

Title page

HAMI 3379, a CysLT₂ receptor antagonist, attenuates ischemia-like neuronal injury by inhibiting microglial activation

Xia-Yan Zhang, Xiao-Rong Wang, Dong-Min Xu, Shu-Ying Yu, Qiao-Juan Shi, Li-Hui Zhang, Lu Chen, San-Hua Fang, Yun-Bi Lu, Wei-Ping Zhang, Er-Qing Wei

Department of Pharmacology, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058 (X.Y.Z., X.R.W., D.M.X., S.Y.Y., Q.Y.S., S.H.F., Y.B.L., W.P.Z., E.Q.W.) and Hangzhou Key Laboratory of Neurobiology and Department of Pharmacology, School of Basic Medicine, Hangzhou Normal University, Hangzhou 310036, China (L.H.Z., L.C.)

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HAMI 3379 attenuates ischemia-like neuronal injury

Corresponding author:

Er-Qing Wei

Department of Pharmacology, School of Medicine, Zhejiang University, 866 Yuhangtang Road,
Hangzhou 310058, China.

Phone: +86-571-8820-8224; Fax: +86-571-8820-8022;

E-mail: weiq2006@zju.edu.cn

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Non-standard Abbreviations and Acronyms

CysLTs: cysteinyl leukotrienes; LTD₄: leukotriene D₄; NMLTC₄: N-methyl-leukotriene C₄;

CysLT₁R: cysteinyl leukotriene receptor 1; CysLT₂R: cysteinyl leukotriene receptor 2; OGD:

oxygen-glucose deprivation; siRNA: small interfering RNA; shRNA: short hairpin RNA; IL-1β:

interleukin-1β; TNF-α: tumor necrosis factor-α HAMI 3379:

3-([(1S,3S)-3-carboxycyclohexyl]amino)carbonyl)-4-(3-{4-[4-(cyclo-hexyloxy) butoxy] phenyl}
propoxy) benzoic acid.

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Abstract

The cysteinyl leukotrienes are inflammatory mediators closely associated with neuronal injury after brain ischemia through the activation of their receptors, CysLT₁R and CysLT₂R. Here we investigated the involvement of both receptors in oxygen-glucose deprivation/recovery (OGD/R)-induced ischemic neuronal injury and the effect of the novel CysLT₂R antagonist HAMI 3379 in comparison with the CysLT₁R antagonist montelukast. In primary neurons, neither the non-selective agonist leukotriene D₄ (LTD₄) nor the CysLT₂R agonist N-methyl-leukotriene C₄ (NMLTC₄) induced neuronal injury, and HAMI3379 did not affect OGD/R-induced neuronal injury. However, in addition to OGD/R, LTD₄ and NMLTC₄ induced cell injury and neuronal loss in mixed cultures of cortical cells, and neuronal loss and necrosis in neuron-microglial co-cultures. Moreover, they induced phagocytosis and cytokine release (IL-1 β and TNF- α) from primary microglia, and conditioned medium from the treated microglia induced neuronal necrosis. HAMI 3379 inhibited all these responses, and its effects were the same as those of CysLT₂R interference by CysLT₂R shRNA, indicating CysLT₂R dependence. In comparison, montelukast moderately inhibited OGD/R-induced primary neuronal injury, and most OGD/R- and LTD₄-induced (but not NMLTC₄-induced) responses in mixed cultures, co-cultures, and microglia. The effects of montelukast were both dependent and independent of CysLT₁Rs because interference by CysLT₁R siRNA had limited effects on neuronal injury in neuron-microglial co-cultures and on cytokine release from microglia. Our findings indicated that HAMI 3379 effectively blocked CysLT₂R-mediated microglial activation, thereby indirectly attenuating ischemic neuronal injury. Therefore, CysLT₂R antagonists may represent a new type of therapeutic agent in the treatment of ischemic stroke.

Introduction

Cysteinyl leukotrienes (CysLTs), namely the leukotrienes C₄ (LTC₄), LTD₄ and LTE₄, are arachidonic acid-derived lipid mediators (Back et al., 2011). CysLTs act on two G protein-coupled receptors, CysLT₁R and CysLT₂R, and play important regulatory roles (Back et al., 2011; Rovati and Capra, 2007; Singh et al., 2010). In the periphery, CysLT₁Rs are involved in various inflammatory diseases such as bronchial asthma and allergic rhinitis (Back et al., 2011; Rovati and Capra, 2007; Singh et al., 2010), and CysLT₂Rs increase vascular permeability and aggravate myocardial ischemia/reperfusion injury (Heise et al., 2000; Hui et al., 2001; Kamohara et al., 2001; Moos et al., 2008; Sarau et al., 1999; Takasaki et al., 2000). In the central nervous system (CNS), the production of CysLTs increases after ischemic injury in rat brain (Zhou et al., 2006), primary neurons (Ge et al., 2006) and astrocytes (Huang et al., 2008). Increased CysLTs induce CNS responses by activating their receptors. It has been reported that the expression of CysLT₁Rs and CysLT₂Rs is up-regulated in the brain after focal cerebral ischemia. These up-regulated receptors are distributed in injured neurons in the acute phase (~24 h), and in activated microglia and proliferating astrocytes in the late phases (3-28 days) (Fang et al., 2006; Fang et al., 2007; Zhao et al., 2011b). These findings suggest that CysLT₁Rs and CysLT₂Rs mediate acute ischemic neuronal injury and sequential microgliosis and astrogliosis *in vivo*.

However, whether ischemic neuronal injury *in vitro* is mediated by activation of CysLT₁Rs or CysLT₂Rs is poorly understood. In primary neurons, oxygen-glucose deprivation (OGD) induces injury but the agonist LTD₄ does not induce ischemia-like neuronal injury (Hu et al., 2007). This suggests that CysLTs may induce ischemic neuronal injury by complex intercellular interactions, especially those between neurons and glia. Astrocytes protect neurons against ischemic or other

injury (Barreto et al., 2011b; Terashvili et al., 2012), and are regulated by CysLTs after OGD-induced ischemic injury in different ways. CysLT₁Rs mediate astrocyte proliferation after mild ischemia (OGD 1 h), while CysLT₂Rs mediate astrocyte death after more severe ischemia (OGD 4 h) (Huang et al., 2008), and this may be associated with ischemic neuronal injury. However, whether CysLTRs regulate microglial activation and thereby mediate ischemic neuronal injury is not clear. Although neuron-like PC12 cells transfected with CysLT₁Rs and CysLT₂Rs show distinct sensitivities to ischemic injury (Sheng et al., 2006), it is necessary to investigate whether and how these receptors regulate ischemic neuronal injury.

Pharmacologically, the CysLT₁R antagonists pranlukast and montelukast protect against focal and global cerebral ischemia (Chu et al., 2006; Fang et al., 2006; Yu et al., 2005a; Yu et al., 2005b; Zhang and Wei, 2003; Zhang et al., 2002), NMDA-induced brain injury (Zhang and Wei, 2005), and brain cold injury (Qian et al., 2006). They attenuate neuronal injury, blood-brain barrier disruption, inflammatory responses, chronic brain injury and the associated glial scar formation (Chu et al., 2006; Yu et al., 2005a; Yu et al., 2005b; Zhang et al., 2002). However, montelukast has no effect on the OGD-induced reduction in neuronal viability (Hu et al., 2007), and a merely moderate effect on the morphological neuronal changes after OGD (Wang et al., 2012). On the other hand, because of the previous lack of selective CysLT₂R antagonists (other than the non-selective antagonist Bay u9773), their effects on ischemic neuronal injury are unknown. Recently, Bay CysLT₂ and HAMI 3379 have been reported to be selective CysLT₂R antagonists (Ni et al., 2011; Wunder et al., 2010). Bay CysLT₂ protects mice from myocardial infarction and inhibits LTD₄-induced Evans blue leakage in mouse ear vasculature (Ni et al., 2011); it also protects astrocytes from *in vitro* ischemic injury (Huang et al., 2008). HAMI 3379,

3-([(1S,3S)-3-carboxycyclohexyl]amino)carbonyl)-4-(3-{4-[4-(cyclo-hexyloxy) butoxy] phenyl} propoxy) benzoic acid, is devoid of CysLT receptor agonism, and shows >10,000-fold affinity for CysLT₂Rs *versus* CysLT₁Rs (Wunder et al., 2010). We have reported that intracerebroventricular injection of HAMI 3379 protects rats from acute brain injury after focal cerebral ischemia (Shi et al., 2012), but its effects on ischemic neuronal injury *in vitro* need investigation.

