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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.

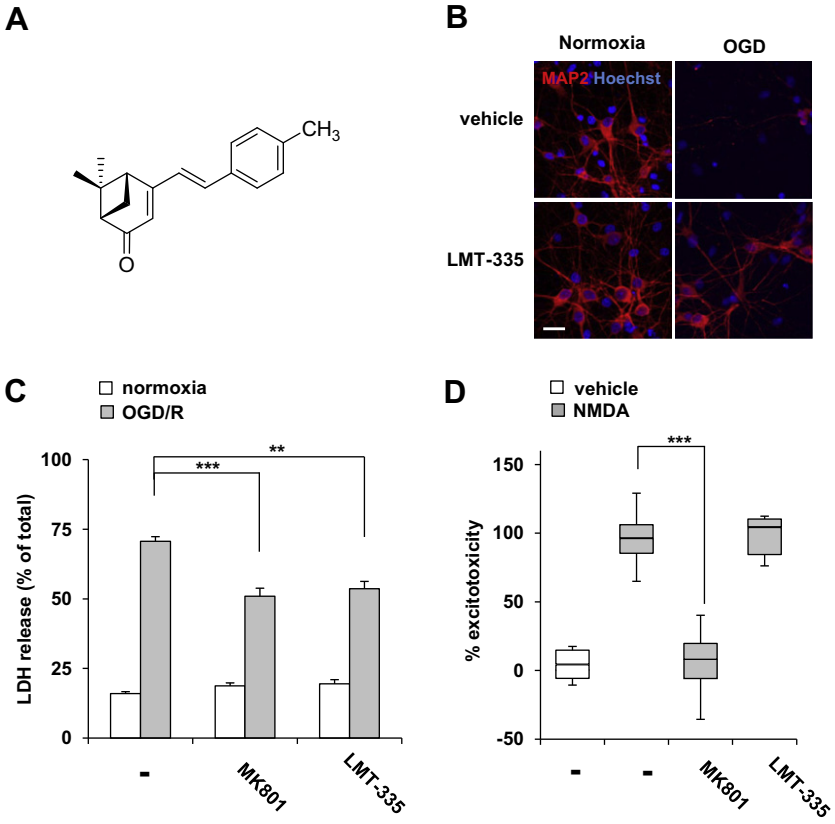

Fig. 1. Neuroprotective effects of LMT-335. (A) The structure of LMT-335. Cortical neurons were pre-treated with LMT-335 or MK-801 (10 μM each) for 30 min and further exposed to OGD/R (B and C). LMT-335 reduces OGD/R-induced neuronal injury. Neuronal cell injury or death was assessed by morphological 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.