

Original Article

Neuroprotective effect of HAMI 3379, a CysLT₂R receptor antagonist, on chronic brain injury after focal cerebral ischemia in rats

Ling-Qun Lu¹, Qi Lou¹, Hong-Gang Guo¹, Wen-Wei Zhou¹, Hua-Zhong Ying¹, Er-Qing Wei², Qiao-Juan Shi^{1,2}

¹Experimental Animal Center, Zhejiang Academy of Medical Sciences, Hangzhou, China; ²Department of Pharmacology, School of Medicine, Zhejiang University, Hangzhou, China

Received October 8, 2016; Accepted December 21, 2016; Epub April 1, 2017; Published April 15, 2017

Abstract: Cysteinyl leukotrienes induce inflammatory responses by activating receptors namely CysLT₁R and CysLT₂R. Recently, the study reported that CysLT₂R is involved in neuronal injury such as astrocytosis and microgliosis after focal cerebral ischemia in rats, and intracerebroventricular injection of HAMI 3379, a selective CysLT₂R antagonist, protects against acute brain injury induced by middle cerebral artery occlusion (MCAO) in rats. In this study, the effect of HAMI 3379 is long lasting and related to the formation of a glial scar in rats with cerebral ischemia has been further determined. Focal cerebral ischemia was induced by MCAO. After ischemia, HAMI 3379 (0.1 mg/kg) was injected intraperitoneally for six consecutive days. Neurological deficits and sensorimotor function were determined during 14 days after ischemia. Brain lesion, neuronal injury, macrophage-microglia, and glial scar formation were detected at the end of the experiment. HAMI 3379 improved neurological deficits and holding angles in the inclined board test, ameliorated brain atrophy and lesion, increased neurons density in the ischemic border zone, inhibited microglia activation, and glial scar formation (astrocyte proliferation). Hence, these results have confirmed the long-term neuroprotective effects of HAMI 3379, providing further evidence of the therapeutic potential of HAMI 3379 in the treatment of ischemic stroke.

Keywords: HAMI 3379, cysteinyl leukotriene receptor 2 (CysLT₂R), antagonist, cerebral ischemia, chronic brain injury

Introduction

Cysteinyl leukotrienes (CysLTs) including leukotriene C₄ (LTC₄), leukotriene D₄, and E₄ are the 5-lipoxygenase metabolites of arachidonic acid, they are potent inflammatory mediators predominantly synthesized by inflammatory cells such as macrophages, mast cells, microglial, astrocytes, and polymorphonuclear leukocytes [1-3]. CysLTs are involved in pathophysiology of many inflammatory diseases such as allergic rhinitis and asthma [2, 4, 5]. In addition, CysLTs have been implicated in different central nervous diseases such as brain trauma [6-8], cerebral ischemia [9-13], intracerebral hemorrhage [14], epileptic seizures [15, 16], brain tumor [7, 8], and aging [17]. CysLTs effects are mediated by two different G protein coupled receptors namely the CysLT₁R and the CysLT₂R, respectively [1, 2].

In the rat brain, CysLTs are increased with two peaks at 3 to 24 h and 7 days after focal cerebral ischemia [18]. Moreover, CysLT₁R receptor antagonists such as montelukast and pranlukast alleviate brain ischemia injury including neurological dysfunction, neuron loss and degeneration, damage of the blood-brain barrier, neutrophil infiltration, and glial scar formation [9, 19-22]. The recent study has shown that the spatiotemporal profiles of CysLT₂R expression are similar to those of CysLT₁R in rat brain after focal cerebral ischemia. It plays an important role in acute neuron injury and in astrocytosis and microgliosis in the late phase [11-13]. In addition, the study found that HAMI 3379, which is one of selective CysLT₂R antagonist recently reported that can effectively reverse the increase in LTC₄-induced perfusion pressure and decrease in contractility in isolated Langendorff-perfused guinea pig heart [23],

attenuates oxygen-glucose deprivation/recovery (OGD/R)-induced ischemic neuronal injury by inhibiting microglial activation [24]. It has been reported that intracerebroventricular injection of HAMI 3379 protects against acute brain injury after focal cerebral ischemia in rats. The evidence is that it attenuates the neurological deficits, and reduced infarction volume, brain edema, immunoglobulin (Ig)G exudation, neuronal degeneration, and loss [13]. These results are also consistent with the effects of HAMI 3379 by intraperitoneal injection on acute brain injury after focal cerebral ischemia in rats [12, 13]. These findings supported that the antagonism of CysLT₂R activation by HAMI 3379 may be a novel therapeutic strategy to improve the sequelae of ischemic stroke. However, only short-term neuroprotective effects of HAMI 3379 have been evaluated within 24-72 h in MCAO rat models, but the long lasting effects are still unknown.

Ischemic brain injury can be separated into three serial phases namely acute injury (energy failure, excitotoxicity, and oxidative stress; minutes to hours), subacute injury (inflammation and cell death; hours to days), and chronic injury (repair and regeneration; days to months) [25, 26]. The angiogenesis, neurogenesis, and gliosis/glial scar formation are the morphological changes (brain remodeling) in the chronic phase after cerebral ischemia [27, 28]. Among these changes, the formation of a glial scar results from gliosis, in which the reactive astrogliosis (the hypertrophy and proliferation on astrocytes) is predominant [29]. Astrocytes can regulate remyelination and axon sprouting of neurons in the early phase after stroke, whereas it can exclude remyelination and prevent axon regeneration in chronic phase [30], hence gliosis has been shown to have both beneficial and detrimental effects, and the balance between these effects is due to a complex array of factors and molecular signaling mechanisms.

It has been reported that the CysLTs, promote astrocyte proliferation through the CysLT₁R receptor after mild ischemia (1 h OGD), whereas CysLT₂R mediate astrocyte death after more severe ischemia (4 h OGD) [31, 32]. The CysLT₁R receptor antagonist pranlukast inhibits glial scar formation in the ischemic brain [19]. The CysLT₂R receptor antagonist HAMI 3379 had

no effect on the reactive astrogliosis at 24-72 h after cerebral ischemia in rats (data on publish). However, its effects on the glial scar formation at 14 days after MCAO need investigation.

The present study examined whether post-ischemic treatment with HAMI 3379, a selective CysLT₂R receptor antagonist, at the most effective dose of 0.1 mg/kg has a long-lasting neuroprotective effect, and inhibits the glial scar formation after transient focal cerebral ischemia in rats.

Methods and materials

Selection of animals

A total of male Sprague-Dawley rats weighing 250-300 g [Experimental Animal Center, Zhejiang Academy of Medicine Sciences, Hangzhou, China, Certificate NO.: SCXK (Zhe) 2008-0033] were used in this study. The animals were housed in a breeding-room with a controlled temperature (20-24°C) and a 12-hour light/dark cycle; animals were allowed free access to food and water with the exception of preoperative fasting.

The experimental protocols were approved by the Ethics Committee of Laboratory Animal Care and Welfare, School of Medicine, and Zhejiang University. All experiments were carried out per the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The efforts were made to minimize the number of animals used and their suffering.

Chemicals

HAMI 3379 was purchased from Cayman Chemical Corporation [Michigan, United States of America (USA)], and Pranlukast was provided by Dr. Masami Tsuboshima (Ono Pharmaceutical Co. Ltd, Osaka, Japan). Chloral hydrate, cresyl violet, and 2, 3, 5-Triphenyltetrazolium chloride (TTC) were from Sigma Chemical Co., St. Louis, USA. All the other reagents were commercially available and analytically pure. HAMI 3379 and Pranlukast were dissolved in absolute ethyl alcohol and dimethyl sulphoxide, respectively. The solution was freshly diluted with normal saline to a final concentration of 20 µg/mL before using. HAMI 3379 and Pranlukast (0.1 mg/kg, 0.5 mL per 100 g bodyweight) diluted

solution was injected intraperitoneally 30 min before and 0, 1, 2 h after MCAO on the first day, then twice daily from 2nd to 6th day and injuries were observed after 14 days of reperfusion. An equal volume (5 ml/kg) of saline was injected intraperitoneally as the control.