Therefore, here we clarified the roles of CysLT₁Rs and CysLT₂Rs in ischemic neuronal injury *in vitro*, and determined the effects of HAMI 3379 and the related cellular mechanisms in comparison with the CysLT₁R antagonist montelukast. To assess whether OGD/R and CysLTs affect neurons directly or indirectly, the experiments were performed in primary neurons, mixed cortical cells and neuron-glia co-cultures as well as in primary microglia.

Materials and Methods

Primary cultures of cortical neurons, astrocytes and microglia, and mixed cultures of cortical cells:

Primary cortical neurons were isolated from neonatal Sprague-Dawley rats of either sex within 24 h of birth (Laboratory Animal Center of Zhejiang University, China) as described previously with modifications (Meloni et al., 2001). Briefly, after the quick decollation using mechanical shearing the cerebral cortices were dissected and digested with 0.25% trypsin (Sangon, Shanghai, China) for 10 min at 37°C. Then the dissociated cells were immediately seeded onto 96-well, 24-well or 6-well plates (Falcon, Franklin Lakes, NJ, USA) coated with poly-L-lysine (0.1 mg/ml, Aldrich-Sigma, St Louis, MO, USA) at 10^5 , 5×10^5 or 2×10^6 cells per well. Cells were cultured in plating medium [high glucose DMEM (Gibco, Temecula, CA, USA) supplemented with 10% fetal bovine serum, 10% horse serum, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Aldrich-Sigma)] for 24 h. Thereafter, the plating medium was exchanged for feeding medium [high glucose DMEM supplemented with 5% horse serum, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.01% N₂ (Gibco) and 0.04% B₂₇ (Gibco)]. The day of plating was counted as 0 days *in vitro* (DIV). On DIV 3, cytosine arabinoside (10 µM, Aldrich-Sigma) was added for 24 h to prevent the proliferation of non-neuronal cells. Cultures were maintained at 37°C in a humidified atmosphere (5% CO₂ and 95% air) and the feeding medium was renewed every 2-3 days. On DIV 10, ~95% of the cultured cells were neurons (data not shown) as identified by immunofluorescence staining with rabbit monoclonal antibody against microtubule-associated protein 2 (MAP2, 1:200, Chemicon). All neuronal experiments were performed at DIV 10.

Primary cortical astrocytes were prepared as described previously with modifications (Meloni et al., 2001). Briefly, the cerebral cortices were digested with 0.25% trypsin for 15 min at 37°C. Then the dissociated cells were immediately seeded onto 150-cm² flasks (one brain per flask) coated with poly-L-lysine (0.1 mg/ml, Aldrich-Sigma). Cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere (5% CO₂ and 95% air) and the medium was renewed every 3 days. On DIV 14, the confluent cultures were shaken on an orbital shaker at 250 rpm for 12-16 h. Then the adherent cells were trypsinized and replated onto poly-L-lysine-coated 24-well plates at 3×10⁵ cells/ml. More than 95% of the cultured cells were astrocytes (data not shown) as identified by immunostaining with the astrocyte marker mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:600, Chemicon).

Primary microglial cells were prepared as described previously (Candelario-Jalil et al., 2007; Ni and Aschner, ; 2010). Briefly, the cerebral cortices were digested with 0.25% trypsin for 10 min at 37°C. Then, the dissociated cells were plated in MEM containing 10% fetal bovine serum onto 150-cm² flasks (one brain per flask) pretreated with poly-L-lysine (0.1 mg/ml). Cultures were grown for 7–9 days at 37°C and the microglial cells were removed by shaking on an orbital shaker at 250 rpm for 30 min. Cells were then centrifuged for 5 min at 1500 g and replated onto poly-L-lysine-coated 24-well plates at 3×10⁵ cells/mm². The cultures contained >95% microglial cells as determined by immunostaining with the microglial marker rabbit polyclonal anti-ionized calcium binding adaptor molecule 1 (Iba-1) antibody (1:1000, Wako, Osaka, Japan).

The procedures for the mixed cultures of cortical cells were the same as for primary neuron culture except for the addition of cytosine arabinoside (10 µM). On DIV 10, ~15% of the cultured

cells were neurons, ~70% were astrocytes and ~15% were microglia (data not shown) as identified by immunofluorescence staining with rabbit monoclonal anti-MAP2 (1:200), mouse monoclonal anti-GFAP (1:600) and rabbit polyclonal anti-Iba1 (1:1000) antibodies.

All the animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Ethics Committee of Laboratory Animal Care and Welfare, School of Medicine, Zhejiang University.

Transwell co-cultures:

The primary neurons (DIV 10) plated on coverslips were transferred onto the upper compartment of a transwell (0.4- μ m pore size polycarbonate membrane coated with poly-L-lysine, Corning, NY, USA). Harvested primary microglia or astrocytes were plated on coverslips and transferred into the lower compartment of the transwell. Then, the transwell cultures were used in experiments.

RNA interference of CysLT₁R and CysLT₂R:

Small interfering RNA (siRNA) duplexes of 21 nucleotides specific for the rat CysLT₁R sequence were chemically synthesized, together with a non-silencing negative control (NC) siRNA. The rat CysLT₁R siRNA sense sequence was 5'-CAG CCU UCC AAG UAU ACA UTT-3'; the NC siRNA sense sequence was 5'-CCU ACG CCA CCA AUU UCG UTT-3' (Genechem Co., Shanghai, China). Transfection of siRNA duplexes was performed according to the instructions of the manufacturer. Briefly, the medium was renewed with an appropriate medium with 10% FBS but without antibiotics 24 h before transfection. Then CysLT₁R siRNA or NC siRNA was transiently transfected using LipofectamineTM 2000 (Invitrogen, USA) at a final concentration of 100 nM for 6 h, and cells were incubated for 48 h before exposure to OGD, leukotriene D₄ (LTD₄) and

N-methyl-leukotriene C₄ (NMLTC₄). CysLT₁R siRNA reduced CysLT₁R mRNA expression by 65.66% and protein expression by 62.77% as assessed by RT-PCR and Western blot analysis (Supplemental Figure 1, A and C).

The rat CysLT₂R short hairpin RNA (shRNA) sense sequence was 5'-GAT CCC CCC GTC AAC ATG TAT ACT AGC ATT TTC AAG AGA AAT GCT AGT ATA CAT GTT GAC TTT TTG GA A C-3'; the negative control (NC) shRNA sense sequence was 5'-GAT CCC CCC TTC TCC GAA CGT GTC ACG TTT CAA GAG ATT CTC CGA ACG TGT CAC GTT TTT TGG AAC-3'. The cDNA of double-stranded shRNA oligo was cloned into pFU-GW-RNAi-GFP lentivirus vector (Genechem Co.) using the Hpa I and Xho restriction enzymes. Cells were infected 72 h before exposure to OGD, LTD₄ and NMLTC₄. CysLT₂R shRNA reduced CysLT₂R mRNA expression by 43.84% and protein expression by 50.71% as assessed by RT-PCR and Western blot analysis (Supplemental Figure 1, B and 1D).

Oxygen-glucose deprivation (OGD) and pharmacological treatments:

In vitro ischemia was induced by OGD as described previously (Ge et al., 2007; Ge et al., 2006; Goldberg and Choi, 1993; Hu et al., 2006; Song et al., 2004). Briefly, cells were rinsed twice and incubated in Earle's solution without glucose (concentrations in mM: NaCl, 117; KCl, 5.3; CaCl₂, 1.8; NaHCO₃, 26; MgSO₄, 0.8; NaH₂PO₄, 1.0). Then, the cells were moved into an anaerobic chamber filled with 95% N₂ and 5% CO₂ at 37°C for 1, 2 or 4 h. This procedure decreased the pO₂ in the medium from 151.8 ± 6.9 (mean ± SD, n = 5, control) to 26.7 ± 4.5 (n = 5, 1-h OGD), 22.3 ± 6.7 (n = 4, 2-h OGD) and 23.8 ± 7.4 mmHg (n = 4, 4-h OGD). The cells were then fed with high-glucose DMEM supplemented with 1% horse serum, and returned to the normal incubator for recovery (24, 48 or 72 h).

The non-selective CysLT₁R/CysLT₂R agonist LTD₄ (Aldrich-Sigma) and the selective CysLT₂R agonist NMLTC₄ (Cayman Chemical Co., Ann Arbor, MI, USA) (Yan et al., 2011) were used to activate the receptors. LTD₄ and NMLTC₄ were added into the culture medium at final concentrations of 0.1-1000 nM for 24, 48 or 72 h. The selective CysLT₁R antagonist montelukast (0.0001-1 μ M, Merck Pharmaceutical Co., Wilmington, Del., USA) and the selective CysLT₂R antagonist HAMI3379 (0.0001-1 μ M, Cayman) were continuously applied from 30 min before exposure (OGD, LTD₄ and NMLTC₄) to the end of experiments. Control cells received the same treatment, except for the exposure to stimuli.

