to the 2^−ΔΔCT method. The primers used in this study are shown in Table 1.

**Western Blot.** Potential pharmacodynamic validation of protein expression in isolated PC12 and the brain tissue was explored by WB analysis. For the in vitro detection, the 2 × 10^5 PC12 cells were plated in six-well plates. After 24 h of incubation with HSYA, H_2O_2_ was added for another 4 h. Then, PC12 was harvested using a cell scraper and PBS and centrifuged at 12,000 g for 5 min at 4 °C, followed by discarding of the supernatant. Afterward, the total protein was extracted with buffer containing 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1% Triton X-100, and a protease inhibitor cocktail (Roche, Penzberg, Germany). The total protein concentration was measured by using the BCA kit (KeyGEN), according to manufacturer’s protocol. In total, 30 μg of total protein was loaded on 10% denaturing SDS gel and transferred to a 0.22 mm polyvinylidene difluoride membrane (Millipore) for blotting. The membrane was blocked with 5% nonfat dry milk and probed with primary antibodies and HRP-labeled secondary antibodies. Proteins were detected by a chemiluminescence substrate (Thermo Fisher). The results were documented on the Chemo-star imaging system (Bio-Rad). Moreover, for the brain tissues, the isolated brains in each group were homogenized at ~80 °C. Next, the samples were lysed in RIPA, and the supernate and equal amounts of protein were loaded on an SDS-PAGE gel. The following steps are consistent with the cell assay method.

**Extracorporeal BBB Model Construction.** The cerebral vascular endothelial cells (b.End3 cells) (5 × 10^5/cm^2) were plated in the upper chamber of a transwell chamber (Corning, six plates), and C6 cells (2 × 10^5/cm^2) were plated in the lower chamber, and these were then cultured for 5–7 days until the upper chamber cells reached the fusion state. After the observation of a fusion state, the cell culture solution was added to both the upper chamber and lower chamber, so that the liquid level difference between the two chambers was >0.5 cm. After 4 h, the liquid level difference between the two pools was observed to change. Most importantly, the resistance values (TEER) across the transwell plates were measured with a resistance meter at 3, 5, 7, and 10 d after the inoculation of b.End3 cells. In addition, the BBB permeability in vitro was evaluated by the tracer HRP throughput, and the HRP-containing medium was added to the upper chamber at the set points 0, 2, 5, 8, 10, 15, 30 min, 1, 2, 4, and 6 h. The 100 μL of chamber culture medium was taken out in a 96-well plate, and after the HRP substrate was added for the reaction, the absorbance was measured at 450 nm.

**Immunofluorescence Assay.** The b.End3 cells were grown on transwell in six-well plates and treated with HSYA (20 μM) and Lex (0.39 μg/mL) for 24 h. Then, the cells were rinsed with PBS and fixed with 4% PFA for 15 min, followed by permeabilization for 30 min. After blocking with 3% BSA for 1 h, the cells were incubated with a primary antibody against F-actin (1:50) or P-gp (1:100) at 4 °C overnight. After rinsing, cells were incubated with a secondary antibody for 2 h at room temperature. The nuclei were stained with DAPI (0.5 μg/mL, Vector Laboratories, Burlingame, CA) for 5 min.

Rats were anesthetized and transcranial perfused with ice-cold PBS immediately at 5 d after reperfusion, and the tissues were harvested and fixed with 4% paraformaldehyde (PFA). In brief, the brain sections (20 μm) were washed twice in PBS, permeabilized (in PBS with 0.1% Triton X-100), and blocked (in PBS containing 1% BSA and 0.3% Triton X-100) for 1 h at room temperature. The sections were then incubated with specific primary antibodies, as follows: rabbit anti-BDNF, for 3 d at 4 °C. After being rinsed with PBS, the sections were incubated with appropriate secondary antibodies overnight at 4 °C. After several rinses with PBS, sections were mounted on
slides with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA). Immunofluorescence confocal microscopy was performed with a confocal laser scanning microscope (A1si+/A1Rsi+, Nikon, Tokyo, Japan).

**EB Leakage.** EB leakage was used to assess BBB permeability. In brief, 5 d after reperfusion, the rats were injected with 2% EB (4 mL/kg) via the tail vein to evaluate the permeability change of BBB. Rats were perfused 2 h later with PBS until the perfusate turned clear. Brains were rapidly removed for the following imaging.

**Transmission Electron Microscopy.** After CIR, the rats were sacrificed at the scheduled time. Then, the brains were trimmed to a dimension of about 0.2 mm × 0.5 mm and fixed with 2.5% glutaraldehyde for 3–4 h. Specimens were rinsed with 1% citric acid · 0.1 M phosphate buffer (pH 7.4), with was fixed at room temperature (20 °C) for 2 h, and rinsed with 0.1 M phosphate buffer. A bloc was stained with uranyl acetate (2% aqueous for 48 h at 48 °C), dehydrated with ethanol, and embedded in epoxy resin. Sections 60 to 80 nm in thickness were stained with lead citrate and examined with a FEI Tecnai G20 TWIN electron microscope.

