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Up-regulation of astroglial heme oxygenase-1 by a synthetic (S)-verbenone derivative LMT-335 ameliorates oxygen–glucose deprivation-evoked injury in cortical neurons

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ABSTRACT

Excessive generation of free radicals is regarded as a major detrimental factor in cerebral ischemic insults. Neurons are particularly vulnerable to oxidative stress due to their limited anti-oxidant capacity. As an important source of antioxidants in the brain, astroglia are now thought to be attractive targets for pharmacological interventions to reduce neuronal oxidative stress in ischemic stroke. In the present study, we synthesized a novel antioxidant, the (1S)-(-)-verbenone derivative LMT-335, and investigated its anti-ischemic activities. In rat cortical neuronal/glial co-cultures, LMT-335 significantly reduced oxygen–glucose deprivation (OGD)/reoxygenation (R)-induced neuronal injury. Although it did not inhibit N-methyl-D-aspartate-induced excitotoxicity, LMT-335 significantly reduced OGD/R-evoked intracellular oxidative stress. However, the oxygen radical absorbance capacity assay and 1,1-diphenyl-2-picrylhydrazyl assay showed that the free radical scavenging activities of LMT-335 were lower than those of trolox. Instead, LMT-335 significantly increased the astroglial expression of heme oxygenase-1 (HO-1), a well-known anti-oxidant stress protein, as evidenced by immunocytochemistry and immunoblot analyses. Moreover, a selective HO-1 inhibitor, tin protoporphyrin IX (SnPP), significantly blocked the anti-ischemic effect of LMT-335. The present findings indicate that LMT-335 exerts neuroprotective effects against OGD/R by up-regulation of HO-1 in astroglial cells. Our data suggest that astroglial HO-1 represents a potential therapeutic target for the treatment of ischemic stroke.

1. Introduction

Ischemic stroke is caused by cerebrovascular occlusion, often associated with a thrombus or an embolus [1]. The resulting ischemic energy failure immediately causes perturbation of mitochondrial membrane potential and ATP synthesis, resulting in massive generation of reactive oxygen/nitrogen species (ROS/RNS). A concomitant activation of glutamate receptors evokes intracellular calcium overload, further increasing oxidative/nitrosative stress. Importantly, neurons are especially vulnerable to oxidative stress due to their high metabolic rate and limited anti-oxidant capacity. Neurons can be further damaged by free radicals released from activated microglia and infiltrating inflammatory cells. It is therefore not surprising that many preclinical and clinical studies have proven that antioxidant therapy enhances neuronal survival after ischemic stroke [2].

Heme oxygenase (HO) is an anti-oxidant stress protein mediating the degradation of heme [3]. Among three isoforms, HO-1 is an inducible isoform present in the brain, which is up-regulated by various stress stimuli including oxidative stress. Considerable evidence supports the neuroprotective function of HO-1 in ischemic injury. HO-1 knockout mice show increased susceptibility to cerebral ischemic injury [4]. Also, over-expression of HO-1 in transgenic mice or by treatment with an adenoviral vector decreases cerebral infarcts [5,6]. However, excessive HO activity and subsequent release and accumulation of free iron ions and bilirubin also results in neurotoxicity [7,8]. Thus, a critical need remains to find a small molecule inducer that can sufficiently activate HO-1 to achieve neuroprotection without producing potential side effects.

Abbreviations: OGD/R, oxygen–glucose deprivation/re-oxygenation; HO-1, heme oxygenase-1; SnPP, tin protoporphyrin IX; ROS, reactive oxygen species; RNS, reactive nitrogen species; CNS, central nervous system; NMDA, N-methyl-D-aspartate; LDH, lactate dehydrogenase; PI, propidium iodide; H_2DCF-DA, 2,7-dihydrodichlorofluorescein diacetate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ORAC, oxygen radical absorbance capacity; AAPH, 2,2′-azobis-(2-methylpropionamide)-dihydrochloride.

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Astroglia are the most abundant cell type in the central nervous system (CNS), outnumbering neurons by a factor of 5–10 to 1. In addition to organizing the structural architecture, astrocytes play various other vital roles in energy metabolism, regulation of neural activity, and antioxidant protection. Although few studies have specifically investigated targeting astroglia to treat ischemic stroke, recent reports support their potential role in promoting neuronal survival during ischemia [9,10]. For example, astroglial overexpression of antioxidant enzymes such as SOD2 and glutathione peroxidase, or cytoprotective proteins such as BDNF and excitatory amino acid transporter 2 (EAAT2), protect neurons from ischemic insult [11–14]. Likewise, upregulation of HO-1 expression has also been observed in astroglia following rodent and human cerebral infarctions [15,16]. It is still unclear, however, whether the induction of astroglial HO-1 can offer neuroprotection in cerebral ischemia.

(1S)-(-)-verbenone is a naturally occurring anti-aggregation pheromone produced by bark beetles from a host tree resin precursor, α-pinene [17]. Verbenone is generated as either a biotransformation or auto-oxidation product of verbenol [18], which our laboratory previously identified as a lead compound with anti-ischemic and anti-inflammatory effect [19]. To develop a small molecule with potent anti-ischemic activities, we recently synthesized a series of novel (1S)-(-)-verbenone derivatives. Among the derivatives synthesized, we found that LMT-335 [(1S,5R)-6,6-dimethyl-4-((E)-4-methylstyryl)bicyclo[3.1.1]hept-3-en-2-one] induces HO-1 expression selectively in astroglia and exhibits potential anti-ischemic activities (Fig. 1A).