Transient focal cerebral ischemia

The rats were anaesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). Transient focal cerebral ischemia was induced by a modified method of MCAO per a previously reported method [33]. Briefly, after anesthesia, a midline incision was made in the neck, the left external carotid artery (ECA), and the left internal carotid artery (ICA) was carefully exposed and dissected, and a 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co., Ltd, Beijing, China) with a round poly-L-lysine coated tip was inserted from the ECA into the ICA to occlude the origin of the left middle cerebral artery. After 1 h of occlusion, the suture was withdrawn to allow reperfusion, the ECA was ligated and the incision was closed. Sham-operated rats underwent identical surgery except inserting the intraluminal filament. Once animals regained complete consciousness they were moved to their cages in an environmentally controlled breeding-room. Animals' weights were monitored.

Regional cerebral blood flow (rCBF) was continuously measured using a laser Doppler flow meter (Moor Instruments, Devon, United Kingdom). The laser Doppler flow probe was fixed on the left forebrain skull at 2.0 mm posterior and 4.5 mm lateral to bregma [34]; continuous monitoring of flow was performed before and during transient forebrain ischemia lasting for 10 min after reperfusion. Since the baseline resting CBF varied from one animal to another, the recorded flow during the experiment was expressed as a percentage of the pre-occlusion CBF value in each animal. Hence the rats that showed a decrease in percentage of rCBF less than 70% of the initial value were excluded.

Determination of brain infarction volume

At 14 d after MCAO, rats were deeply anesthetized and killed. The brains were rapidly removed. Infarction volumes were measured as described previously [13]. Briefly, brains were cut into 2-mm-thick coronal sections in a cut-

ting block and stained with 0.5% TTC for 20 min at 37°C, followed by overnight immersion in 10% formalin. The stained sections were photographed with a digital camera (FinePix S602 Zoom, Fuji, Japan). The infarction zone was demarcated and analyzed by Image J software (NIH Image, Version 1.61, Bethesda, Maryland, USA). The areas of the infarction and both hemispheres of each section were measured, and the total infarction volume of each brain was calculated by summation of the infarction volumes [infarction area × thickness (2 mm)] of all brain sections. To detect brain atrophy, the percentage decrease in ischemic hemisphere volume was calculated as (ipsilateral volume - contralateral volume)/contralateral volume × 100.

Behavioral assessments

Neurological deficit: To examine the effects of HAMI 3379 on the neurological deficits of rats after cerebral ischemia/reperfusion, behavioral tests were performed by a physician blinded to the treatment status of the rats with neurological deficit scoring and modified inclined board test. The neurological deficit scores were initially evaluated 24 h after MCAO as follows [33]: 0, no deficit; 1, flexion of contralateral forelimb upon lifting of the whole animal by the tail; 2, circling to the contralateral side; 3, falling to contralateral side; and 4, no spontaneous motor activity. Those rats subjected to MCAO but did not show neurological deficits were excluded, since their neuroanatomical damage and behaviors were not expected to reflect typical effects of transient MCAO. Then, rats were scored at 3, 7, 10, and 14 days after MCAO.

Inclined board test: The inclined board test was performed to assess balance and coordination as reported, based on modification of a method developed [35]. Rats were placed on a board (50×30 cm), once they stayed stably the board was inclined from horizontal to vertical. The degree at which the animal fell from the board was recorded. The test was repeated for three times and the average was obtained. This task was performed at time points as the same as neurological deficit scoring.

Histopathology

In another series, after anesthesia, rats were perfused transcardially with normal saline fol-

Long-term neuroprotective effects of HAMI 3379

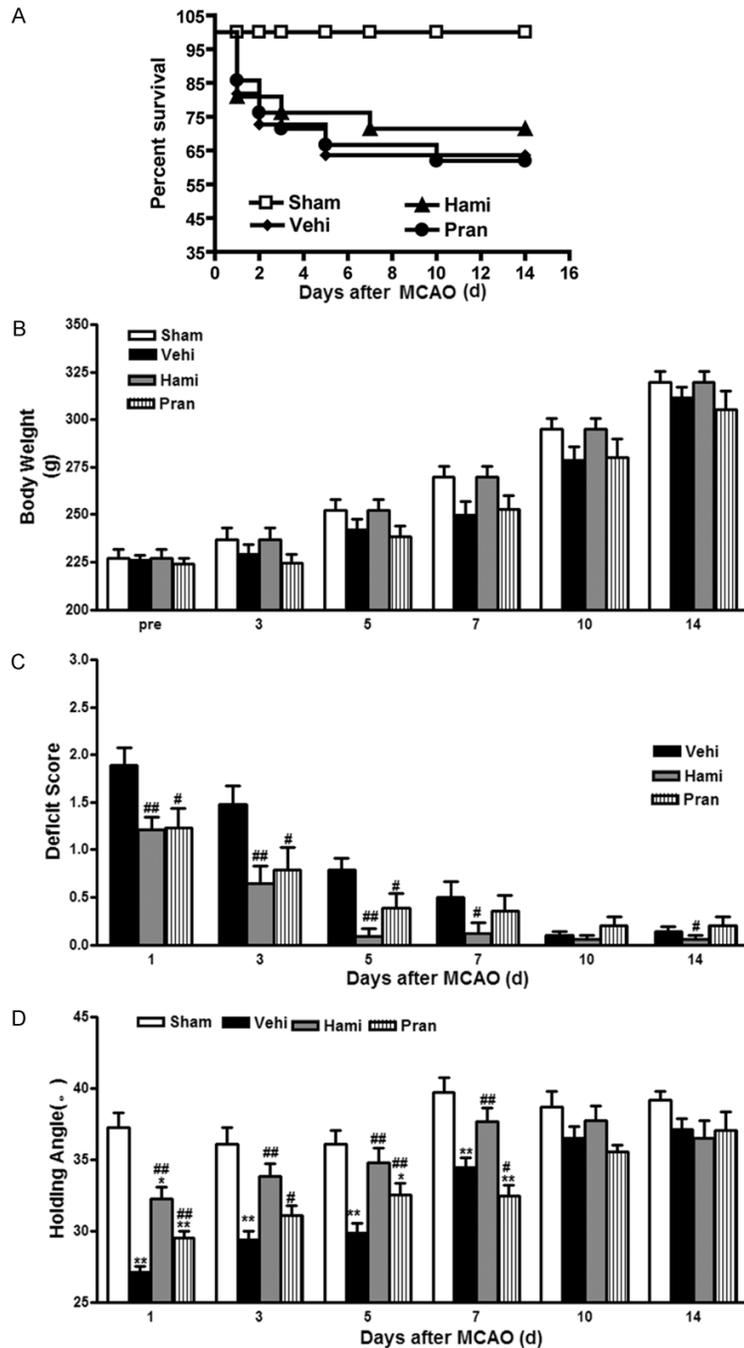


Figure 1. Effect of HAMI 3379 on percent of survival rate, changes in body weight and behaviors during 14 days after MCAO in rats. (A) Percent survival of rats over 14 days after cerebral ischemia. Animals received post-ischemic treatment with HAMI 3379, and Pranlukast (0.1 mg/kg) or normal saline for 5 consecutive days, respectively. There is no significant difference between normal saline- and Pranlukast-treated rats with ischemia ($P > 0.05$, chi-square test). Body weight (B), neurological deficit scores (C), and the holding angle in the inclined board test (D) were summarized before MCAO and 3, 5, 7, 10, and 14 d after reperfusion. HAMI 3379 and Pranlukast (0.1 mg/kg, i.p.) have no effect on body weight during treatment, but significantly improved the neurological dysfunctions. Data are presented as mean \pm S.E.M; $n = 13-21$ rats; $\#P < 0.05$ and $\##P < 0.01$ compared with ischemic control, analyzed by one-way ANOVA. n.d = not detectable.

followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) 24 h after ischemia. Then, the brains were post-fixed overnight in the same fixative, and immersed in 30% sucrose solution in the phosphate buffer. The brains were frozen and cut into coronal sections with the thick of 10 μ m by cryomicrotomy (CM1900; Leica, Wetzlar, Germany). The sections were prepared for the histopathological examination.

Cresyl violet is a cationic dye and used for the detection of Nissl body in the cytoplasm of neurons on tissue sections. The Nissl body would be stained purple-blue. This stain is commonly used for identifying the basic neuronal structure in brain. Cresyl violet staining was performed per modifications of the described protocol. Briefly, the brain sections were deparaffinized by soaking in a 1:1 alcohol/chloroform mixture for 15 min followed by 15 min in 100% alcohol, and 95% alcohol/5% deionized water mixture for rehydration. The sections were transferred to 0.1% cresyl violet solution for 10 minutes, rinsed quickly in distilled water and differentiated in 95% ethyl alcohol for 2-30 minutes, then the sections were dehydrated in 100% alcohol, immersed in xylene and covered with mounting medium.

