Inhibition of immunoproteasome subunit low molecular mass polypeptide 7 with ONX-0914 improves hypoxic-ischemic brain damage via PI3K/Akt signaling

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Introduction

Hypoxic-ischemic brain damage (HIBD) can cause acute death and chronic central nervous system (CNS) damage. The prevalence of HIBD is approximately 3 per 1000 in full-term newborns; approximately 25–30% of survivors suffer from long-term sequelae, such as motor, sensory, cognitive, learning and memory impairments [1,2]. Hypothermia is the only approved therapy for neonatal HIBD in the clinic. However, the therapeutic window is limited to 6 hours after birth, and more than 40% of infants die or survive with a series of impairments despite hypothermia treatment [3]. Hypoxia-ischemia (HI) directly results in inflammation and is a major trigger of secondary brain injury [4]. Therefore, inhibiting inflammation is a promising strategy to address HIBD sequelae.

The immunoproteasome is linked to the development of inflammatory responses, and it harbors low molecular mass polypeptide 2 (LMP2/β1i), multicatalytic endopeptidase complex subunit-1 (MECL-1/ LMP10/β2i) and low molecular mass polypeptide 7 (LMP7/β5i) [5]. LMP7 expression was increased in a cerebral ischemic stroke model and autoimmune neuritis model, inflammatory responses in these conditions were alleviated by LMP7 inhibition [6,7]. Further, cytokine release was reduced by the knock-out of LMP7 in an Alzheimer’s disease mouse model [8]. Hence, LMP7 may be involved in HIBD inflammation and progression. The PI3K/Akt pathway plays a key role in regulating cell growth, inflammation, and metabolism [9]. Notably, activation of the PI3K/Akt pathway inhibits inflammation and neuronal apoptosis and stimulates neuroprotection against HI [10]. Administration of ONX-0914, a specific LMP7 inhibitor, suppresses osteoclast apoptosis via activation of the PI3K/Akt pathway [11]. However, it remains unclear whether ONX-0914 exerts its effects via the PI3K/Akt pathway in HIBD.

This study aimed to identify the specific mechanisms underpinning the involvement of LMP7 in HIBD. We hypothesized that inhibiting LMP7 with ONX-0914 would improve HIBD by alleviating inflammation via PI3K/Akt signaling in a neonatal rat model following HIBD.
Methods

Animals
Postnatal day (PND) 7 Sprague–Dawley rats weighing 15±2 g were purchased from Chengdu Dashuo Experimental Animal Company (License No: SCXK (CHUAN)2015-030, Chengdu, China); both male and female rats were used. According to the US National Institutes of Health guidelines, all procedures were approved by the Animal Ethics Committee of Chengdu Medical College (Ethics approval number: CDXY-EM-20180416). All work described in this article was carried out in accordance with the EC Directive 86/609/EEC for animal experiments.

Establishment of the hypoxic-ischemic brain damage model
A neonatal HIBD rat model was established according to the modified Rice–Vannucci method [12]. Briefly, PND7 rats were anesthetized with isoflurane. The left common carotid artery was then exposed, double ligated and cut between the ligatures. After 1 h of recovery, all rats were placed into a transparent hypoxia chamber with 8% oxygen and 92% nitrogen for 2.5 h. The weakness of the rat's left hind limb with fixed left rotation indicated that the model was successfully established. The Sham group underwent separation of the left common carotid artery without ligation and did not experience hypoxia.

Group allocation
Rats that died or those in which the model was not successfully established were excluded, the remaining rats were randomly divided into the following groups: Sham operation (Sham, N = 60), model (HIBD, N = 108), dimethyl sulfoxide (DMSO) treatment (intraperitoneally injected with 15 µL DMSO solvent, N = 30), 10 mg/kg ONX-0914 treatment (intraperitoneally injected with 15 µL ONX-0914 at a dose of 10 mg/kg, N = 6), 20 mg/kg ONX-0914 treatment (intraperitoneally injected with 15 µL ONX-0914 at a dose of 20 mg/kg, N = 30), 30 mg/kg ONX-0914 treatment (intraperitoneally injected with 15 µL ONX-0914 at a dose of 30 mg/kg, N = 6), LY294002 treatment (intraperitoneally injected with 15 µL 0.3 mg/kg of LY294002, N = 6), and ONX-0914 + LY294002 treatment (intraperitoneally injected with 15 µL 20 mg/kg ONX-0914 and 15 µL 0.3 mg/kg of LY294002, N = 6). ONX-0914 (MedChemExpress Co., Ltd.) and LY294002 (MedChemExpress Co., Ltd.) were dissolved in DMSO (Absin Bioscience Inc.) to the same volume, and the timing of injection is 2 h after the model was established.