In the primary cultures of microglia, after exposure to OGD/R, LTD₄ and NMLTC₄ for 48 h, the medium was collected and centrifuged at 2000 rpm for 10 min to remove cells and debris. The medium (conditioned medium) was immediately applied to neuronal cultures to induce necrosis.

Assessments of cell viability and death:

At the end of experiments, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Aldrich-Sigma) was added to each well to reach a final concentration of 0.5 mg/ml for 4 h at 37°C. Then, the medium was removed and 100 μ l dimethyl sulfoxide (Sangon) was added. After 5-min incubation, the product formazan was measured at 570 nm (OD₅₇₀) in a Microplate Recorder (Elx800, Bio-TEK Instruments Inc., USA). To detect cell death, the lactate dehydrogenase activity in the medium was determined according to the protocols of the LDH kit (Jiancheng Bioengineering Institute, Nanjing, China) (Hui et al., 2004). Briefly, an aliquot of the medium was mixed with nicotinamide adenine dinucleotide and lactate solution, and the LDH product was measured at 450 nm (OD₄₅₀) in the Microplate Recorder. One unit represents an increase of 1 μ mol pyruvate catalyzed by LDH in 100 μ l recovery medium at 37°C for 15 min. Results are

reported as percentages of control.

To detect apoptosis and necrosis, cells grown on coverslips were stained with 10 µg/ml Hoechst 33342 (Sigma-Aldrich) and 10 µg/ml propidium iodide (PI, Aldrich-Sigma) for 10 min at 37°C. After repeated washing, the cells were fixed in cold methanol (-20°C). Then, the cells were photographed under a fluorescence microscope (Olympus BX51, Japan). The apoptotic cells were determined as condensed or fragmented nuclei with strong bright Hoechst 33342 staining, and the necrotic cells as condensed nuclei with red PI staining. Apoptotic or necrotic cells were counted by a researcher who was blind to the treatments, and reported as percentages of total cells.

Immunocytochemical examination:

Neurons were immunostained with rabbit monoclonal anti-MAP2 antibody (1:200, Chemicon), astrocytes with mouse monoclonal anti-GFAP antibody (1:600, Chemicon), and microglia with rabbit polyclonal anti-Iba-1 antibody (1:1000, Wako). Goat anti-rabbit Cy3 (1:200, Chemicon) and goat anti-mouse FITC (1:200, Chemicon) antibodies were used as secondary antibodies. Cells cultured on coverslips were fixed in cold methanol (-20°C) for 5 min and incubated in PBS containing 10% normal goat serum for 2 h to block non-specific binding of IgG. Then, the cells were incubated at 4°C overnight with the primary antibodies; after washing three times, the cells were incubated with the secondary antibodies for 2 h at room temperature. Finally, the stained cells were observed by fluorescence microscopy (Olympus BX51, Japan). Control slips were treated with normal goat serum instead of the primary antibody, and no positive immunostaining was detected (data not shown).

Microglial phagocytosis:

To determine the phagocytic activity of microglia, fluorescent microspheres (1-µm diameter

fluorescent carboxylate-modified microspheres, Millipore F8819, Invitrogen, Carlsbad, CA, USA) were added for 1 h at the end of experiments. Then, microglia were harvested and washed with PBS, resuspended in 0.5 ml PBS, and transferred into cytometric tubes. The fluorescence was analyzed on the FL-3 channel of a flow cytometer (BD Biosciences, San Jose, CA, USA).

Cytokine measurement:

The medium was collected from microglial cultures after 6-h recovery following OGD or 6-h exposure to LTD₄ and NMLTC₄, and centrifuged at 2000 rpm for 10 min to remove cells and debris. The cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) in the medium were measured using ELISA kits for IL-1 β (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) and TNF- α (R&D Systems, MN, USA) according to the manufacturers' instructions.

Statistical Analysis:

Data are reported as mean \pm S.E.M. The significance of differences was analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (SPSS 10.0 for Windows, 1999, SPSS, USA). A value of $P < 0.05$ was considered statistically significant.

Results

OGD/R, but not CysLT receptor agonists, induces injury in primary neurons

First, we determined whether the agonists of CysLT receptors have a directly injurious effect on neurons, similar to OGD/R-induced ischemia-like injury. As confirmation of ischemic neuronal injury, we found that OGD for 1, 2 and 4 h decreased neuronal viability by 18%, 30% and 50%, and increased LDH release by 50%, 90% and 169%, respectively (Figure 1A). These time-dependent changes were gradually aggravated after 24, 48 and 72 h of recovery (Figure 1A). An optimal OGD/R condition was confirmed as 1-h OGD followed by 24-h recovery, in which neuron viability decreased by ~20% and LDH release increased by ~70%. Thus, the next experiments were performed under this condition. Since the production of CysLTs (LTC₄, LTD₄ and LTE₄) is elevated after OGD/R in both cultured neurons and astrocytes (Ge et al., 2007; Ge et al., 2006; Huang et al., 2008), we next determined whether CysLTs induce OGD/R-like neuronal injury by activating their receptors. The results showed that neither the non-selective CysLT₁R/CysLT₂R agonist LTD₄ nor the selective CysLT₂R agonist NMLTC₄ (0.1-1000 nM) affected neuronal viability and LDH release (Figure 1, B and C).

To elucidate the involvement of CysLTRs in ischemic neuronal injury, we further determined whether the antagonists and RNA interference of CysLT₁R and CysLT₂R affect OGD/R-induced neuronal injury. The results showed that the CysLT₁R antagonist montelukast (0.001-1 μM) attenuated the reduction of OGD/R-induced neuronal viability and the increased LDH release (Figure 2A), but CysLT₁R siRNA, the CysLT₂R antagonist HAMI 3379 and CysLT₂R shRNA had no such effect (Figure 2, A and B). OGD/R-induced reduction in the number of MAP2-positive neurons (Figure 2C) and the increase in necrotic neurons (Figure. 2D) were partly reversed by

montelukast (0.01 μ M), but not by CysLT₁R siRNA, HAMI 3379 (0.01 μ M) or CysLT₂R shRNA. OGD/R induced little neuronal apoptosis (Hoechst 33342 staining, <10%), and none of the treatments affected apoptosis (data not shown).

These results suggested that neither CysLT₁Rs nor CysLT₂Rs might induce neuronal injury directly, nor mediate ischemic neuronal injury. HAMI 3379 did not directly protect neurons from ischemic injury. Montelukast might inhibit the ischemic injury through receptor-independent mechanisms because CysLT₁R siRNA had no inhibitory effect.

OGD/R- and agonist-induced neuronal injury in mixed cultures of cortical cells

To confirm the roles of CysLT₁Rs and CysLT₂Rs in animal experiments *in vivo*, we investigated neuronal injury in mixed cultures of cortical cells, a cellular environment mimicking the intact brain. We found that 1-h, 2-h and 4-h OGD increased LDH release by 40%, 70% and 80%, respectively; this increase was aggravated after 24, 48 and 72 h of recovery (Figure 3A, upper panel). On the other hand, the agonists LTD₄ (0.1-1000 nM; Figure 3B, upper panel) and NMLTC₄ (0.1-1000 nM; Figure 3C, upper panel) concentration- and time-dependently reduced cell viability and increased LDH release. The moderate exposures were used as the optimal conditions in the next experiments, i.e. 1-h OGD and 24-h recovery, and 48-h exposure to 100 nM LTD₄ or NMLTC₄. In addition, the time- and concentration-dependent pattern of cell viability reduction was the same as that of the increase in LDH release (data not shown). Montelukast (0.01-1 μ M), but not CysLT₁R siRNA, attenuated the OGD/R-increased LDH release (Figure 3A, middle panel), while both of them slightly inhibited the LTD₄-increased LDH release (Figure 3B, middle panel) but not the NMLTC₄-increased release (Figure 3C, middle panel). HAMI 3379 (0.0001-1 μ M, except 0.0001 μ M for OGD/R) and CysLT₂R shRNA inhibited the increased LDH

release by all three stimuli (Figure 3, lower panels). The effects of these treatments on cell viability reduction were the same as those on increased LDH release (data not shown).

To characterize the cellular damage, we examined the cell morphology by immunostaining the mixed cultures. After exposure to OGD/R, LTD₄ (100 nM) and NMLTC₄ (100 nM), the number of MAP2-positive neurons was reduced (Figure 4A). The number of GFAP-positive astrocytes was slightly increased (but reduced by NMLTC₄), and the microglia had an activated appearance (from ramified to round and ameboid) was found (Supplemental Figure 2). The reduction in neuron number induced by OGD/R and LTD₄ was slightly inhibited by montelukast (0.01-1 μ M for OGD/R; 0.001-1 μ M for LTD₄), HAMI 3379 (0.001-1 μ M for OGD/R; 0.01-1 μ M for LTD₄) and CysLT₂R shRNA, but not by CysLT₁R siRNA (Figure 4, B and C). The NMLTC₄-induced reduction was remarkably inhibited by HAMI 3379 (0.01-1 μ M) and CysLT₂R shRNA, but not by montelukast and CysLT₁R siRNA (Figure 4C).