2. Materials and methods

2.1. Mixed cortical neuronal/glial co-cultures

Primary mixed cortical neuronal/glial co-cultures were prepared from embryonic (E16–17 day-old) Sprague–Dawley rats. In brief, dissociated cerebrocortical cells (1.8×10^3 cells/mm^2) were added to pre-coated culture plates and maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 2% B27 supplements (Invitrogen, Grand Island, NY). All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by Korea University Institutional Animal Care & Use Committee. Experiments were performed 13–16 days after initial plating of cultures.

2.2. Oxygen–glucose deprivation (OGD) followed by re-oxygenation (R)

For an in vitro model of hypoxic/ischemic insult, cells were placed in an anaerobic chamber (partial pressure of oxygen <2 mm Hg), while the culture medium was replaced with a glucose-free DMEM bubbled with an anaerobic gas mix (95% N_2, 5% CO_2) for 30 min to remove residual oxygen. Cells were maintained in the anaerobic chamber at 37 °C for 1.5 h to produce oxygen deprivation. OGD was terminated by replacing the exposure solution with oxygenated DMEM supplemented with 25 mM glucose, and returning cells to the incubator under normoxic conditions.

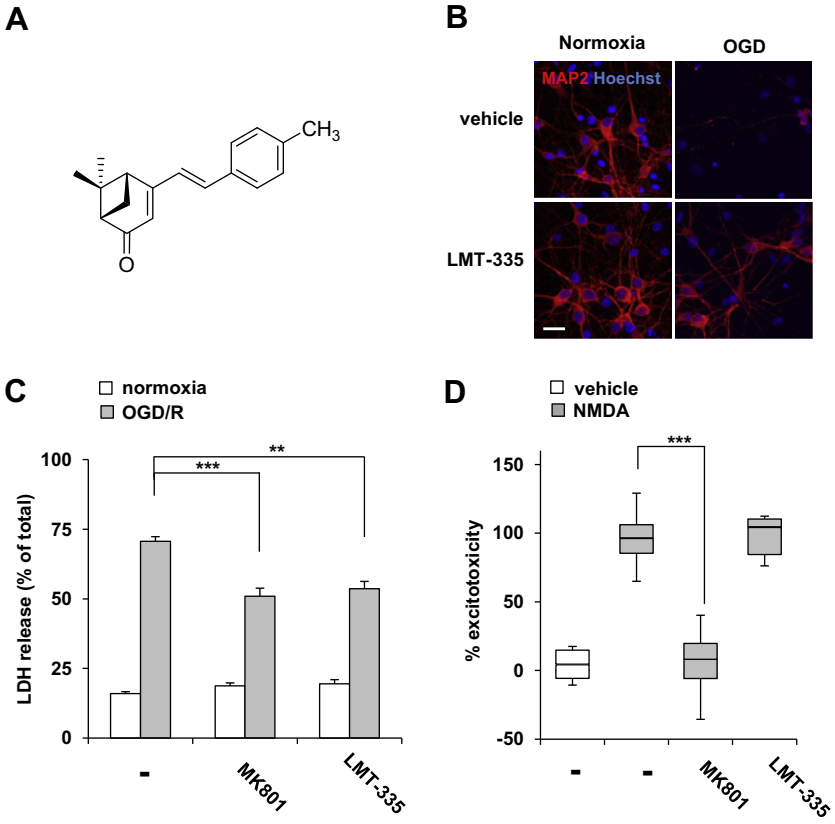

Fig. 1. Neuroprotective effects of LMT-335. (A) The structure of LMT-335. Cortical neurons were pre-treated with LMT-335 or MK-801 (10 μM each) for 30 min and further exposed to OGD/R (B and C). LMT-335 reduces OGD/R-induced neuronal injury. Neuronal cell injury or death was assessed by morphological observation (B) and by measuring LDH release (C, 9 h after reoxygenation). (B) Representative immunostaining images. $N = 3$. Scale bar = 50 μm. (C) LDH release. Neuronal injury was assessed as % of total LDH release. Data are expressed as the mean ± SEM. $N = 27–54$. $^{**}P < 0.01$, $^{***}P < 0.001$: vs. OGD/R group. (D) LMT-335 did not block NMDA-evoked excitotoxicity. Cortical neurons were treated with 100 μM NMDA for 15 min and further incubated for 9 h. LMT-335 or MK801 (10 μM each) was pretreated for 30 min and maintained during experiments. Data were expressed as the median (bar), interquartile range (Q1–Q3, vertical column), and min–max (whisker plots), and analyzed by Kruskal–Wallis test followed by Mann–Whitney test. $N = 6–12$. $^{***}P < 0.001$: vs. NMDA-treated group.

Cells were treated with LMT-335 or a well-known NMDA receptor blocking agent MK801 (10 μM each) 30 min before, and during the entire period of OGD/R in the absence or presence of tin protoporphyrin IX (SnPP, 10 μM).