Neurons were immunostained with a mouse monoclonal antibody against neuronal nuclear antigen [NeuN, a specific marker of neurons; Chemicon, California (CA), USA]. The sections were incubated overnight at 4°C with a mouse anti-NeuN antibody (1:200), then with Cy3-conjugated go-

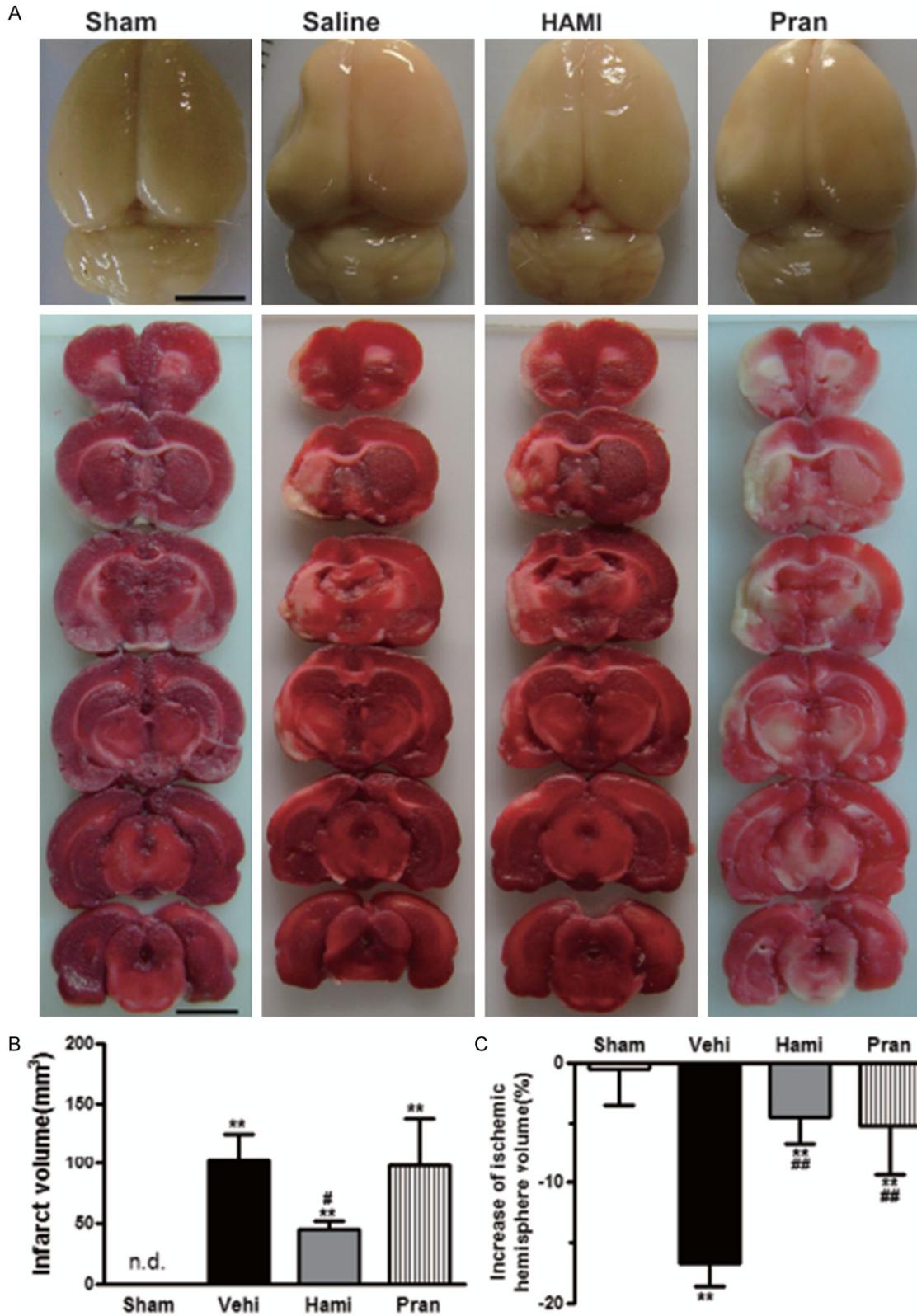


Figure 2. Effect of HAMI 3379 on brain injury 14 days after MCAO in rats. (A) Photographs of whole brain and TTC-stained coronal sections showing brain lesions 14 d after MCAO. (B and C) Infarction volume (B) and percentage increase in ischemic hemisphere volume (C) were reduced by HAMI 3379 and pranlukast (0.1 mg/kg, i.p.). Data are presented as mean \pm S.E.M; n=6-9 rats for (B and C); *P<0.05 and **P<0.01 compared with sham operation, #P<0.05 and ##P<0.01 compared with ischemic control (saline), analyzed by one-way ANOVA. Scale bars, 5 mm.

at anti-mouse IgG (1:200, Chemicon) for 2 h at room temperature. Phosphate buffered saline (PBS) was used instead of the primary antibody in negative control sections. A fluorescence microscope (Olympus BX51, Tokyo, Japan) was used for the observation of stained sections. The neurons in the cortex, both the ischemic core and the boundary zone, were counted by Image J.

For immunohistochemical examination, 10 μ m sections were rinsed with 0.01 M PBS, followed by incubation with 3% hydrogen peroxide (H_2O_2) for 30 min to eliminate endogenous peroxidase activity. Non-specific binding of IgG was blocked by incubation with 5% normal goat serum for 2 h at room temperature. Then, the sections were incubated overnight at 4°C with rabbit anti-gial fibrillary acidic protein (GFAP) (1:200, Zhongshan Biotechnology Co., Beijing, China), rabbit anti-ionized calcium binding adaptor molecule-1 (Iba-1) antibodies (1:2000, Wako, Osaka, Japan), and mouse anti-ED1 (1:500, Millipore, Billerica, Massachusetts, USA). Then the sections were washed extensively with 0.01 M PBS and sequentially incubated with biotinylated IgG (1:200, Vectorlab, Burlingame, CA, USA), and with streptavidin horse radish peroxidase (1:200, Vectorlab, Burlingame, CA, USA) for 2 h. Finally, the sections were exposed to 0.05% 3,3-diaminobenzidine and 0.03% H_2O_2 for 2 min. For negative controls, PBS was used instead of the primary antibodies.

Data were presented as mean \pm standard error mean (S.E.M). Statistical analyses were performed using one-way analysis of variance, followed by Newman-Keuls post-hoc multiple comparison (The GraphPad Prism Software 4.02, GraphPad Software Inc., San Diego, CA, USA). Kruskal-Wallis and log-rank tests were performed for neurological deficit scores and survival rates, respectively. $P < 0.05$ was considered as statistically significant.

Results

Total mortality rate was 34.37 (22/64) over 14 days after reperfusion, and all sham-operated rats were survived. No significant differences in the survival rates were observed between normal saline (63.64%, 14/22), HAMI 3379 (71.43%, 15/21), and Pranlukast groups (61.90%, 13/21) (**Figure 1A**).

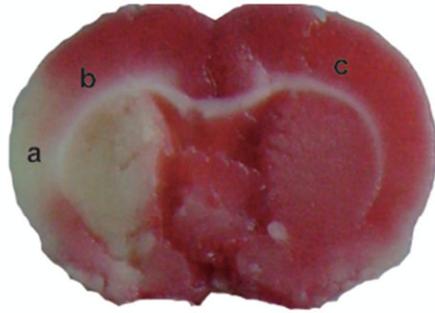
Sham-operated rats did not show any neurological deficit throughout the observation period. The ischemic rats showed apparent neurological deficits with the score being maximal at 24 h after MCAO, then rapidly being reduced within 7 days, as well as almost being recovered within 10 days after reperfusion. Intraperitoneal exposure to HAMI 3379 and Pranlukast for 6 days significantly reduced the neurological deficit scores at the first 7 days after ischemia (**Figure 1B**). In the inclined board test, the inclined degree of sham operated rats were kept at constant over 14 days after surgery, and ischemic rats showed a decreased holding angle within 7 days after reperfusion and later recovered, both agents significantly increased the holding angles from the first to 7 days after ischemia (**Figure 1C**).