These results suggested that CysLT₂R might mediate ischemic injury in the mixed cultures, especially the neuronal injury, and this injury might result from interactions between neurons and glia. HAMI 3379 showed a remarkable protective effect on the ischemic injury. Montelukast might exert the inhibitory effect on neuronal loss and necrosis *via* receptor-independent mechanisms, because of the lack of support from the CysLT₁R knockdown results.

OGD/R- and agonist-induced neuronal injury in neuron-glial transwell co-cultures

To clarify the interactions between neurons and glia, we performed neuron-microglial and neuron-astrocyte transwell co-cultures. In neuron-microglial co-cultures, we found that OGD/R, LTD₄ (100 nM) and NMLTC₄ (100 nM) reduced the numbers of neurons (Figure 5A) from 860 ± 32 (control) to 700 ± 35 cells/mm² (18.6%, $n = 10$, $P < 0.05$, OGD/R), 918 ± 29 to 745 ± 31

cells/mm² (18.8%, $n = 10$, $P < 0.05$, LTD₄), and 889 ± 33 to 657 ± 45 cells/mm² (26.1%, $n = 10$, $P < 0.05$, NMLTC₄). Montelukast (0.1 and 1 μ M) and CysLT₁R siRNA inhibited the neuron number reduction induced by LTD₄ (but not NMLTC₄); montelukast also showed a non-significant trend to inhibit the OGD/R-induced reduction (Figure 5B). However, HAMI3379 (0.01-1 μ M) and CysLT₂R shRNA significantly inhibited the neuron number reduction induced by all three stimuli (Figure 5C).

PI/Hoechst 33342 staining showed that OGD/R, LTD₄ (100 nM) and NMLTC₄ (100 nM) induced little apoptosis (data not shown) but remarkable necrosis in the co-cultured neurons (Figure 6A). Necrotic neurons increased from $4.78 \pm 0.89\%$ (control) to $17.82 \pm 0.78\%$ ($n = 10$, $P < 0.001$, OGD/R), $5.10 \pm 0.45\%$ to $16.52 \pm 0.98\%$ ($n = 10$, $P < 0.001$, LTD₄), and $5.60 \pm 0.35\%$ to $20.53 \pm 0.42\%$ ($n = 10$, $P < 0.001$, NMLTC₄). Montelukast (0.1 and 1 μ M) inhibited the OGD/R- and LTD₄-induced neuronal necrosis, and CysLT₁R siRNA inhibited the LTD₄-induced necrosis; but neither affected the NMLTC₄-induced necrosis (Figure 6B). HAMI3379 (0.01-1 μ M) and CysLT₂R shRNA significantly inhibited the necrosis induced by all three stimuli (Figure 6C).

In neuron-astrocyte co-cultures, we found that OGD/R, LTD₄ (100 nM) and NMLTC₄ (100 nM) reduced the number of neurons from 920 ± 23 (control) to 760 ± 25 cells/mm² (17.4%, $n = 10$, $P < 0.05$, OGD/R), 874 ± 24 to 764 ± 31 cells/mm² (12.6%, $n = 10$, $P < 0.05$, LTD₄), and 859 ± 31 to 723 ± 27 cells/mm² (15.8%, $n = 10$, $P < 0.05$, NMLTC₄). These reductions were not inhibited by montelukast or HAMI 3379 (0.0001-1 μ M) as well as CysLT₁R siRNA and CysLT₂R shRNA (data not shown). OGD/R, LTD₄ and NMLTC₄ also induced neuronal necrosis, which was not significantly inhibited by montelukast, HAMI 3379, CysLT₁R siRNA or CysLT₂R shRNA (Supplemental Figure 3).

These results suggested that CysLT₂R might be a determinant in ischemic neuronal injury in mixed cultures and co-cultures, and this injury can be inhibited by HAMI 3379. CysLT₁R and montelukast also somewhat affected the neuronal injury. Microglia might be the target cells of CysLT₂R-regulated ischemic neuronal injury, while the roles of astrocytes in CysLT₁R- and CysLT₂R-mediated neuronal injury remain unclear.

OGD/R- and agonist-induced phagocytosis and cytokine release in primary microglia, and effect of conditioned microglial medium on neuronal necrosis

To elucidate the regulation of microglial activation by CysLT₁R and CysLT₂R, we assessed phagocytosis and pro-inflammatory cytokine release as indicators of microglial activation. On microscopic examination, we found that OGD/R, LTD₄ and NMLTC₄ significantly induced microglial phagocytosis (Supplemental Figure 4A). OGD/R increased the phagocytosis by 125%, and this increase was significantly reduced by montelukast (0.1 and 1 μ M), HAMI 3379 (0.01-1 μ M) and CysLT₂R shRNA, but not by CysLT₁R siRNA (Supplemental Figure 4 , B and C, left panels). LTD₄ (100 nM) increased the phagocytosis by 73% and NMLTC₄ (100 nM) by 93%. These increases were significantly reduced by HAM I 3379 (0.01-1 μ M for LTD₄ and 0.0001-1 μ M for NMLTC₄) and CysLT₂R shRNA, but not by montelukast or CysLT₁R siRNA (Supplemental Figure 4 , B and C, middle and right panels). Flow cytometry showed that OGD/R, LTD₄ (100 nM) and NMLTC₄ (100 nM) enhanced microglial phagocytosis (Figure 7A). Montelukast (0.01 μ M) inhibited the OGD/R-induced phagocytosis, and HAMI 3379 and CysLT₂R shRNA inhibited the phagocytosis induced by all 3 stimuli, but CysLT₁R siRNA had no effect (Figure 7B).

Moreover, OGD/R, LTD₄ and NMLTC₄ increased the release of TNF- α by 379%, 353% and

355%, and that of IL-1 β by 239%, 208% and 236%, respectively. The OGD/R- and LTD₄-induced TNF- α and IL-1 β increases were significantly inhibited by montelukast (0.01 μ M), HAMI 3379 (0.01 μ M), CysLT₁R siRNA and CysLT₂R shRNA (Figure 8 , A and B, upper and middle panels). The NMLTC₄-induced TNF- α and IL-1 β increases were significantly inhibited by HAMI 3379 and CysLT₂R shRNA, but not by CysLT₁R siRNA; only the NMLTC₄-increased TNF- α release was slightly inhibited by montelukast (Figure 8 , A and B, lower panels).

Finally, we measured the necrosis in primary neurons induced by the conditioned medium from microglia that had been pretreated with OGD/R, LTD₄ and NMLTC₄. The results showed that, after these pretreatments, the conditioned medium induced neuronal necrosis, which was significantly inhibited by HAMI 3379 and CysLT₂R shRNA (Figure 8C). However, montelukast showed an inhibitory trend but no significant effect on OGD/R- and LTD₄-induced necrosis, and CysLT₁R siRNA had no effect (Figure 8C).

These results indicated that HAMI 3379 inhibits CysLT₂R-mediated microglial phagocytosis and cytokine release, which might cause neuronal injury. CysLT₁Rs partially mediate the cytokine release, and montelukast might inhibit microglial activation through CysLT₁R-independent and -dependent mechanisms.

Discussion

In the present study, we found that the selective CysLT₂R antagonist HAMI 3379 attenuated OGD/R-induced ischemic neuronal injury which was indirectly mediated by inhibiting microglial activation. Our findings also revealed the regulatory roles of CysLT₂Rs in microglial activation and the resultant ischemic neuronal injury for the first time. In comparison, the CysLT₁R-selective antagonist montelukast attenuated OGD/R-induced ischemic neuronal injury, possibly mediated by both receptor-independent and -dependent mechanisms.