2.3. N-methyl-D-aspartate (NMDA)-induced excitotoxicity

Cortical co-cultures were exposed to NMDA (100 μM) for 10 min in nominally Mg^{2+}-free Earle's balanced salt solution (EBSS) containing 1.8 mM $CaCl_2$ and 10 μM glycine. After NMDA exposure, cells were washed with EBSS containing $MgSO_4$ and then maintained in glucose (25 mM)-containing DMEM at 37 °C. Cells were treated with LMT-335 or MK-801 (10 μM each) 30 min before NMDA treatment.

2.4. Assessment of cell injury or death

Cell injury or death was assessed by observing morphological deterioration or by measuring release of lactate dehydrogenase (LDH) into culture media using a diagnostic kit (Sigma–Aldrich, St. Louis, MO). In experiments using SnPP, cell injury was also assessed by measuring uptake of the cell membrane-impermeable dye propidium iodide (PI), due to interference in the LDH assay by SnPP. In brief, cells were stained with PI (40 μM) and Hoechst 33258 (12 μM; for nuclear counter-staining) for 30 min. After fixation, the number of PI positive cells relative to the total number of nuclei per field was counted by using ImageJ software (NIH).

2.5. Measurement of intracellular levels of reactive oxygen species (ROS): DCF fluorescence

2,7-Dihydrodichlorofluorescein diacetate (H_2DCF-DA; Molecular probes, Grand Island, NY) specifically reacts with ROS/RNS such as peroxyl radical, peroxynitrite, or hydrogen peroxide, generating highly fluorescent DCF [20]. At 1 h after rexoxygenation, cells were loaded with H_2DCF-DA (30 μM; Molecular probe, Eugene, Oregon). Three hours after reoxygenation, the fluorescence of DCF was measured using a fluorescence microscope (Ex = 485 nm, Em = 530 nm DM IL HC Fluo, Leica, Wetzlar, Germany) equipped with a digital camera (DFC420C). Fluorescence intensities were quantified using ImageJ software (NIH) after adjustment for autofluorescence.

2.6. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

Free radical scavenging capacity of LMT-335 was examined by employing an organic nitrogen radical generator, DPPH (Sigma–Aldrich, St. Louis, MO) [21]. After incubation of compounds with 23.6 μg/ml of DPPH solution for 30 min at 37 °C in the dark, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. Vitamin C (Vit. C; Sigma–Aldrich) was used as the standard.

2.7. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed as previously described [21]. In brief, 2,2′-azobis-(2-methylpropionamide)-dihydrochloride (AAPH, 60 mM) and fluorescein (50 nM) were used as a peroxyl radical generator and a fluorescent probe, respectively. After incubation of compounds with fluorescein solution (66 nM, 190 μl) at 37 °C for 10 min, AAPH (500 mM, 30 μl) was added. Decreasing levels of fluorescence were measured every 5 min for 9 h using a fluorescence microplate reader at 37 °C (Ex = 485 nm, Em = 530 nm; SpectraMax GeminiEM, Molecular Devices, Sunnyvale, CA). For quantification, the net area-under-the-curve (AUC) of each sample was calculated by subtracting that of the blank. The trolox equivalents (TE) of each sample, calculated as TE = [net AUC_{sample} at 50 μM]/[net AUC_{trolox} at 50 μM], were determined by generating a standard curve of net AUC relative to increasing concentrations of trolox.

2.8. Western blot analysis

Total cellular proteins from lysates (20 μg/ lane) were separated by electrophoresis on 4–20% SDS/polyacrylamide gels and transferred to PVDF membranes (GE healthcare, Buckinghamshire, UK). The blots were blocked with 5% bovine serum albumin (Millipore, Temecula, CA) in Tris-buffered saline (TBS) containing 0.1% Tween 20 at room temperature for 1 h and then incubated overnight at 4 °C with primary antibodies. The blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) and developed using an enhanced chemiluminescence detection kit (GE healthcare, Buckinghamshire, UK). The following dilutions of primary antibodies were employed: anti-HO-1 (Stressgen Bioreagents, Victoria, BC, Canada; 1:1000), anti-β-actin (Cell signaling Technology, Beverly, MA; 1:1000).

2.9. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and blocked with TBS with 5% normal goat serum for 1 h at room temperature. Cells were then stained with anti-microtubule-associated protein 2 (MAP-2, 1:100; Sigma–Aldrich, St. Louis, MA) or anti-glial fibrillary acidic protein (GFAP, 1:100; Millipore, Temecula, CA) antibodies as well as anti-HO-1 antibodies (1:100; Stressgen Bioreagents, Victoria, BC, Canada) at 4 °C overnight. After washing, cells were incubated with secondary antibody (2 μg/ml) for an additional 1 h at room temperature and counterstained with Hoechst 33258 for 20 min. Fluorescence was quantified using a fluorescence microscope (DM IL HC Fluo, Leica) equipped with a digital camera.

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and analyzed for statistical significance by employing an analysis of variance (ANOVA) followed by the post hoc Bonferroni test for multiple comparisons, unless otherwise stated. A P value < 0.05 after Bonferroni correction was considered significant.