The gross photographs of whole brain showed the change in the surface area, and the infarction tissue in the ischemia hemispheres was showed by the coronal sections stained with TTC. At the end of the experiment (14 days after MCAO), a moderate atrophy in ischemic hemispheres was observed in the gross photographs of whole brain and the brain sections were stained with TTC (**Figure 2A**). Treatment with HAMI 3379 significantly reduced the infarction volume; both HAMI 3379 and Pranlukast could inhibit the reduction in left/right hemispheric ratio, so they could attenuate the atrophy induced by MCAO (**Figure 2B, 2C**).

MCAO induced significant neuronal injury in the ischemic cortex, where the Nissl bodies disappeared; cell bodies were stained and shrunken or completely lost. In the lesion core of cortex, the density of the apparently surviving neurons was almost disappeared 14 days after reperfusion (**Figure 3B, 3C**), in the boundary zone adjacent to the lesion core, the number of NeuN positive neuron were reduced (**Figure 3B, 3D**). HAMI 3379 did not reverse neuron loss in the ischemic core but attenuated neuron loss in the boundary zone 14 days after ischemia. Pranlukast had the same protective effects on the neuronal damage (**Figure 3C, 3D**).

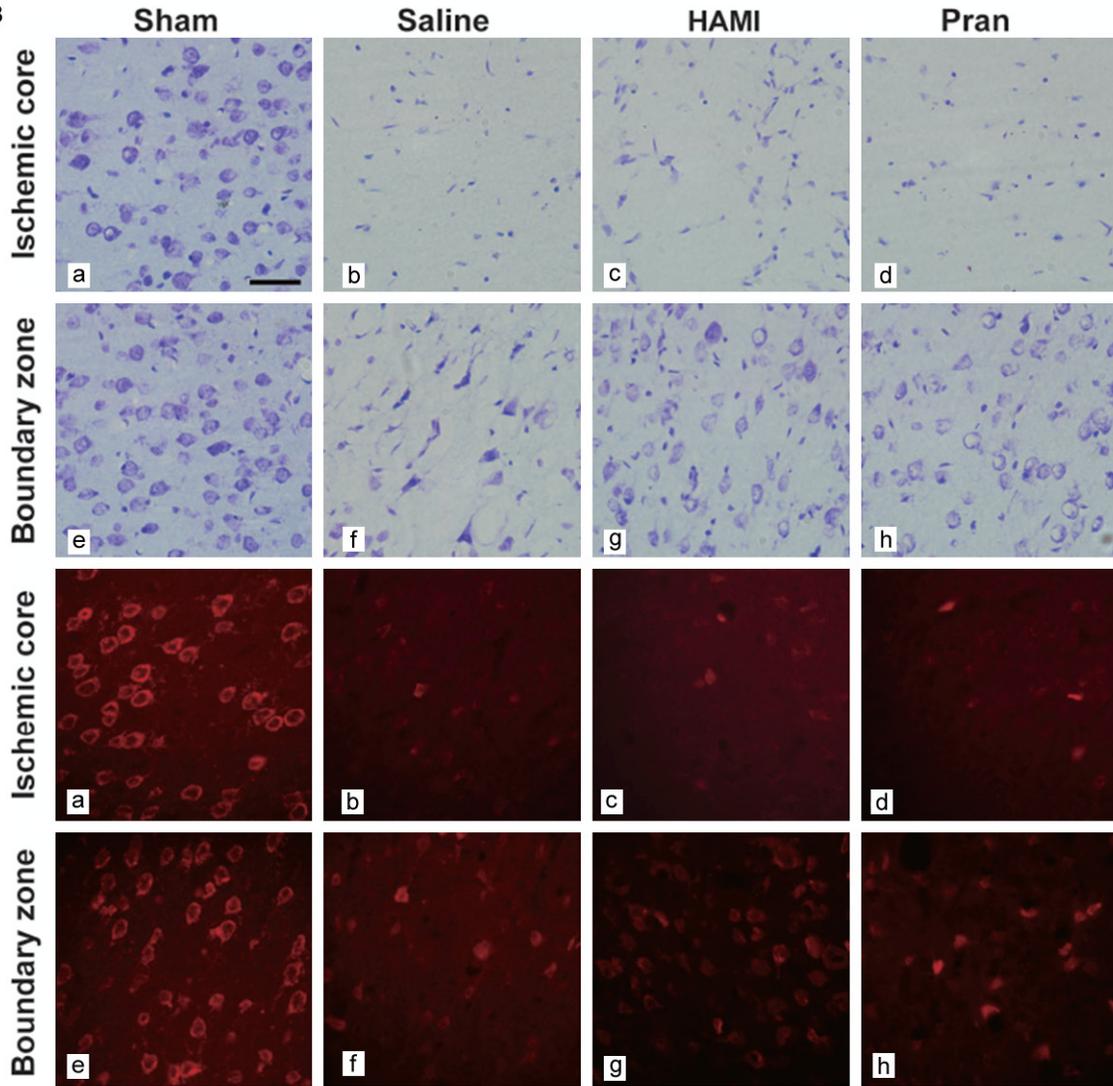
To determine whether HAMI 3379 affected MCAO-induced neuroinflammation, an analysis of macrophage-microglia activation in the ischemic hemisphere was performed by staining with Iba-1 (**Figure 4**) and ED-1 (**Figure 5**). HAMI

A

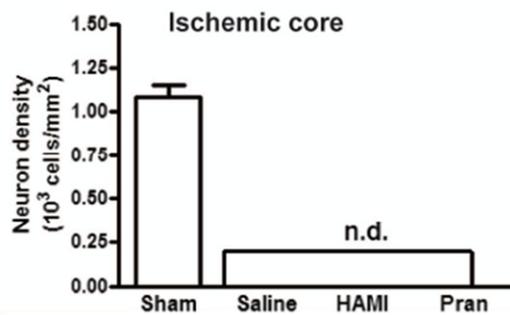


a: Ischemic core
b: Boundary zone
c: Contralateral

B



C



D

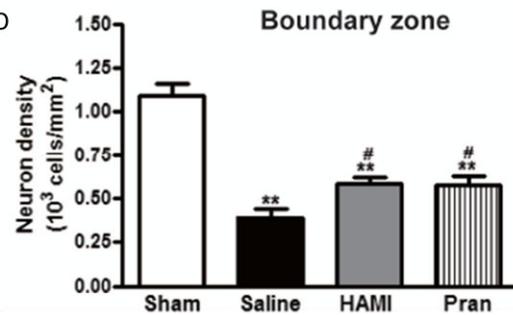


Figure 3. Effect of HAMI 3379 on neuron density 14 days after MCAO in rats. (A) Sampling regions for photomicrographic examination. (B) Cresyl violet staining and NeuN immunostaining show the changes in neuronal density in the ischemic core (a-d) and boundary zone (e-h) 14 days after reperfusion. (C and D) Numbers of NeuN-positive neurons in the ischemic core (C) and boundary zone (D). Neuronal loss in the ischemic core and boundary zone was inhibited by HAMI 3379 and pranlukast (0.1 mg/kg, i.p.). Data are presented as mean \pm S.E.M; n=7-12 rats; **P<0.01 compared with sham operation, #P<0.05 and ##P<0.01 compared with ischemic control, analyzed by one-way ANOVA. Scale bars, 50 μ m.