As the target of HAMI 3379, the CysLT₂R is important and complex in its mediation of ischemic neuronal injury. It seems not to be a direct determining factor for primary neuronal injury although CysLT₂R-transfected PC12 cells exhibit more severe ischemic injury after OGD (Sheng et al., 2006). We showed that neither the non-selective CysLT₁R/CysLT₂R agonist LTD₄ nor the selective CysLT₂R agonist NMLTC₄ injured primary neurons. Moreover, OGD/R-induced ischemic neuronal injury was not attenuated by HAMI 3379 and CysLT₁R or CysLT₂R RNA interference. Therefore, the CysLT₁Rs and CysLT₂Rs may not directly mediate neuronal injury or ischemic neuronal injury. Rather, the CysLT₂R indirectly regulates ischemic neuronal injury through intercellular interactions. The interactions are defined by following findings. First, in mixed cultures of cortical cells, the agonists LTD₄ and NMLTC₄, like OGD/R, actually induced neuronal injury. Second, in the neuron-microglial and neuron-astrocyte co-cultures, neuronal injury was induced by LTD₄ and NMLTC₄. CysLT₂R inhibition or blockade remarkably attenuated the neuronal injury in neuron-microglial co-cultures (not in neuron-astrocyte co-cultures), but CysLT₁R siRNA did not have this effect. These findings suggest that CysLT₂Rs may regulate neuronal injury *via* microglial activation. Third, neuronal injury was induced by the conditioned

medium from microglia pretreated with OGD/R, LTD₄ and NMLTC₄, and this injury was inhibited by HAMI 3379 and CysLT₂R shRNA. This finding demonstrated the role of microglial CysLT₂Rs in the induction of neuronal injury.

Furthermore, in primary microglial cultures, we found that CysLT₂R agonists induced microglial phagocytosis and cytokine release, and its antagonist HAMI 3379 and shRNA inhibited the responses of microglia to the agonists and OGD/R. In contrast, CysLT₁Rs only regulated microglial cytokine release. Therefore, the role of CysLT₂Rs in ischemic neuronal injury could be described as follows: the endogenously released CysLTs during ischemic injury, or exogenously administered agonists, activate microglial phagocytosis and cytokine release, thereby causing neuronal injury (Figure 9).

Currently, the pharmacological effects of HAMI 3379 are poorly understood. It has been reported that HAMI 3379 concentration-dependently inhibits and reverses the increase in perfusion pressure and decrease in contractility induced by LTC₄ in isolated Langendorff-perfused guinea pig heart (Wunder et al., 2010). Recently, we reported that intracerebroventricular injection of HAMI 3379 protects against acute brain injury after focal cerebral ischemia in rats (Shi et al., 2012). In the present study, we found that HAMI 3379, unlike montelukast, had no effect on OGD/R-induced neuronal injury in primary neurons. However, it inhibited OGD/R- or LTD₄-induced injury similar to, or 10- to 100-fold more potently than, montelukast in mixed cultures of cortical cells as well as inhibiting all the responses to NMLTC₄. HAMI 3379 also inhibited neuronal injury in neuron-microglial co-cultures more effectively than montelukast, but had no effect in neuron-astrocyte co-cultures, indicating that it mainly acts on microglial activation. In primarily cultured microglia, HAMI 3379 inhibited the phagocytotic activity and cytokine

release induced by OGD/R, LTD₄ and NMLTC₄, and conditioned medium from the treated microglia induced neuronal necrosis, while montelukast only inhibited the release of cytokines. These results strongly suggest that HAMI 3379 has more potent protective effects than montelukast on ischemic neuronal injury in the relatively intact cellular environment. Because HAMI 3379 had the same effects as CysLT₂R knockdown by shRNA, its effects might be CysLT₂R-dependent. However, HAMI 3379 could not completely reverse some of injurious responses, even those induced by NMLTC₄, to control levels. This might result from insensitive responses mediated by non-CysLT receptors or unknown metabolites (see description below).

However, montelukast and CysLT₁Rs are also partially involved in ischemic neuronal injury. CysLT₁R knockdown by siRNA only attenuated a few responses, i.e. LTD₄-induced injury in mixed cultures, LTD₄-induced neuronal loss and necrosis in neuron-microglial co-cultures, and OGD/R- and LTD₄-induced cytokine release from microglia. Thus, CysLT₁Rs may partially mediate microglial activation possibly through regulating cytokine release, and thereby be involved in neuronal injury. However, montelukast had protective effects on multiple responses to ischemia-like injury and agonist stimulation. It inhibited OGD/R-induced injury in primary neurons, attenuated most of the OGD/R- and LTD₄-induced changes in mixed cultures and neuron-glial co-cultures, but had no effect on NMLTC₄-induced changes (except the increased TNF- α release). Montelukast seems to have both CysLT₁R-independent and -dependent effects; it blocked CysLT₁R responses the same as CysLT₁R siRNA, and also inhibited the other responses to OGD/R and LTD₄. Especially, it attenuated OGD/R injury in primary neurons, which differed from HAMI 3379.

It has been reported that montelukast has a broader spectrum of pharmacological effects than originally thought, including the primary effects of CysLT₁R antagonism, and the secondary effects, for example inhibition of cyclic nucleotide phosphodiesterases (PDEs) and nucleotide P2Y receptor signaling (Tintinger et al., 2010). Inhibition of PDEs by montelukast may be beneficial to ischemic neuronal injury, because the resultant accumulation of cyclic AMP protects neurons from ischemic brain injury (Lin et al., 2009; Tsukada et al., 2004), and inhibitors of PDE3 (cilostazol) and PDE4 (rolipram) have protective effects on neurons (Schaal et al., 2012; Tanaka et al., 2010). Also, its inhibitory effects on P2Y receptors (Lau et al., ; 2011; Mamedova et al., 2005; Pugliese et al., 2009) may be protective, because down-regulation of the novel P2Y-like receptor GPR17 protects from ischemic neuronal injury after focal cerebral ischemia in rats (Ciana et al., 2006; Zhao et al., 2011a). Moreover, montelukast has anti-oxidative effects in peripheral tissues (Coskun et al., ; 2011; Mohamadin et al., 2011; Muthuraman and Sood, 2010); in a preliminary study, we also found its moderate CysLT₁R-independent anti-oxidative activity in primary neurons (unpublished data). This may be another beneficial effect of montelukast on ischemic neuronal injury because oxidative stress is a major cause of the ischemic injury (Gan et al., 2012; Perez Velazquez et al., 1997; Zhou et al., ; 2012).

Microglia are immunologically active residents in the brain, and play dual roles under physiological and pathological conditions (Gomes-Leal, 2012; Saijo and Glass, 2011; Yenari et al., 2010). Activated microglia protect neurons against ischemic injury by phagocytosis of debris or dead cells and by releasing anti-inflammatory cytokines and neurotrophic factors (Denes et al., 2007; Imai et al., 2007; Lamberts et al., 2009). However, over-activated microglia contribute to neuronal damage by releasing harmful substances, including inflammatory cytokines, reactive

oxygen species and proteinases (Dheen et al., 2007; Lv et al., 2011; Zhang et al., 2012). Our results revealed that CysLTs-activated microglia mediated ischemic neuronal injury in our experimental conditions, and this might have resulted from releasing harmful cytokines, such as IL-1 β and TNF- α (Amantea et al., 2010; Lambertsen et al., 2012). This response was mainly mediated by CysLT₂Rs, and somewhat by CysLT₁Rs through regulating cytokine release as well. However, the neuronal injury induced by conditioned medium from pretreated microglia was not significantly inhibited by montelukast and CysLT₁R siRNA; this could be explained by the possibility that they also inhibit the CysLT₁R-mediated release of protective substances (Denes et al., 2007; Imai et al., 2007; Lambertsen et al., 2009). Because microglia-mediated inflammation occurs in several brain diseases (Gomes-Leal, 2012; Saijo and Glass, 2011; Yenari et al., 2010), CysLT₂R antagonists such as HAMI 3379 may represent a potential therapeutic agent for these diseases as well as ischemic stroke.

Unexpectedly, it is not yet clear whether CysLT₁Rs or CysLT₂Rs on astrocytes play regulatory roles in ischemic neuronal injury. Astrocytes in the mixed cultures only showed slight changes in number after exposure to the three stimuli. In neuron-astrocyte co-cultures, OGD/R induced relatively severe neuronal necrosis, which might result from both direct ischemic injury and astrocytes-mediated injury. Generally, astrocytes play protective roles in the ischemic injury of the neurons by releasing protective molecules like neurotrophic factors and removing toxic substances (Barreto et al., 2011a; Takano et al., 2009). On the other hand, proliferating or activated astrocytes may also be associated with neuronal injury (Katayama et al., 2010; Qu et al., 2011; Sullivan et al., 2010), which may explain our OGD/R results. In addition, LTD₄ and NMLTC₄ also induced milder neuronal necrosis (~3%) in the neuron-astrocyte co-cultures. Since neither agonist

had effects on neurons, this effect might result from astrocytes. Astrocytes might mediate neuron injury *via* acting on their CysLT₂Rs, because NMLTC₄ is a selective CysLT₂R agonist (Yan et al., 2011) and LTD₄ at 100 nM mainly acts on CysLT₂Rs in astrocytes (Huang et al., 2008). In spite of this, the mild neuronal necrosis induced by both agonists was not affected by the antagonist or RNA interference of CysLT₂Rs as well as those of CysLT₁Rs.