3. Results

3.1. LMT-335 inhibits OGD/R-evoked neuronal injury

We first examined the neuroprotective effects of LMT-335 by using morphological examination and LDH measurement. Exposure to OGD (1.5 h) and subsequent reoxygenation induced morphological deterioration of cortical neurons, as reflected by the rapid loss of MAP2 immunoreactivity, a neuronal somatodendritic marker (within 2 h after reoxygenation; Fig. 1B). LMT-335 ameliorated the deterioration of neuronal dendritic structures occurring after OGD/R (Fig. 1B). In addition, LMT-335 significantly reduced OGD/R-induced LDH release to a similar extent as MK801, a well-known NMDA receptor antagonist (Fig. 1C). The LDH release measured most likely reflects neuronal and not astroglial injury, because astroglial cells are very resistant to OGD/R under the experimental conditions employed here, as previously reported by our laboratory and others [22,23]. Although excitotoxicity is considered to play a key role in OGD/R-evoked neuronal injury, LMT-335 did not inhibit NMDA-induced excitotoxicity (Fig. 1D).

3.2. LMT-335 reduces OGD/R-evoked intracellular oxidative stress

Results obtained from the H_2DCFDA assay demonstrated that LMT-335 significantly reduced intracellular oxidative stress during OGD/R (Fig. 2A and B). To examine additional antioxidant capacities of LMT-335, we next determined the activity of LMT-335 in two free radical scavenging assays: the single electron transfer-based DPPH assay and the hydrogen atom transfer-based ORAC assay. The DPPH assay showed that LMT-335 did not significantly scavenge organic nitrogen radicals (DPPH·) at a concentration (i.e., 10 μM) used in the OGD/R experiments (Fig. 2C). In contrast, in the ORAC assay, LMT-335 possessed peroxyl radical scavenging activity, but was less efficacious than trolox, a well-known antioxidant: Trolox equivalent (TE) at 50 μM = 0.73 (Fig. 2D).

3.3. Suppression of HO-1 activity antagonizes the neuroprotective effects of LMT-335

As shown by Western blot analysis (Fig. 3A and B), pretreatment with LMT-335 significantly up-regulated protein levels of HO-1 in mixed cortical neuronal/glial co-cultures. Interestingly, double immunostaining with an anti HO-1 antibody and antibodies specific for each cell type examined showed that the LMT-335 up-regulated HO-1 proteins predominantly in astroglial cells (Fig. 3C). We assessed neuronal injury by measuring uptake of the cell membrane-impermeable dye PI. LMT-335 significantly reduced OGD/R-evoked PI uptake, which was prevented by the HO-1 inhibitor, tin protoporphyrin IX (SnPP, 10 μM) (Fig. 4). SnPP alone did not significantly attenuate OGD/R-evoked neuronal injury (Fig. 4). Exposure to OGD/R did not significantly change the number of GFAP-immunopositive astroglia (data not shown). In addition, the uptake of PI was not significantly observed in pure astroglial cultures under our experimental conditions (data not shown), suggesting that increased PI uptake observed in mixed cortical neuronal/glial co-cultures reflects relatively selective injury of neurons and not astroglial cells.

4. Discussion

In the present study, a novel synthetic small molecule LMT-335 ameliorates OGD/R-evoked oxidative stress and neuronal injury in mixed cortical/glial co-cultures. Data obtained from the ORAC and DPPH assays indicate that LMT-335 acts as a free radical scavenger (e.g., peroxyl radicals) but was less efficacious than trolox. However, LMT-335 also significantly up-regulates astroglial HO-1 expression. The specific HO-1 inhibitor SnPP blunted the