**Near-Infrared Fluorescence Imaging.** Balb/c male mice were divided into four groups: the control group, Lex group, Cy7 group, and Cy7-Lex group. First, the mice were fasted 6 h before imaging, and the mice were depilated after anesthesia to reduce background fluorescence interference caused by hair. Second, the control group did not receive treatment, the Lex group was injected with Lex solution (0.39 mg/kg), the Cy7 group was injected with 0.1 mL of free Cy7 solution (1 mg/mL), and the Cy7-Lex group was injected with Lex solution (0.39 mg/kg) and free Cy7 solution (1 mg/mL). After the administration, the mice were anesthetized by intraperitoneal injection with sodium pentobarbital (60 mg/kg) at 2, 4, 8, and 24 h. The fluorescence in brains was obtained by the small animal living imaging system (absorption maximum at 749 nm, emission maximum at 776 nm). Finally, the brain tissue of the mice was dissected for imaging analysis.

**Drug Concentration in Blood and Tissue Distribution.** Pretreated brain tissue homogenate and serum samples were investigated at 405 nm wavelength, and each UV spectrum peak was compared to the standard compounds by an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, United States). Chromatographic analysis was performed at 35 °C with a Phenomenex Luna C18 column, and water-phosphoric acid (100:0.2, v/v) and methanol were used as the mobile phases A and B (the flow comparison is A/B = 65:35). The mobile phase was delivered at a rate of 1 mL/min with a 20 μL injection volume. The data were analyzed by Lab Solutions software version C01.07 (Agilent Technologies, Santa Clara, CA, United States).

**Construction and Neurobehavioral Score of the MCAO Models.** Cerebral ischemia models were induced by transient occlusion of the middle cerebral artery with the intraluminal filament technique, as previously described. Rats were anesthetized by sodium pentobarbital [60 mg/kg, intraperitoneal injection (i.p.)]. The right common and external carotid arteries were isolated and ligated. A microvascular clip was placed on the internal carotid artery. A nylon monofilament coated with silicon resin was introduced through a small incision in the common carotid artery for occlusion of the MCAO. The internal carotid artery was then ligated, and the skin incision was closed. After 2 h, the monofilament was removed to allow reperfusion of the MCAO. Finally, the degree of nerve damage and the neurobehavioral score were evaluated according to the Longa.33 Except for the MCAO and hemostasis from the common vena jugulars, the same operation was performed on the rats in the sham group. In addition to administration once before surgery and in the 2 h after reperfusion, administration once a day for the 3 days after reperfusion was conducted. Tissues were harvested after five administrations for further analysis. Both were administered intravenously.

**Measurement of the Infarct Volume and Water Content.** Rats in each group were sacrificed at 24 h after the last administration, and their brains were collected for measuring the infarct area (n = 6) and water content. The brains were removed immediately and washed with phosphate buffer solution (PBS, 0.1 mol/L) three times. Then, the 2 mm slices of the brain tissue were incubated in a 2% TTC solution, with mitochondria appearing red in color, while the ischemic area remained colorless. Digital photographs of the TTC-stained slices were taken for documentation. The infarct volume was calculated as follows:

\[
\text{infarct volume (\%)} = \frac{\text{weight of white area}}{\text{weight of whole brain}} \times 100\%
\]

For the measurement of water content, the weights of fresh tissues \(\left(W_1\right)\) were measured with an electronic balance. After 8 h of moisture removal, the weights of the samples \(\left(W_2\right)\) were measured again. The ratio of the water content in each group was calculated using the following formula:

\[
\text{the ratio of the water contents (\%)} = (1 - \frac{W_2}{W_1}) \times 100\%
\]

**Histopathological Tests.** The brain samples were fixed and postfixed in 4% PFA for at least 24 h. Then, the samples were embedded in paraffin and coronally sectioned around 7 μm for a series of detection. The thickness were stained with hematoxylin and eosin (H&E staining). The histomorphology of neurons was observed under a microscope. The injured neurons cells exhibited dark staining, shrinkage, or a dysmorphic appearance, and intact cells displayed a distinct nucleus and nucleolus. For Nissl staining, sections were dehydrated in ethanol and chloroform; stained with 1% toluidine blue at 50 °C for 10 min; and then rinsed, cleared in graded ethanol and xylene, and cover-slipped. Furthermore, the TUNEL assay was used to evaluate the DNA fragmentation associated with apoptosis by an in situ cell death detection kit. The counts of cells were performed with a magnification microscope.

**STATISTICAL ANALYSIS**

The comparison of each group was evaluated by statistical analysis using SPSS software with one-way ANOVA. All the statistical results are expressed as the mean ± SD, with an alpha level equal to 0.05.

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Author Contributions
§L.T. and Y.W. are the co-first authors for this work.

Notes
The authors declare no competing financial interest.

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