Our results show that the CysLT₂R antagonist HAMI 3379 protects neurons from ischemic injury by inhibiting microglial activation, and the CysLT₁R antagonist montelukast also has a broad but relatively weaker protective effect that may be both dependent on and independent of CysLT₁Rs. These findings demonstrate that CysLT₂Rs play an important role in microglial activation and the resultant neuronal injury, and CysLT₁Rs are also involved in microglia-mediated responses through regulating microglial cytokine release (Figure 9). Therefore, CysLT₂R antagonists may represent a new type of therapeutic agent in the treatment of diseases with neuronal injury such as ischemic stroke, and may synergize the effects of CysLT₁R antagonists. However, the mechanisms underlying the regulation of microglial activation by CysLT₂Rs need further investigation.

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Authorship Contributions

Participated in research design: Wei, X.Y. Zhang

Conducted experiments: X.Y. Zhang, Wang, Yu, Xu, L.Y. Zhang, Chen, Fang, Lu, W.P. Zhang

Performed data analysis: X.Y. Zhang, Wang, Xu, Yu, Shi

Wrote or contributed to the writing of the manuscript: X.Y. Zhang, Wei

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Footnotes

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Reprint Requests

Er-Qing Wei, Department of Pharmacology, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China.

Phone: +86-571-8820-8224; Fax: +86-571-8820-8022;

E-mail: weiq2006@zju.edu.cn

Figure legends

Figure 1. Viability of primary cultured cortical neurons after exposure to OGD/R and the agonists LTD₄ and NMLTC₄. Neuronal viability was determined by MTT reduction assay and neuronal death by LDH release. (A) After exposure to OGD for 1, 2 and 4 h, neuronal viability was decreased and LDH release was increased; these were gradually aggravated after 24, 48 and 72 h of recovery. (B, C) The CysLT₁R agonist LTD₄ (B) and the CysLT₂R agonist NMLTC₄ (C) altered neither neuronal viability nor LDH release. Data are expressed as mean \pm S.E.M.; $n = 8$; ** $P < 0.01$ compared with control, analyzed by one-way ANOVA.

Figure 2. Effects of the antagonists and RNA interference of CysLT₁Rs and CysLT₂Rs on OGD/R-induced neuronal injury. (A) After 1-h OGD and 24-h recovery (OGD/R), the reduced viability and increased LDH release were significantly attenuated by montelukast (0.001-1 μ M), but not by CysLT₁R siRNA (0.1 μ M). (B) The CysLT₂R antagonist HAMI 3379 (0.0001-1 μ M) and CysLT₂R shRNA had no effect on the OGD/R-induced viability reduction and increase in LDH release. (C) MAP2 immunostaining showing reduced numbers of neurons after OGD/R. This reduction was slightly inhibited by montelukast (0.01 μ M) but not by CysLT₁R siRNA, HAMI 3379 (0.01 μ M) or CysLT₂R shRNA. (D) PI staining showing neuronal necrosis (red nuclear staining) after OGD/R. The necrotic neurons were only reduced by montelukast (0.01 μ M). Data are expressed as mean \pm S.E.M.; $n = 8$; * $P < 0.05$ and ** $P < 0.01$ compared with control; # $P < 0.05$ compared with OGD/R alone, analyzed by one-way ANOVA. Scale bar, 50 μ m.

Figure 3. OGD/R, LTD₄- or NMLTC₄-induced injury and the effects of the antagonists and

RNA interference of CysLT₁R and CysLT₂R in mixed cultures of cortical cells. (A) After the cortical cells were exposed to OGD for 1, 2 and 4 h, LDH release increased time-dependently at 0, 24, 48 and 72 h of recovery. Increased LDH release after 1-h OGD and 24-h recovery (OGD/R) was attenuated by montelukast (0.01-1 μ M), HAMI 3379 (0.001-1 μ M) and CysLT₂R shRNA, but not by CysLT₁R siRNA. (B) LTD₄ (1-1000 nM for 24, 48 and 72 h) increased LDH release in a concentration-dependent and time-dependent manner. The LTD₄ (100 nM for 48 h)-induced increase was inhibited by montelukast (0.01-1 μ M), CysLT₁R siRNA, HAMI 3379 (0.0001-1 μ M) and CysLT₂R shRNA. (C) NMLTC₄ (1-1000 nM, for 24, 48 and 72 h) also increased LDH release. At 100 nM, NMLTC₄ induced a greater increase than LTD₄. The NMLTC₄ (100 nM for 48 h)-induced increase was inhibited by HAMI 3379 (0.0001-1 μ M) and CysLT₂R shRNA, but not by montelukast (0.0001-1 μ M) and CysLT₁R siRNA. Data are expressed as mean \pm S.E.M.; $n = 8$; * $P < 0.05$ and ** $P < 0.01$ compared with control; # $P < 0.05$ compared with OGD/R, LTD₄, NMLTC₄ alone or indicated group, analyzed by one-way ANOVA.

Figure 4. Neuronal morphology and density in mixed cultures of cortical cells after exposure to OGD/R, LTD₄ and NMLTC₄ and effects of the antagonists and RNA interference of CysLT₁R and CysLT₂R. (A) Neurons were immunostained with antibody against MAP2 (a neuronal marker). After exposure to OGD/R (1-h OGD/24-h recovery), LTD₄ or NMLTC₄ (100 nM for 48 h), the numbers of intact neurons with normal morphology were reduced. Scale bar, 50 μ m. (B) Montelukast (0.01-1 μ M), but not CysLT₁R siRNA, attenuated the reduction in neuronal density induced by OGD/R and LTD₄, but not that by NMLTC₄. (C) HAMI 3379 (0.01-1 μ M) and CysLT₂R shRNA inhibited the neuronal density reduction induced by OGD/R, LTD₄ and NMLTC₄.

Data are expressed as mean \pm S.E.M; $n = 10$; $*P < 0.05$ and $**P < 0.01$ compared with control; $^{\#}P < 0.05$ compared with OGD/R, LTD₄ or NMLTC₄ alone, analyzed by one-way ANOVA.

Figure 5. Neurons in neuron-microglia transwell co-cultures after exposure to OGD/R, LTD₄ and NMLTC₄ and the effects of the antagonists and RNA interference of CysLT₁R and CysLT₂R.

Neurons were plated onto the upper compartment and microglia onto the lower compartment of the transwell. After the transwell cultures were exposed to OGD/R, LTD₄ and NMLTC₄, neurons were immunostained with antibody against MAP2. (A) Photomicrographs showing the changes in neuronal morphology and number after various treatments. Scale bar, 50 μ m. (B) Montelukast (0.1 and 1 μ M) attenuated the OGD/R- and LTD₄-induced but not the NMLTC₄-induced reduction in neuron number. CysLT₁R siRNA inhibited the LTD₄-induced, but not the OGD/R- and NMLTC₄-induced, reduction. (C) HAMI 3379 (0.01-1 μ M) and CysLT₂R shRNA (MOI = 5) inhibited the reduction in neuron number induced by OGD/R, LTD₄ and NMLTC₄. Data are expressed as mean \pm S.E.M.; $n = 10$; $*P < 0.05$ and $**P < 0.01$ compared with control; $^{\#}P < 0.05$ compared with OGD/R, LTD₄ or NMLTC₄ alone, analyzed by one-way ANOVA.

Figure 6. Neuronal necrosis in neuron-microglial transwell co-cultures after exposure to OGD/R, LTD₄ and NMLTC₄ and the effects of the antagonists and RNA interference of CysLT₁R and CysLT₂R.

After the neuron-microglial co-cultures were exposed to OGD/R, LTD₄ and NMLTC₄, necrosis was detected by PI fluorescence staining. (A) Photomicrographs showing necrotic neurons with nuclei stained red with PI staining after various treatments. Scale bar, 50 μ m.

(B) Montelukast (0.1 and 1 μ M) attenuated the OGD/R- and LTD₄-induced necrosis but not the NMLTC₄-induced necrosis. CysLT₁R siRNA inhibited the LTD₄-induced necrosis, but not the OGD/R- and NMLTC₄-induced necrosis. (C) HAMI 3379 (0.001-1 μ M) and CysLT₂R shRNA (MOI = 5) inhibited the necrosis induced by OGD/R, LTD₄ and NMLTC₄. Data are expressed as mean \pm S.E.M; $n = 10$; * $P < 0.05$ and ** $P < 0.01$ compared with control; # $P < 0.05$ compared with OGD/R, LTD₄ or NMLTC₄ alone, analyzed by one-way ANOVA.