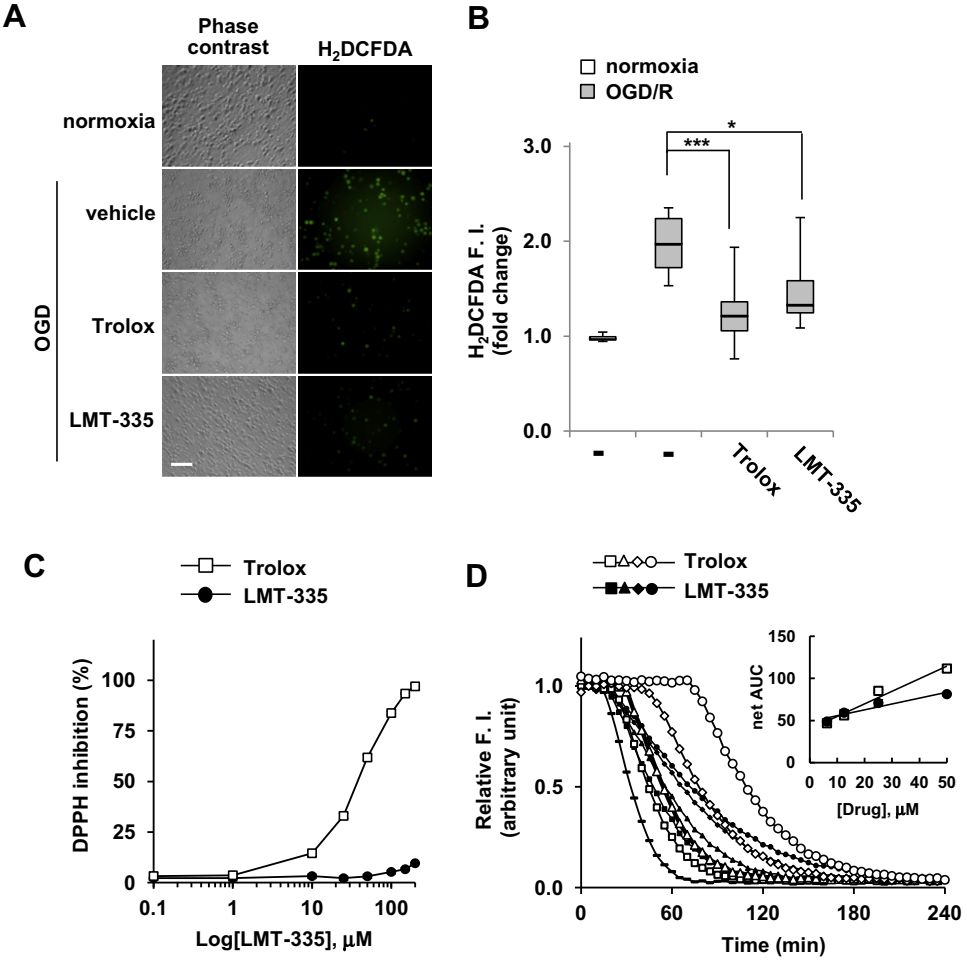

Fig. 2. Antioxidant effects of LMT-335. (A and B) LMT-335 reduced OGD/R-evoked oxidative stress. Cortical cultures were treated with LMT-335 (10 μM) for 30 min before OGD and during OGD/R. Cells were loaded with H_2DCFDA for1 h after reoxygenation, and intracellular oxidative stress was measured at 3 h after initiation of reoxygenation by an increase of fluorescence intensity (F.I.). Representative images (A, scale bar = 50 μm) and quantitative data (B). Data were expressed as the median (bar), interquartile range (vertical column), and min–max (whisker plots) and analyzed by Kruskal–Wallis test followed by Mann–Whitney test. $N = 8–12$. $^{*}P < 0.05$, $^{***}P < 0.001$: vs. OGD/R group. (C and D) Free radical scavenging activities of LMT-335. (C) The DPPH assay. The dose–response curves of LMT-335 in comparison of that of trolox. Data represent mean ± SEM. $N = 6–8$. (D) The ORAC assay. Representative time-dependent decay graphs of relative F.I. in the presence of trolox or LMT-335 at different concentrations (- = untreated; □,■ = 6.25 μM; △,▲ = 12.5 μM; ◇,♦ = 25 μM; ○,● = 50 μM). The inset represents the best linear fit between net AUCs and concentrations of trolox or LMT-335. Data represent mean ± SEM. $N = 6$.

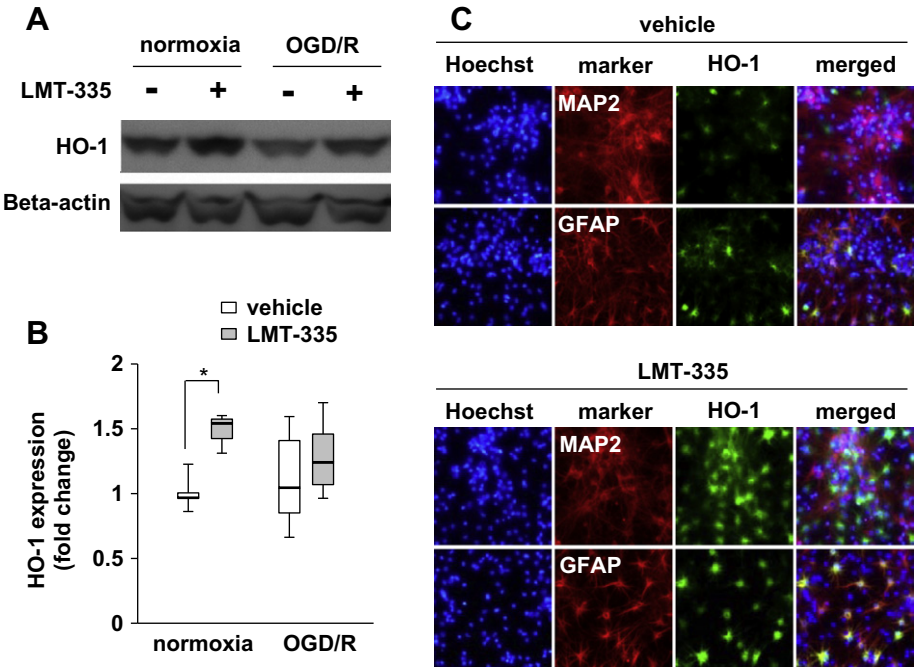

Fig. 3. Up-regulation of astroglial HO-1 by LMT-335. (A) LMT-335 increases the expression of HO-1 proteins in cortical cultures. Cultured rat cortical neurons were subjected to OGD/R. LMT-335 (10 μM) was pretreated for 30 min and maintained during OGD/R. Cell lysates were collected 3 h after reoxygenation and analyzed by Western blot analysis. Representative images (A) and quantification (B). Levels of the HO-1 proteins were normalized with respect to the density of β-actin and expressed as relative fold changes in comparison with vehicle-treated normoxia group. $N = 4–6$. Data were expressed as the median (bar), interquartile range (vertical column), and min–max (whisker plots), and analyzed by Kruskal–Wallis test followed by Mann–Whitney test. $^{*}P < 0.05$: vs. indicated group. (C) Cellular distribution of HO-1 showing astroglial distribution of HO-1. Double immunostaining against HO-1 (green) and each cell-type marker (red) with nuclear Hoechst 33258 counterstaining. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