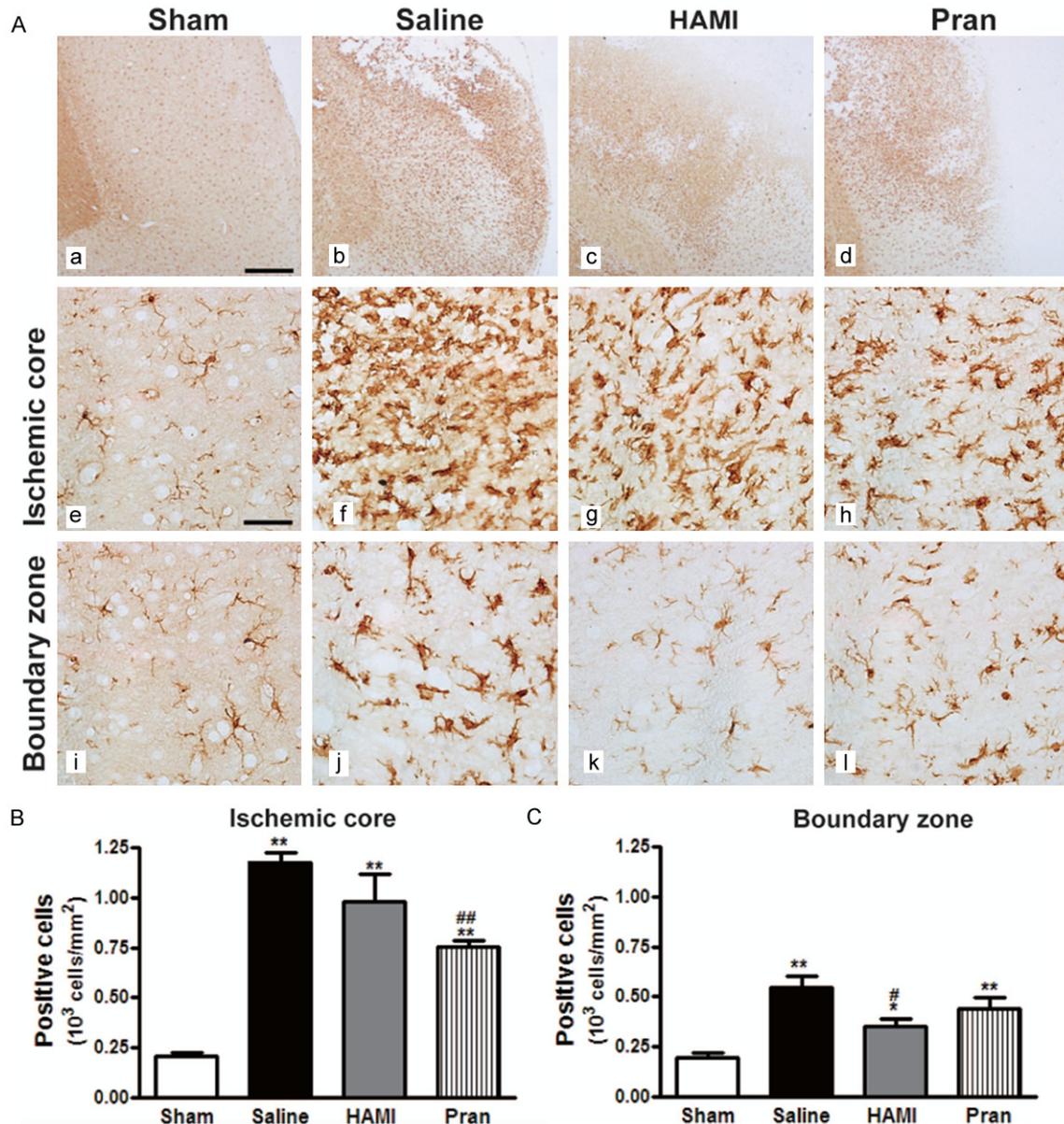


Figure 4. Effect of HAMI 3379 on microglial number 14 days after MCAO in rats. (A) Microphotographs from ischemic hemispheres showing marked increase in Iba-1 immunopositive microglia 14 days after MCAO both in the ischemia core and in the boundary zone in rats, and HAMI 3379 and pranlukast (0.1 mg/kg, i.p.) significantly reduced the number of Iba-1-immunopositive cells. Scale bars for a-d, 200 μ m; Scale bars for g-k, 50 μ m. (B and C) Numbers of Iba-1 immunopositive cells in the ischemic core (B) and boundary zone (C) were summarized. Data are presented as mean \pm S.E.M; n=7-12 rats; **P<0.01 compared with sham operation, #P<0.05 and ##P<0.01 compared with ischemic control, analyzed by one-way ANOVA.

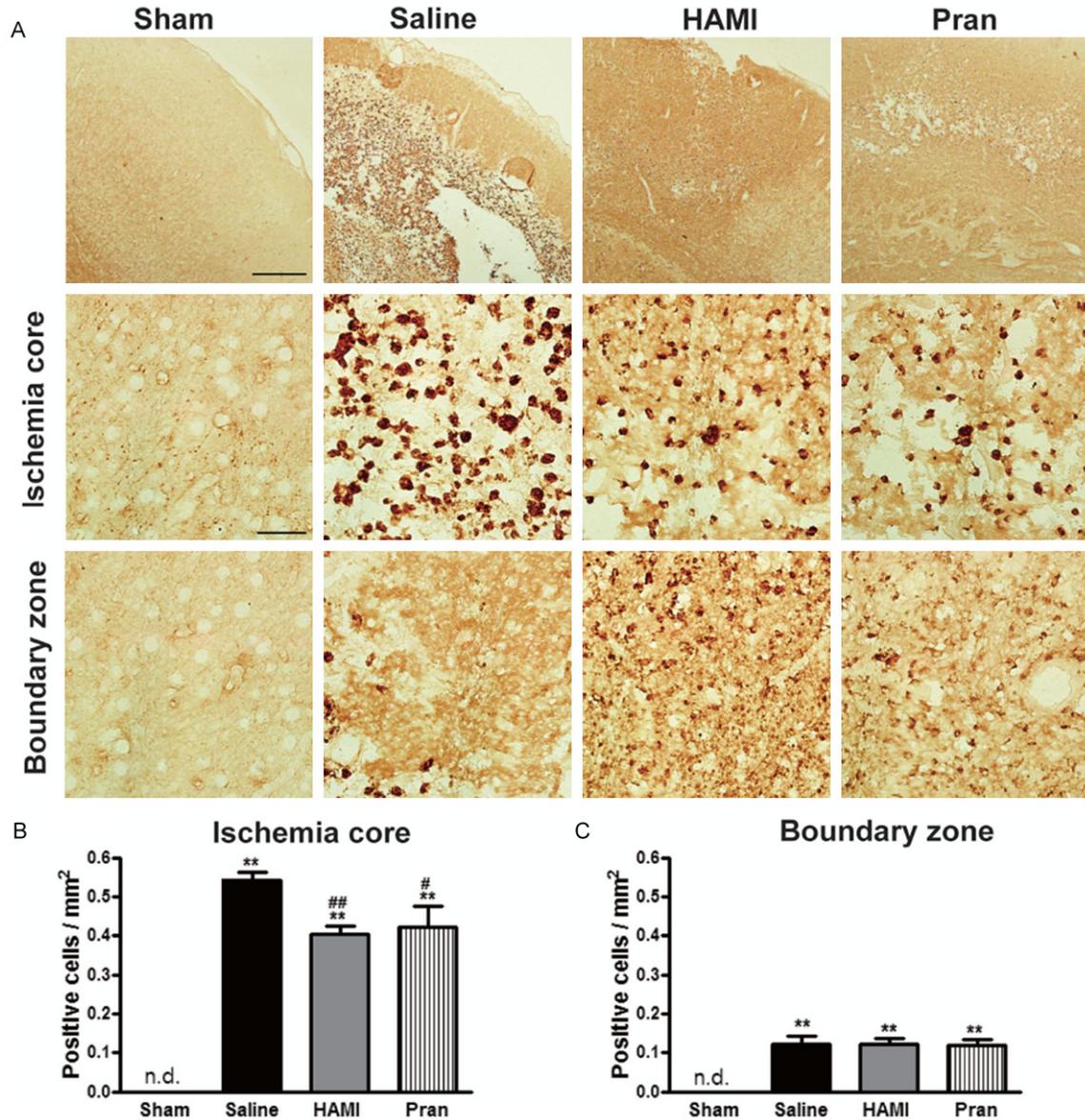


Figure 5. Effect of HAMI 3379 on number of ED1-immunopositive cells 14 days after MCAO in rats. A: Microphotographs from ischemic hemispheres showing marked increase in ED-1 immunopositive activated microglia 14 days after MCAO both in the ischemia core and in the boundary zone in rats, and HAMI 3379 and pranlukast (0.1 mg/kg, i.p.) significantly reduced the number of ED-1-immunopositive cells. Scale bars, 50 μ m. B, C: Numbers of ED-1 immunopositive cells were summarized. Data are presented as mean \pm S.E.M.; n=7-12 rats; **P<0.01 compared with sham operation, #P<0.05 and ##P<0.01 compared with ischemic control, analyzed by one-way ANOVA.