Figure 7. Flow cytometry of microglial phagocytosis in primary microglia. After exposure to OGD/R, LTD₄ or NMLTC₄, microglia were incubated with fluorescent microspheres for 1 h, and then phagocytosis was detected by flow cytometry. (A) Representative results showing increased phagocytotic activity of microglia. (B) HAMI 3379 (0.01 μ M) and CysLT₂R shRNA inhibited the microglial phagocytosis induced by OGD/R, LTD₄ and NMLTC₄. Montelukast (0.01 μ M) only attenuated the OGD/R-induced phagocytosis, but CysLT₁R siRNA had no effect. Data are expressed as mean \pm S.E.M.; $n = 3$; ** $P < 0.01$ compared with control; # $P < 0.05$ compared with OGD/R, LTD₄ or NMLTC₄ alone, analyzed by one-way ANOVA.

Figure 8. Pro-inflammatory cytokine release from primary microglia, the neuronal necrosis induced by conditioned medium from microglia pretreated with OGD/R, LTD₄ and NMLTC₄, and the effects of the antagonists and RNA interference of CysLT₁R and CysLT₂R. (A and B) After exposure to OGD/R, LTD₄ or NMLTC₄, cytokine release in the culture medium of microglia was detected by ELISA. Montelukast and HAMI 3379 (0.1 μ M) as well as CysLT₁R siRNA and CysLT₂R shRNA significantly inhibited the release of TNF- α (A) and IL-1 β (B) induced by

OGD/R and LTD₄. HAMI 3379 and CysLT₂R shRNA also blocked the NMLTC₄-induced release of TNF- α (A) and IL-1 β (B), and montelukast attenuated the NMLTC₄-induced TNF- α release (A, lower panel). (C) Conditioned medium from microglia pretreated with OGD/R, LTD₄ and NMLTC₄ was added into primary neuron cultures, and neuronal necrosis was induced as determined by PI staining. HAMI 3379 (0.01 μ M) and CysLT₂R shRNA inhibited the necrosis induced by all 3 stimuli. However, montelukast (0.01 μ M) showed a trend to attenuate OGD/R- and LTD₄-induced necrosis, but CysLT₁R siRNA had no effect. Data are expressed as mean \pm S.E.M.; $n = 6$; $**P < 0.01$ and $***P < 0.001$ compared with control; $^{\#}P < 0.05$ compared with OGD/R, LTD₄ or NMLTC₄ alone, analyzed by one-way ANOVA.

Figure 9. Diagram showing the roles of CysLT₂Rs and CysLT₁Rs in ischemic neuronal injury as well as the effects of their antagonists. During brain ischemia or OGD/R in a mixed culture system, CysLTs are produced by brain tissue (Zhou et al., 2006), or astrocytes (Huang et al., 2008) and neurons (Ge et al., 2006), and the increased CysLTs activate CysLT₁Rs and CysLT₂Rs. In the main pathway, activated CysLT₂Rs on microglia evoke microglial phagocytosis and cytokine release, thereby inducing ischemic neuronal injury. On the other hand, CysLT₁R activation also induces cytokine release and aggravates the neuronal injury. Many other events are also involved in the process of ischemic neuronal injury. The CysLT₂R antagonist HAMI 3379 inhibits microglial activation, which indirectly attenuates ischemic neuronal injury, whereas, the CysLT₁R antagonist montelukast inhibits ischemic neuronal injury in receptor-dependent and –independent manners.

Figure 1

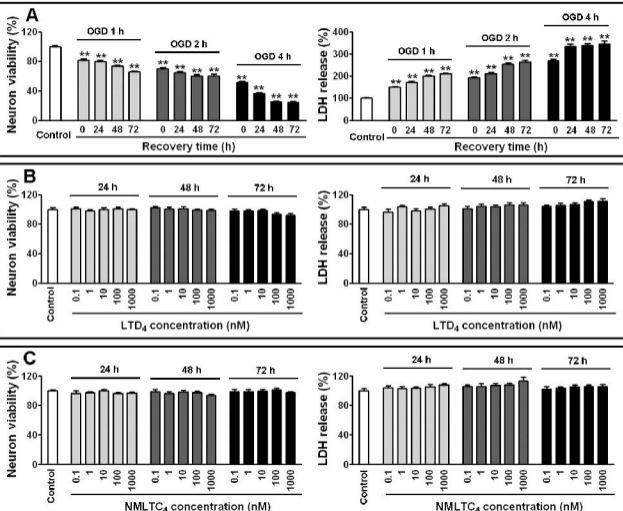


Figure 2

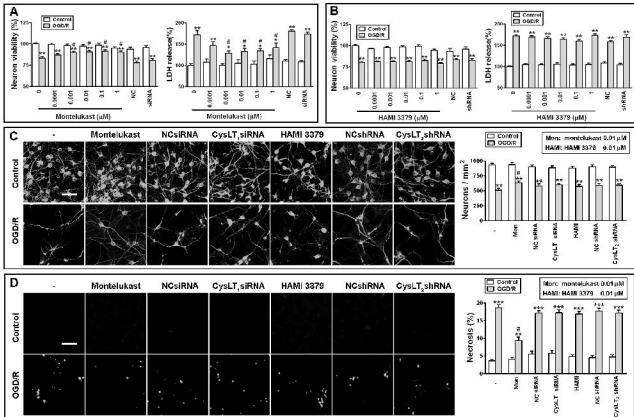


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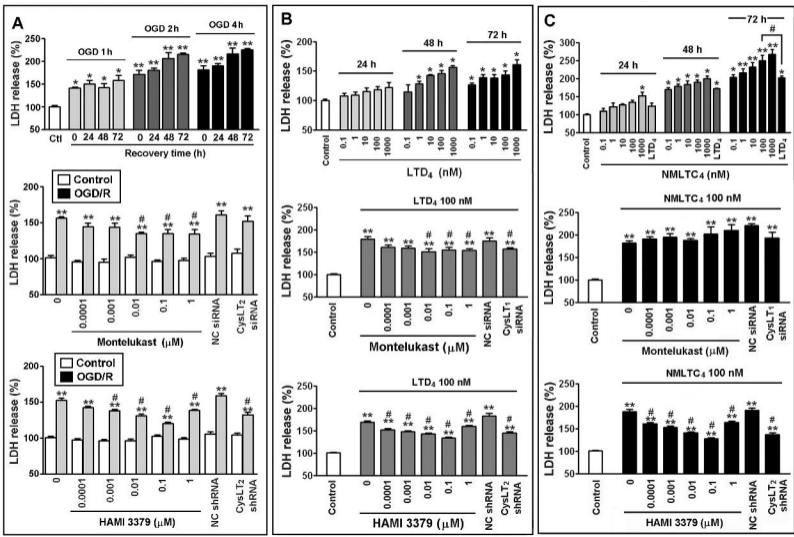
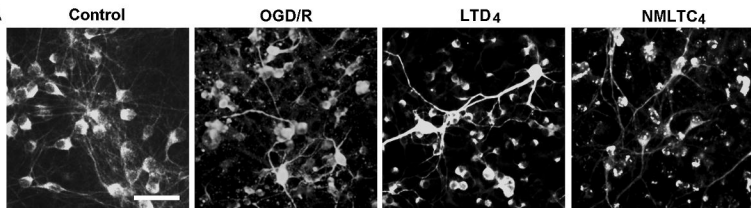
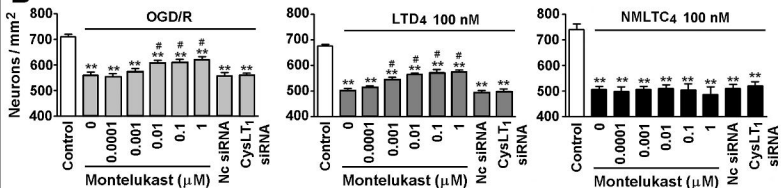