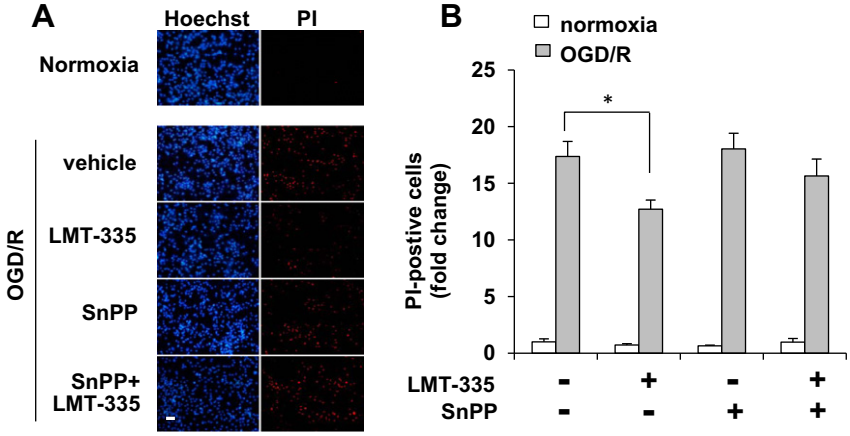

Fig. 4. Neuroprotective effects of LMT-335 mediated by astroglial HO-1. The treatment with a HO-1 inhibitor, SnPP blocked the neuroprotective effects of LMT-335 (A and B). Cortical neurons were pretreated with LMT-335 in the presence or absence of SnPP (10 μM) for 30 min and subjected to OGD/R. Cells were then loaded with propidium iodide (PI) and Hoechst 33258 for 30 min. After fixation, the number of PI positive cells upon injury (red) was counted using a fluorescent microscopy and normalized to the total cell numbers measured by Hoechst 33258 staining (blue). Scale bar = 50 μm. $N = 8–9$. $^{*}P < 0.05$: vs. vehicle-treated OGD/R group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neuroprotective effects of LMT-335, suggesting that the neuroprotective effect of LMT-335 is likely mediated by astroglial HO-1.

HO-1 represents an important endogenous defense mechanism in brain disorders including ischemic stroke [3]. Genetic or pharmacological up-regulation of HO-1 at early stages of ischemic injury may provide significant potential therapeutic benefit for treatment of cerebral ischemia. Up-regulation of HO-1 using an adenoviral delivery system attenuates ischemic brain damage [6]. Treatment with HO-1 inducers such as Ginkgo bibloba, polyphenols, and triterpenoids also exert neuroprotective effects against ischemic injury [24–26]. Furthermore, HO-1 knockout worsens cerebral infarct [24]. In the present study, we identified LMT-335 as a prototype for a potential new class of HO-1 inducers that are structurally distinct from previously identified compounds. Our data suggest that the neuroprotective effects of LMT-335 are mediated by upregulation of HO-1 expression selectively in astroglia. Interestingly, when compared to other HO-1 inducers that generally require long-term pretreatment (several hours to overnight) to achieve neuroprotection [26,27], LMT-335 exerts significant neuroprotection when administered for a much shorter pretreatment period (i.e., 30 min) (Figs. 1 and 4). Although the *in vivo* effects of LMT-335 are not yet known, the present studies suggest that agents producing specific induction of astroglial HO-1 expression could be promising candidates for the treatment of ischemic stroke.

In astroglia, up-regulation of HO-1 expression is modulated by several redox-sensitive transcription factors, particularly nuclear factor-erythroid 2-related factor2 (Nrf2) [28]. Hypoxia-inducible factor-1 and endoplasmic reticulum (ER) stress proteins such as activating transcription factor 6α have also been associated with HO-1 upregulation in astroglia [29–31]. However, the mechanisms responsible for astroglial HO-1 expression under ischemic conditions are still unclear. Moreover, some noxious stimuli, such as H_2O_2, enhance HO-1 expression in astrocytes but not in neurons [32,33]. The present study demonstrates that the small molecule LMT-335 also increases HO-1 expression particularly in astrocytes and not in neurons. Thus, these data suggest that regulation of HO-1 expression is cell-type dependent.