3379 reduced the infiltration of monocytes/macrophages at the lesion site in terms of the immunoreactivity of Iba-1 in the boundary zone by 36.38% but not in ischemic core, Pranlukast reduced Iba-1 positive cell in ischemic core by 36.05% but not in the boundary zone (Figure 4A-C). HAMI 3379 and Pranlukast treatment induced a significant decrease in the number of activated microglia (ED-1 immunoreactive cells)

in boundary areas, suggesting that MCAO-induced microglial activation was inhibited by HAMI 3379 and Pranlukast (Figure 5A-C). These results have indicated that HAMI 3379 exerts an inhibitory effect on the MCAO-induced microgliosis.

At 14 days after reperfusion, a glial scar was observed in the ischemic hemispheres of MCAO

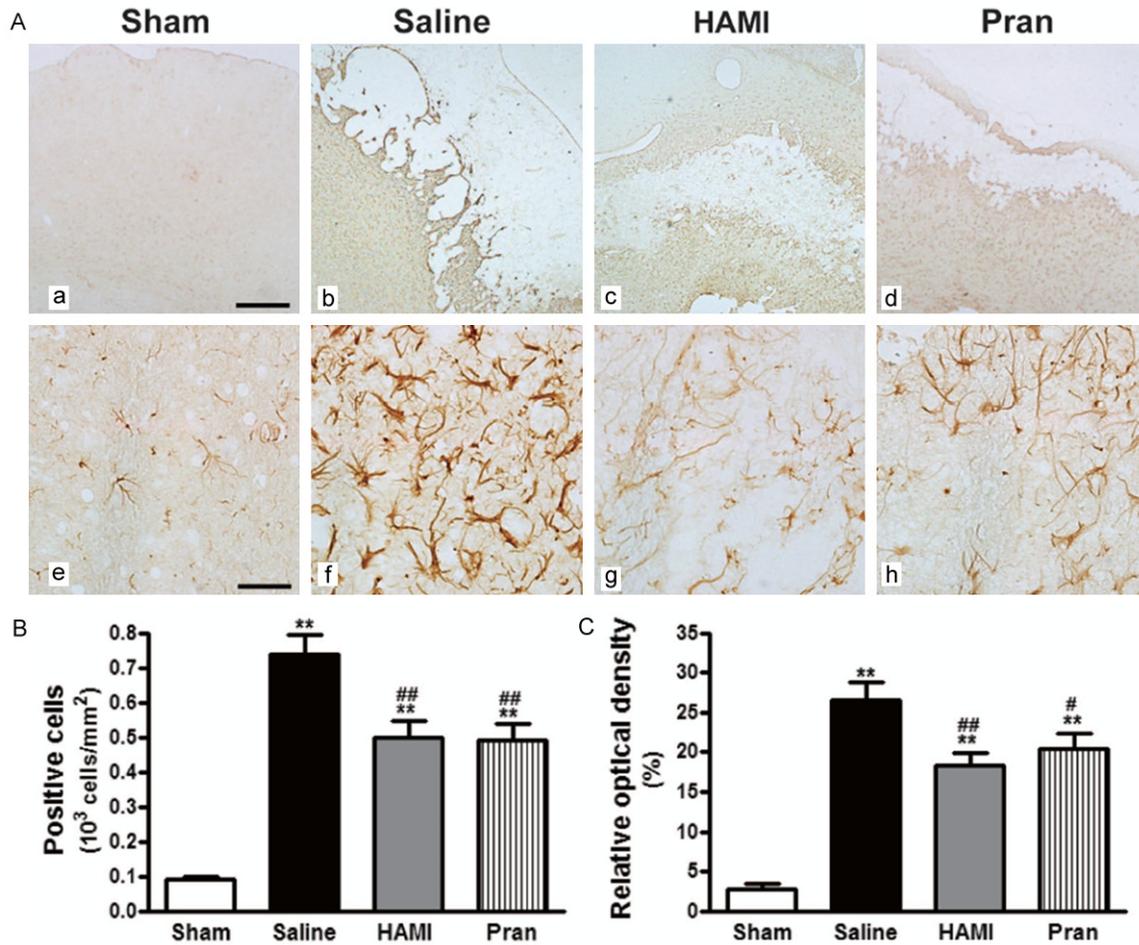


Figure 6. Effect of HAMI 3379 on astrocyte density 14 days after MCAO in rats. (A) Microphotographs from ischemic hemispheres showing marked increase in GFAP immunopositive astrocytes in the boundary zone 14 days after MCAO, and HAMI 3379 and pranlukast (0.1 mg/kg, i.p.) significantly reduced the number of GFAP-immunopositive cells. Scale bars for a-d, 200 μ m; Scale bars for g-h, 50 μ m. (B) Numbers of GFAP-immunopositive cells and (C) the relative optical density of GFAP immunostaining were summarized. Data are presented as mean \pm S.E.M.; n=7-12 rats; **P<0.01 compared with sham operation, #P<0.05 and ##P<0.01 compared with ischemic control, analyzed by one-way ANOVA.

in all rats. Ischemia tissues transformed into the center cavitation of a scar. Several GFAP-positive astrocytes were present in the scar wall parallel to the cavitation (Figure 6). Reactive astrocytes with hypertrophy/hyperplasia were dominant in the scar. HAMI 3379 significantly reduced the GFAP proliferation by 56.4% at 14 days and it also inhibits the increase in percentage of the GFAP gray intensity in the ischemic hemisphere (Figure 6A-C).

Discussion

In the present study, HAMI 3379, a CysLT₂R receptor antagonist has a long-lasting protective effect on focal cerebral ischemia in rats.

The evidence showed that treatment with HAMI 3379 for six consecutive days after ischemia significantly improved neurological deficits and holding angles in the inclined board test, ameliorated brain atrophy and lesion, increased neurons density in the ischemic border zone, and inhibited microglia activation, and glial scar formation (astrocyte proliferation), these results have confirmed the long-term neuroprotective effects of HAMI 3379.

Long-lasting neuroprotective effects of drugs are more important in evaluating clinical conditions in patients because dysfunction or repair is more lasting in ischemic stroke candidates and functional scores are typically assessed

between 3 and 6 months, while the protective effects of drugs are rarely determined beyond 7 days after ischemia; this may partly explain a reason for variety of effective drugs in animal models failed in clinical trials [36, 37]. Therefore, the present results in the long-term study provided further evidence for the therapeutic potentials of CysLT₂R receptor antagonists in the treatment of cerebral ischemic injury.

HAMI 3379 administrated by intracerebroventricular or intraperitoneal injection is shown to have neuroprotective effects [13]. The most effective dose of HAMI 3379 by intraperitoneal injection was about 0.1 mg/kg with a therapeutic window of 2 h and the duration of administration (six consecutive days after MCAO) was chosen [12] to maintain its effect in the acute phase of ischemic injury. Using this regimen, the study found the efficacy of post-ischemic HAMI 3379 and Pranlukast on both morphological and functional outcomes. In addition to the lesion volume and neuron density, HAMI 3379 and Pranlukast accelerated the recovery of long-lasting neurological dysfunctions after ischemia. Neurological scoring has been chosen as an indicator for neurological deficits, and inclined board as an indicator for sensorimotor dysfunction after focal cerebral ischemia. Neurological deficits, which are related to shrinkage of caudate putamen and cortex [38, 39], are most frequently assessed in focal cerebral ischemic animals including those in chronic experiments [19, 40]. Sensorimotor dysfunctions can be assessed by several methods. Among these methods, inclined board test is a simple and quantitative method and has been used in acute experiments in rats [9, 13]. Thus, these methods can be reasonably used in evaluating chronic dysfunctions after focal cerebral ischemia. Here, both neurological deficits and the dysfunction detected by inclined board test that occurred only within 7 days and spontaneously returned to baseline level within 10 days after ischemia. HAMI 3379 and Pranlukast had a significant improvement in the neurological deficits and sensorimotor dysfunction from 3 to 7 days after MCAO.