Figure 4

A



B



C

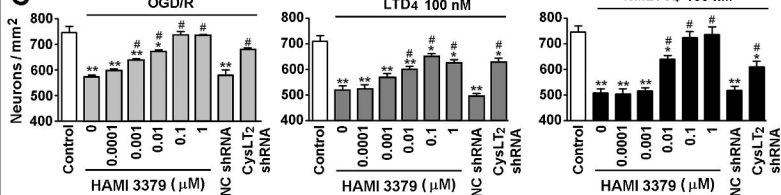


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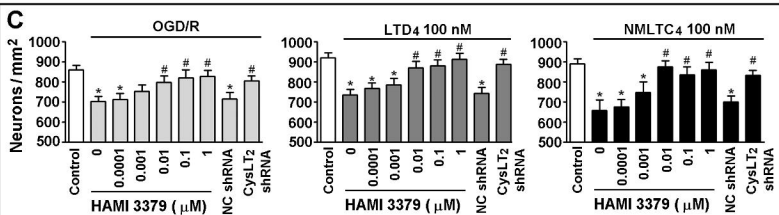
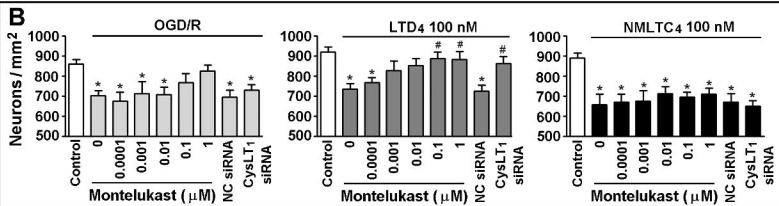
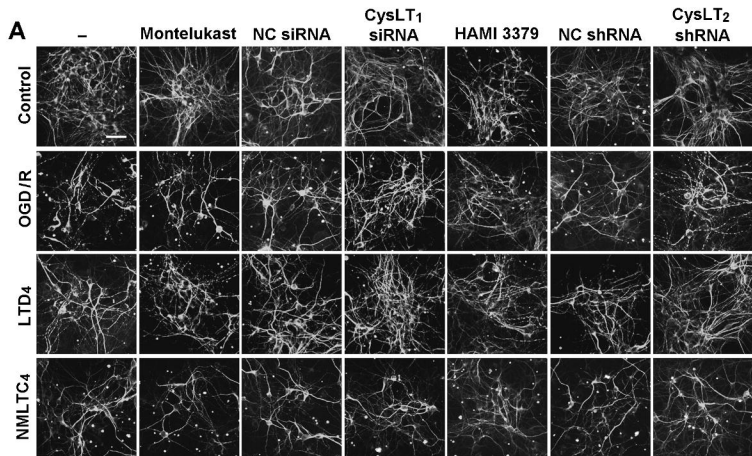


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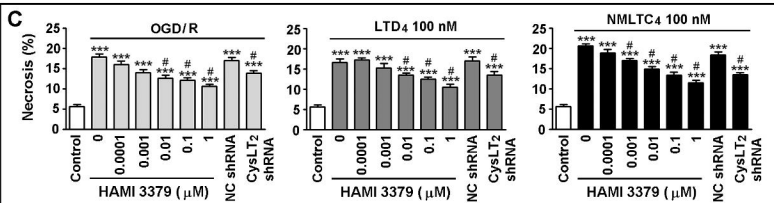
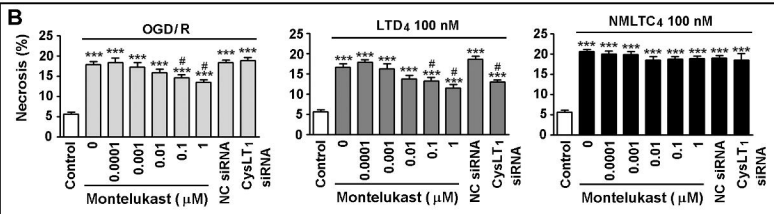
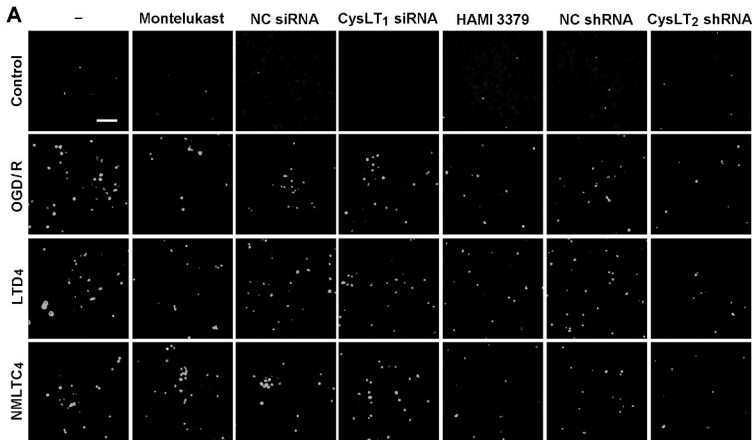


Figure 7

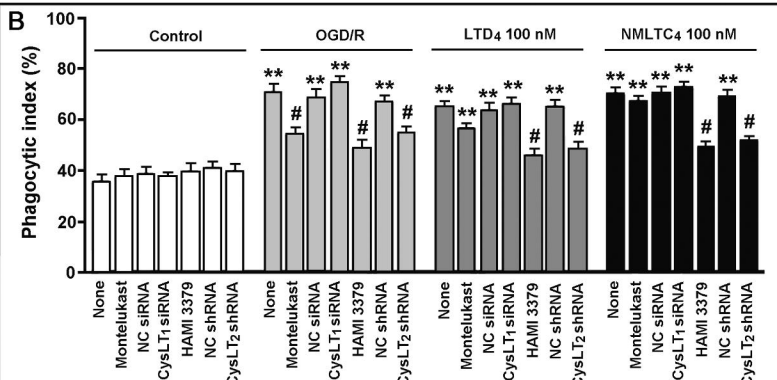
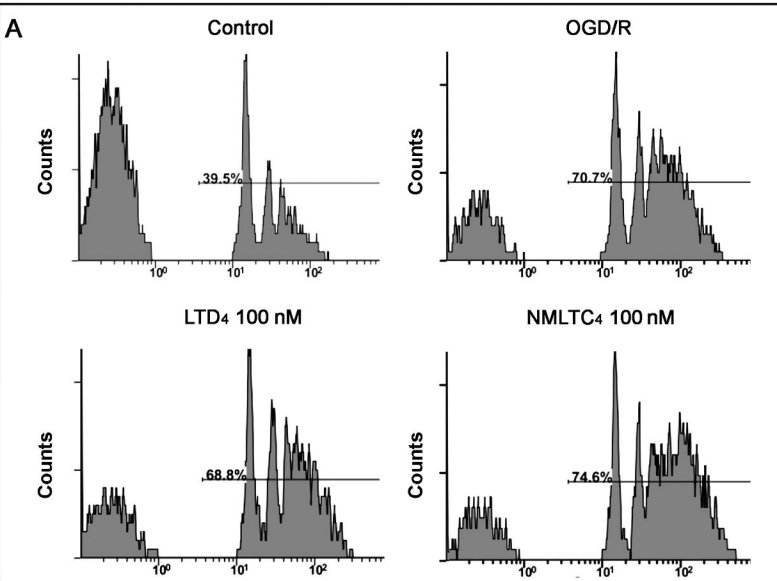


Figure 8

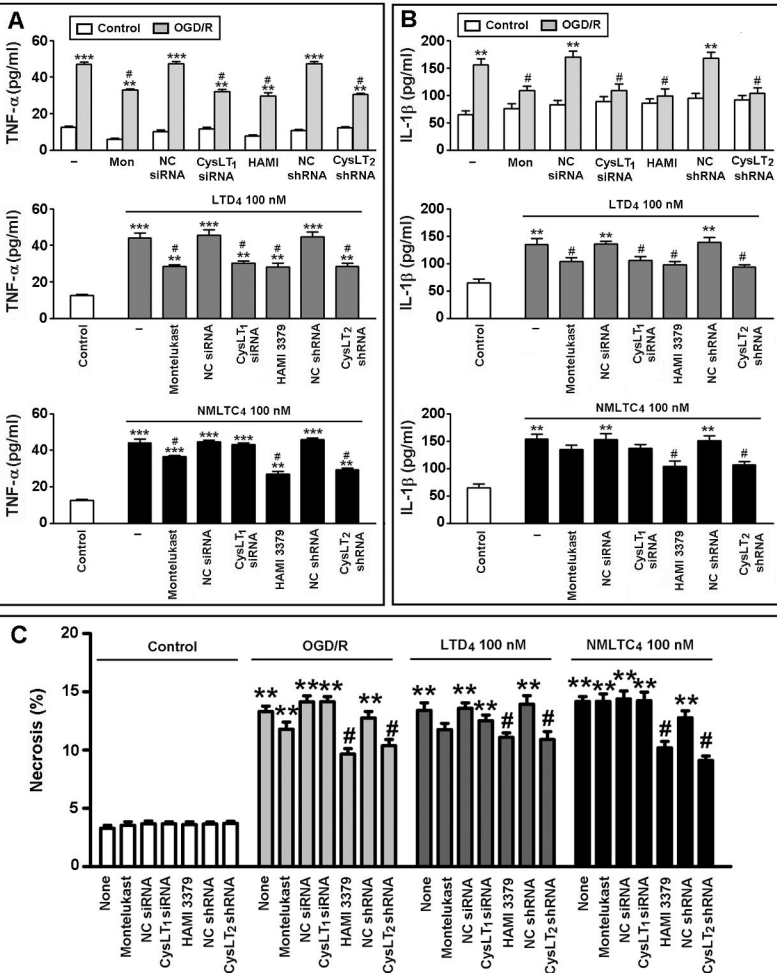


Figure 9