Neuroprotection produced by astroglial HO-1 may be attributed to rapid catabolism of cytotoxic free heme and the generation of cytoprotective end products such as carbon monoxide (CO), iron (Fe, which upregulates the expression of H-ferritin), and biliverdin (which is converted to bilirubin by biliverdin reductase). These end products are freely diffusible from cell to cell and exhibit potent antioxidant, anti-inflammatory and/or cytoprotective activity [3]. In ischemic animal models, treatment with low concentrations of CO or biliverdin reduces ischemic infarct [30,34,35]. Thus, upregulation of astroglial HO-1 by LMT-335 may reduce oxidative stress in astroglia as well as in neighboring neurons.

Taken together, the present results indicate that LMT-335 may be therapeutically useful for treatment of cerebral ischemic injury due to its ability to upregulate astroglial HO-1 expression and scavenge peroxyl radicals. Further delineation of the molecular target(s) of LMT-335 and determining a more defined mechanism of its action may provide new insights for design of more efficacious, but less toxic, drugs to treat cerebral ischemia.

Acknowledgments

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References

[1] M.A. Moskowitz, E.H. Lo, C. Iadecola, The science of stroke: mechanisms in search of treatments, Neuron 67 (2010) 181–198.
[2] T.R. Doeppner, D.M. Hermann, Free radical scavengers and spin traps – therapeutic implications for ischemic stroke, Best Pract. Res. Clin. Anaesthesiol. 24 (2010) 511–520.
[3] M.P. Soares, F.H. Bach, Heme oxygenase-1: from biology to therapeutic potential, Trends Mol. Med. 15 (2009) 50–58.
[4] E. Zeynalov, Z.A. Shah, R.C. Li, S. Dore, Heme oxygenase 1 is associated with ischemic preconditioning-induced protection against brain ischemia, Neurobiol. Dis. 35 (2009) 264–269.
[5] N. Panahian, M. Yoshiura, M.D. Maines, Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice, J. Neurochem. 72 (1999) 1187–1203.
[6] X.D. Chao, Y.H. Ma, P. Luo, L. Cao, W.B. La, B.C. Zhao, F. Han, W. Liu, W.D. Ning, N. Su, L. Zhang, J. Zhu, Z. Fei, Y. Qu, Up-regulation of Heme oxygenase-1 attenuates brain damage after cerebral ischemia via simultaneous inhibition of superoxide production and preservation of NO bioavailability, Exp. Neurol. 239 (2013) 163–169.
[7] D.M. Suttner, P.A. Dennery, Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron, FASEB J. 13 (1999) 1800–1809.
[8] W. Song, H. Zukor, S.H. Lin, A. Liberman, A. Tavitian, J. Mui, H. Vali, C. Fillebeen, K. Pantopoulos, T.D. Wu, J.L. Guerquin-Kern, H.M. Schipper, Unregulated brain iron deposition in transgenic mice over-expressing HMOX1 in the astrocytic compartment, J. Neurochem. 123 (2012) 325–336.
[9] G.E. Barreto, J. Gonzalez, Y. Torres, L. Morales, Astrocytic–neuronal crosstalk: implications for neuroprotection from brain injury, Neurosci. Res. 71 (2011) 107–113.
[10] T. Takano, N. Oberheim, M.L. Cotrina, M. Nedergaard, Astrocytes and ischemic injury, Stroke 40 (2009) S8–S12.
[11] L. Xu, J.F. Emery, Y.B. Ouyang, L.A. Voloboueva, R.G. Giffard, Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia, Glia 58 (2010) 1042–1049.
[12] W.S. Qu, Y.H. Wang, J.P. Wang, Y.X. Tang, Q. Zhang, D.S. Tian, Z.Y. Yu, M.J. Xie, W. Wang, Galectin-1 enhances astrocytic BDNF production and improves functional outcome in rats following ischemia, Neurochem. Res. 35 (2010) 1716–1724.
[13] M.L. Weller, I.M. Stone, A. Goss, T. Rau, C. Rova, D.J. Poulsen, Selective overexpression of excitatory amino acid transporter 2 (EAAT2) in astrocytes enhances neuroprotection from moderate but not severe hypoxia-ischemia, Neuroscience 155 (2008) 1204–1211.
[14] X. Wu, Y. Liu, X. Chen, Q. Sun, R. Tang, W. Wang, Z. Yu, M. Xie, Involvement of TREK-1 activity in astrocyte function and neuroprotection under simulated ischemia conditions, J. Mol. Neurosci. (2012), http://dx.doi.org/10.1007/s12031-012-9875-5.
[15] R. Beschorner, D. Adjodah, J.M. Schwab, M. Mittelbronn, I. Pedal, R. Mattern, H.J. Schluesener, R. Meyermann, Long-term expression of heme oxygenase-1 (HO-1, HSP-32) following focal cerebral infarctions and traumatic brain injury in humans, Acta Neuropathol. 100 (2000) 377–384.