Gliosis is a nonspecific reactive change of glial cells in response to damage such as cerebral ischemia, brain trauma, Alzheimer's and Parkinson's diseases [41]. Gliosis involves the proliferation or hypertrophy of several different

types of glial cells including astrocytes, microglia, and oligodendrocytes [42, 43]. In the most extreme form, the proliferation associated with gliosis leads to the formation of a glial scar [29]. The studies have proved HAMI 3379 can inhibit glial scar formation in the ischemic brain, which is similar to Pranlukast, but the hidden mechanism may both have similarities and differences. Microglia, another type of glial cell, act as macrophage-like cells in the central nervous system when activated. Unlike other glial cell types, microglia is extremely sensitive and rapid to even small changes in the cellular environment. Due to their short response time, microgliosis, or the activation of microglia, is commonly the first observed stage of gliosis [43, 44]. In vitro studies have indicated that activated microglia play an important role in initiating and modulating astrogliosis [45]. Microglia upon activation released the pro-inflammatory cytokines and chemokines including macrophage inflammatory protein-1, and colony stimulating factor, the interleukins (IL-1, IL-6, and IL-8), and tumor necrosis factor- α [46]. The receptors for these molecules have been identified on astrocytes, and the molecules, when exogenously introduced, have been shown to induce, enhance, and accompany astrogliosis. Astrocytes themselves produce cytokines, which may be used for self-regulation or for the regulation of microglia, which contain similar cytokine receptors. This phenomenon creates a feedback loop, allowing both microglia and astrocytes to regulate one another. In addition, the evidence suggests microglial regulation of astrogliosis may also include inhibitory effects [44]. Reduced levels of microgliosis have been associated with reduced astrocyte numbers, which also suggests that microglia are important regulators for the degree of astrocyte activation [12, 44].

HAMI 3379 inhibited microglia activation (both IBA-1 and ED-1 positive cells) in the ischemic cerebral hemisphere 72 h after MCAO [12], while Pranlukast significantly decreased infarction volume, and inhibited neuron degeneration, IgG extravasation, and neutrophil infiltration, but not microglia/macrophage accumulation in the mice ischemic cortex [21]. In the cerebral ischemia chronic phase, both HAMI 3379 and Pranlukast could inhibit macrophage-microglia activation at the lesion site, but the effect of HAMI 3379 seems more significant

than Pranlukast, thus the inhibition of HAMI 3379 glial scar formation in the ischemic brain is more dependent on the regulation of microglia activation than Pranlukast. Of course, HAMI 3379 reduced the release of inflammation mediator such as interferon (IFN)- γ may also contribute to the above effects (data on publish), because the inflammatory cytokines IFN- γ is responsible for the induction of gliosis. In culture, the molecule act as mitogens, prompting the proliferation of astrocytes. Moreover, addition of IFN- γ to brain lesion sites has resulted in an increase in glial scarring [47, 48].

The effect of Pranlukast on glial scar formation may be mediated by transforming growth factor (TGF)- β , these evidence confirmed the TGF- β 1/CysLT₁R system might be associated with astrocyte activation induced by mild ischemic injury [49], and TGF- β is a potential trigger of gliosis, TGF- β 2, whose expression is gradually increased as gliosis occurs, and has been shown to increase astrocyte production of scar-forming proteoglycans. Experimental reduction of both TGF- β 2 and TGF- β 1, which is expressed immediately after injury, has resulted in reduced glial scarring [47].

Conclusion

In summary, intraperitoneal injection of HAMI 3379, has a long-lasting protective effect on late injuries 14 days after focal cerebral ischemic injury in rats, it can recover neurological dysfunction, decrease brain atrophy and lesion, and neuron loss degeneration, and inhibited microglia activation, and glial scar formation (astrocyte proliferation), the neuroprotective effect of HAMI 3379 and Pranlukast is the almost same but with some slight differences. Thus prevention of CysLT₂R activation through pharmacological antagonism as HAMI 3379 may be a novel therapeutic strategy to improve the outcomes of stroke.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (3130-1933, 81401566, 81573591), the Zhejiang Provincial Natural Science Foundation of China (Q15H090023), the Science and Technology Planning Project in Zhejiang Province (2014-C37011), and the Medical Science and Technology Planning Project in Zhejiang Province (201477310).

Disclosure of conflict of interest

None.

Address correspondence to: Qiao-Juan Shi, Experimental Animal Center, Zhejiang Academy of Medical Sciences, 182 Tianmushan Road, Hangzhou 310-013, China; Department of Pharmacology, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China. Tel: +86-571-8821-5497; Fax: +86-571-8821-5499; E-mail: shiqiaojuan@163.com

References

- [1] Singh RK, Gupta S, Dastidar S and Ray A. Cysteinyl leukotrienes and their receptors: molecular and functional characteristics. *Pharmacology* 2010; 85: 336-349.
- [2] Back M, Dahlen SE, Drazen JM, Evans JF, Serhan CN, Shimizu T, Yokomizo T and Rovati GE. International Union of Basic and Clinical Pharmacology. LXXXIV: leukotriene receptor nomenclature, distribution, and pathophysiological functions. *Pharmacol Rev* 2011; 63: 539-584.
- [3] Savari S, Vinnakota K, Zhang Y and Sjolander A. Cysteinyl leukotrienes and their receptors: bridging inflammation and colorectal cancer. *World J Gastroenterol* 2014; 20: 968-977.
- [4] Rovati GE and Capra V. Cysteinyl-leukotriene receptors and cellular signals. *ScientificWorldJournal* 2007; 7: 1375-1392.
- [5] Coffey MJ, Torretti B and Mancuso P. Adipokines and Cysteinyl Leukotrienes in the Pathogenesis of Asthma. *J Allergy (Cairo)* 2015; 2015: 157919.
- [6] Dhillon HS, Dose JM and Prasad MR. Regional generation of leukotriene C4 after experimental brain injury in anesthetized rats. *J Neurotrauma* 1996; 13: 781-789.
- [7] Zhang WP, Hu H, Zhang L, Ding W, Yao HT, Chen KD, Sheng WW, Chen Z and Wei EQ. Expression of cysteinyl leukotriene receptor 1 in human traumatic brain injury and brain tumors. *Neurosci Lett* 2004; 363: 247-251.
- [8] Hu H, Chen G, Zhang JM, Zhang WP, Zhang L, Ge QF, Yao HT, Ding W, Chen Z and Wei EQ. Distribution of cysteinyl leukotriene receptor 2 in human traumatic brain injury and brain tumors. *Acta Pharmacol Sin* 2005; 26: 685-690.
- [9] Fang SH, Wei EQ, Zhou Y, Wang ML, Zhang WP, Yu GL, Chu LS and Chen Z. Increased expression of cysteinyl leukotriene receptor-1 in the brain mediates neuronal damage and astrogliosis after focal cerebral ischemia in rats. *Neuroscience* 2006; 140: 969-979.
- [10] Fang SH, Zhou Y, Chu LS, Zhang WP, Wang ML, Yu GL, Peng F and Wei EQ. Spatio-temporal ex-