[16] A. Takeda, H. Onodera, A. Sugimoto, Y. Itoyama, K. Kogure, S. Shibahara, Increased expression of heme oxygenase mRNA in rat brain following transient forebrain ischemia, Brain Res. 666 (1994) 120–124.
[17] G.J. Blomquist, R. Figueroa-Teran, M. Aw, M. Song, A. Gorzalski, N.L. Abbott, E. Chang, C. Tittiger, Pheromone production in bark beetles, Insect Biochem. Mol. Biol. 40 (2010) 699–712.
[18] J.A. Byers, Bark beetle conversion of a plant compound to a sex-specific inhibitor of pheromone attraction, Science 220 (1983) 624–626.
[19] I.Y. Choi, J.H. Lim, S. Hwang, J.C. Lee, G.S. Cho, W.K. Kim, Anti-ischemic and anti-inflammatory activity of (S)-cis-verbenol, Free Radic. Res. 44 (2010) 541–551.
[20] C. Rota, C.F. Chignell, R.P. Mason, Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescin to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements, Free Radic. Biol. Med. 27 (1999) 873–881.
[21] D. Huang, B. Ou, R.L. Prior, The chemistry behind antioxidant capacity assays, J. Agric. Food Chem. 53 (2005) 1841–1856.
[22] A. Almeida, M. Delgado-Esteban, J.P. Bolanos, J.M. Medina, Oxygen and glucose deprivation induces mitochondrial dysfunction and oxidative stress in neurones but not in astrocytes in primary culture, J. Neurochem. 81 (2002) 207–217.
[23] J.H. Lim, J.C. Lee, Y.H. Lee, I.Y. Choi, Y.K. Oh, H.S. Kim, J.S. Park, W.K. Kim, Simvastatin prevents oxygen and glucose deprivation/reoxygenation-induced death of cortical neurons by reducing the production and toxicity of 4-hydroxy-2E-nonenal, J. Neurochem. 97 (2006) 140–150.
[24] Z.A. Shah, S.E. Nada, S. Dore, Heme oxygenase 1, beneficial role in permanent ischemic stroke and in *Gingko biloba* (EGb 761) neuroprotection, Neuroscience 180 (2011) 248–255.
[25] Z.A. Shah, R.C. Li, A.S. Ahmad, T.W. Kensler, M. Yamamoto, S. Biswal, S. Dore, The flavanol (-)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway, J. Cereb. Blood Flow Metab. 30 (2010) 1951–1961.
[26] F. Zhang, S. Wang, M. Zhang, Z. Weng, P. Li, Y. Gan, L. Zhang, G. Cao, Y. Gao, R.K. Leak, M.B. Sporn, J. Chen, Pharmacological induction of heme oxygenase-1 by a triterpenoid protects neurons against ischemic injury, Stroke 43 (2012) 1390–1397.
[27] S. Saleem, H. Zhuang, S. Biswal, Y. Christen, S. Dore, Ginkgo biloba extract neuroprotective action is dependent on heme oxygenase 1 in ischemic reperfusion brain injury, Stroke 39 (2008) 3389–3396.
[28] K.F. Bell, B. Al-Mubarak, J.H. Fowler, P.S. Baxter, K. Gupta, T. Tsujita, S. Chowdhry, R. Patani, S. Chandran, K. Horsburgh, J.D. Hayes, G.E. Hardingham, Mild oxidative stress activates Nrf2 in astrocytes, which contributes to neuroprotective ischemic preconditioning, Proc. Natl. Acad. Sci. USA 108 (2011) E1–E2. author reply E3–4.
[29] D. Schubert, T. Soucek, B. Blouw, The induction of HIF-1 reduces astrocyte activation by amyloid beta peptide, Eur. J. Neurosci. 29 (2009) 1323–1334.
[30] J.H. Shin, S.W. Kim, Y. Jin, I.D. Kim, J.K. Lee, Ethyl pyruvate-mediated Nrf2 activation and hemeoxygenase 1 induction in astrocytes confer protective effects via autocrine and paracrine mechanisms, Neurochem. Int. 61 (2012) 89–99.
[31] K. Hashida, Y. Kitao, H. Sudo, Y. Awa, S. Maeda, K. Mori, R. Takahashi, M. Iinuma, O. Hori, ATF6alpha promotes astroglial activation and neuronal survival in a chronic mouse model of Parkinson's disease, PLoS One 7 (2012) e47950.
[32] B.E. Dwyer, R.N. Nishimura, S.Y. Lu, Differential expression of heme oxygenase-1 in cultured cortical neurons and astrocytes determined by the aid of a new heme oxygenase antibody. Response to oxidative stress, Brain Res. Mol. Brain Res. 30 (1995) 37–47.
[33] L. Cabell, C. Ferguson, D. Luginbill, M. Kern, A. Weingart, G. Audesirk, Differential induction of heme oxygenase and other stress proteins in cultured hippocampal astrocytes and neurons by inorganic lead, Toxicol. Appl. Pharmacol. 198 (2004) 49–60.
[34] B. Wang, W. Cao, S. Biswal, S. Dore, Carbon monoxide-activated Nrf2 pathway leads to protection against permanent focal cerebral ischemia, Stroke 42 (2011) 2605–2610.
[35] K. Deguchi, T. Hayashi, S. Nagotani, Y. Sehara, H. Zhang, A. Tsuchiya, Y. Ohta, K. Tomiyama, N. Morimoto, M. Miyazaki, N.H. Huh, A. Nakao, T. Kamiya, K. Abe, Reduction of cerebral infarction in rats by biliverdin associated with amelioration of oxidative stress, Brain Res. 1188 (2008) 1–8.