- pression of cysteinyl leukotriene receptor-2 mRNA in rat brain after focal cerebral ischemia. *Neurosci Lett* 2007; 412: 78-83.
- [11] Zhao CZ, Zhao B, Zhang XY, Huang XQ, Shi WZ, Liu HL, Fang SH, Lu YB, Zhang WP, Tang FD and Wei EQ. Cysteinyl leukotriene receptor 2 is spatiotemporally involved in neuron injury, astrogliosis and microgliosis after focal cerebral ischemia in rats. *Neuroscience* 2011; 189: 1-11.
- [12] Shi QJ, Wang H, Liu ZX, Fang SH, Song XM, Lu YB, Zhang WP, Sa XY, Ying HZ and Wei EQ. HAMI 3379, a CysLT2R antagonist, dose- and time-dependently attenuates brain injury and inhibits microglial inflammation after focal cerebral ischemia in rats. *Neuroscience* 2015; 291: 53-69.
- [13] Shi QJ, Xiao L, Zhao B, Zhang XY, Wang XR, Xu DM, Yu SY, Fang SH, Lu YB, Zhang WP, Sa XY and Wei EQ. Intracerebroventricular injection of HAMI 3379, a selective cysteinyl leukotriene receptor 2 antagonist, protects against acute brain injury after focal cerebral ischemia in rats. *Brain Res* 2012; 1484: 57-67.
- [14] Ji X, Trandafir CC, Wang A and Kurahashi K. Effects of the experimental subarachnoid hemorrhage on the eicosanoid receptors in nicotine-induced contraction of the rat basilar artery. *J Stroke Cerebrovasc Dis* 2013; 22: 1258-62.
- [15] Takahashi Y, Imai K, Ikeda H, Kubota Y, Yamazaki E and Susa F. Open study of pranlukast add-on therapy in intractable partial epilepsy. *Brain Dev* 2013; 35: 236-244.
- [16] Simmet T and Tippler B. Cysteinyl-leukotriene production during limbic seizures triggered by kainic acid. *Brain Res* 1990; 515: 79-86.
- [17] Gangemi S, Pescara L, D'Urbano E, Basile G, Nicita-Mauro V, Davi G and Romano M. Aging is characterized by a profound reduction in anti-inflammatory lipoxin A4 levels. *Exp Gerontol* 2005; 40: 612-614.
- [18] Zhou Y, Wei EQ, Fang SH, Chu LS, Wang ML, Zhang WP, Yu GL, Ye YL, Lin SC and Chen Z. Spatio-temporal properties of 5-lipoxygenase expression and activation in the brain after focal cerebral ischemia in rats. *Life Sci* 2006; 79: 1645-1656.
- [19] Yu GL, Wei EQ, Wang ML, Zhang WP, Zhang SH, Weng JQ, Chu LS, Fang SH, Zhou Y, Chen Z, Zhang Q and Zhang LH. Pranlukast, a cysteinyl leukotriene receptor-1 antagonist, protects against chronic ischemic brain injury and inhibits the glial scar formation in mice. *Brain Res* 2005; 1053: 116-125.
- [20] Yu GL, Wei EQ, Zhang SH, Xu HM, Chu LS, Zhang WP, Zhang Q, Chen Z, Mei RH and Zhao MH. Montelukast, a cysteinyl leukotriene receptor-1 antagonist, dose- and time-dependently protects against focal cerebral ischemia in mice. *Pharmacology* 2005; 73: 31-40.
- [21] Chu LS, Wei EQ, Yu GL, Fang SH, Zhou Y, Wang ML and Zhang WP. Pranlukast reduces neutrophil but not macrophage/microglial accumulation in brain after focal cerebral ischemia in mice. *Acta Pharmacol Sin* 2006; 27: 282-288.
- [22] Zhao R, Shi WZ, Zhang YM, Fang SH and Wei EQ. Montelukast, a cysteinyl leukotriene receptor-1 antagonist, attenuates chronic brain injury after focal cerebral ischaemia in mice and rats. *J Pharm Pharmacol* 2011; 63: 550-557.
- [23] Wunder F, Tinel H, Kast R, Geerts A, Becker EM, Kolkhof P, Hutter J, Erguden J and Harter M. Pharmacological characterization of the first potent and selective antagonist at the cysteinyl leukotriene 2 (CysLT(2)) receptor. *Br J Pharmacol* 2010; 160: 399-409.
- [24] Zhang XY, Wang XR, Xu DM, Yu SY, Shi QJ, Zhang LH, Chen L, Fang SH, Lu YB, Zhang WP and Wei EQ. HAMI 3379, a CysLT2 receptor antagonist, attenuates ischemia-like neuronal injury by inhibiting microglial activation. *J Pharmacol Exp Ther* 2013; 346: 328-341.
- [25] Dirnagl U, Simon RP and Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* 2003; 26: 248-254.
- [26] Mukandala G, Tynan R, Lanigan S and O'Connor JJ. The effects of hypoxia and inflammation on synaptic signaling in the CNS. *Brain Sci* 2016; 6.
- [27] Bramlett HM and Dietrich WD. Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. *J Cereb Blood Flow Metab* 2004; 24: 133-150.
- [28] Seitz RJ and Donnan GA. Recovery potential after acute stroke. *Front Neurol* 2015; 6: 238.
- [29] Logan A and Berry M. Cellular and molecular determinants of glial scar formation. *Adv Exp Med Biol* 2002; 513: 115-158.
- [30] Fok-Seang J, DiProspero NA, Meiners S, Muir E and Fawcett JW. Cytokine-induced changes in the ability of astrocytes to support migration of oligodendrocyte precursors and axon growth. *Eur J Neurosci* 1998; 10: 2400-2415.
- [31] Ciccarelli R, D'Alimonte I, Santavenere C, D'Auro M, Ballerini P, Nargi E, Buccella S, Nicotia S, Folco G, Caciagli F and Di Iorio P. Cysteinyl-leukotrienes are released from astrocytes and increase astrocyte proliferation and glial fibrillary acidic protein via cys-LT1 receptors and mitogen-activated protein kinase pathway. *Eur J Neurosci* 2004; 20: 1514-1524.
- [32] Huang XJ, Zhang WP, Li CT, Shi WZ, Fang SH, Lu YB, Chen Z and Wei EQ. Activation of CysLT receptors induces astrocyte proliferation and death after oxygen-glucose deprivation. *Glia* 2008; 56: 27-37.
- [33] Longa EZ, Weinstein PR, Carlson S and Cummins R. Reversible middle cerebral artery oc-

Long-term neuroprotective effects of HAMI 3379

- clusion without craniectomy in rats. *Stroke* 1989; 20: 84-91.
- [34] Yuzawa I, Yamada M and Fujii K. An oral administration of cilostazol before focal ischemia reduces the infarct volume with delayed cerebral blood flow increase in rats. *J Stroke Cerebrovasc Dis* 2008; 17: 281-286.
- [35] Yonemori F, Yamaguchi T, Yamada H and Tamura A. Evaluation of a motor deficit after chronic focal cerebral ischemia in rats. *J Cereb Blood Flow Metab* 1998; 18: 1099-1106.
- [36] Curry SH. Why have so many drugs with stellar results in laboratory stroke models failed in clinical trials? A theory based on allometric relationships. *Ann N Y Acad Sci* 2003; 993: 69-74; discussion 79-81.
- [37] Richard Green A, Odergren T and Ashwood T. Animal models of stroke: do they have value for discovering neuroprotective agents? *Trends Pharmacol Sci* 2003; 24: 402-408.
- [38] Persson L, Hardemark HG, Bolander HG, Hillered L and Olsson Y. Neurologic and neuropathologic outcome after middle cerebral artery occlusion in rats. *Stroke* 1989; 20: 641-645.
- [39] Yonemori F, Yamaguchi T, Yamada H and Tamura A. Spatial cognitive performance after chronic focal cerebral ischemia in rats. *J Cereb Blood Flow Metab* 1999; 19: 483-494.
- [40] Li X, Blizzard KK, Zeng Z, DeVries AC, Hurn PD and McCullough LD. Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. *Exp Neurol* 2004; 187: 94-104.
- [41] McMillian MK, Thai L, Hong JS, O'Callaghan JP and Pennypacker KR. Brain injury in a dish: a model for reactive gliosis. *Trends Neurosci* 1994; 17: 138-142.
- [42] Fawcett JW and Asher RA. The glial scar and central nervous system repair. *Brain Res Bull* 1999; 49: 377-391.
- [43] Streit WJ, Walter SA and Pennell NA. Reactive microgliosis. *Prog Neurobiol* 1999; 57: 563-581.
- [44] Zhang D, Hu X, Qian L, O'Callaghan JP and Hong JS. Astrogliosis in CNS pathologies: is there a role for microglia? *Mol Neurobiol* 2010; 41: 232-241.
- [45] Rohl C, Lucius R and Sievers J. The effect of activated microglia on astrogliosis parameters in astrocyte cultures. *Brain Res* 2007; 1129: 43-52.
- [46] Barron KD. The microglial cell. A historical review. *J Neurol Sci* 1995; 134 Suppl: 57-68.
- [47] Silver J and Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci* 2004; 5: 146-156.
- [48] DiProspero NA, Meiners S and Geller HM. Inflammatory cytokines interact to modulate extracellular matrix and astrocytic support of neurite outgrowth. *Exp Neurol* 1997; 148: 628-639.
- [49] Huang XQ, Zhang XY, Wang XR, Yu SY, Fang SH, Lu YB, Zhang WP and Wei EQ. Transforming growth factor beta 1-induced astrocyte migration is mediated in part by activating 5-lipoxygenase and cysteinyl leukotriene receptor 1. *J Neuroinflammation* 2012; 9: 145.