Hematoxylin-eosin staining and immunohistochemical staining
Cerebral tissues were extracted from the rats after being anesthetized with isoflurane and euthanized at 72 h after the model was established. Brain tissues, including the left hippocampus, were fixed with 4% paraformaldehyde for 72 h and subsequently sliced into 4-µm thick coronal sections for staining. Coronal sections were stained based on the hematoxylin-eosin (H&E) staining kit protocol (Solarbio Science & Technology Co., Ltd.). Immunohistochemical (IHC) staining was performed according to the SPlink detection kit protocol (Huperzine Bridge Co., Ltd.). After antigen repair, endogenous peroxidase blocking and serum sealing, coronal sections were incubated with primary antibodies at 4°C overnight. The primary antibody dilutions were as follows: rabbit anti-LMP7 antibody (1:200; Abcam) and mouse anti-NeuN (neuronal marker) antibody (1:400; Abcam). After washing, the slices were immersed in a biotin-labeled secondary antibody for 40 min at 37°C followed by incubation with a third antibody (horseradish enzyme-labeled streptomyacin) for 40 min at room temperature. The colorimetric reaction was performed using a diaminobenzidine chromogen kit (Huperzine Bridge Co., Ltd.). Finally, hematoxylin was used for counterstaining. Images were obtained using an upright metallurgical microscope (DM4000B, Leica; Germany).

Reverse transcription-quantitative PCR
Rats in the HIBD group were euthanized at 6, 24, 48, 72 h, and 7 days postoperatively. The left hippocampus was collected for mRNA analysis. Total RNA was extracted with TRIzol reagent for cDNA synthesis and RT-qPCR analysis. Primers were designed by Sichuan Shenggong Technology Co. Ltd. and verified by NCBI Blast. The primer sequences were as follows: LMP7, 5′-TGCAATGGGACTCGCTTCT-3′ (forward) and 5′-CAAGGTTGTAGGCTCCTCA-3′ (reverse); IL-1β, 5′-AATCTGTACCTGTCCGTGGT-3′ (forward) and 5′-GAGAGGTGTCTATGTTCACTTTG-3′ (reverse); IL-6, 5′-CGCAAGAGACTTCAGCCTAG-3′ (forward) and 5′-TGCTCTGTGTGGTTGTACCTC-3′ (reverse); TGF-β, 5′-ACCACCAGGAGAGGACAC-3′ (forward) and 5′-GCCCTTGCCCTTGAGAAGACCG-3′ (reverse); Gapdh, 5′-ATGACATCCAGAGGTTGG-3′ (forward) and 5′-CATTACAAGAAGTTGG-3′ (reverse). RT-qPCR amplification was used to amplify target genes. After amplification, relative expression levels of mRNA were calculated using the 2-ΔΔCt method and normalized with reference to the gapdh gene.

Western blot analysis
The left hippocampus was collected after the rats had been euthanized. Proteins were extracted using RIPA buffer (Solarbio Science & Technology Co., Ltd.), and the concentrations were detected using the BCA protein assay kit (Solarbio Science & Technology Co., Ltd.). Protein samples (90 µg) were separated using 12% sodium dodecyl sulfate gel electrophoresis and transferred to a polyvinylidene fluoride membrane, and, subsequently, the membranes were blocked in 5% skimmed milk for 2 h. The membranes were then incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 2 h at room temperature. Primary antibodies were diluted in western
primary antibody diluent as follows: rabbit anti-LMP7 (1:500; Abcam), rabbit anti-Akt (1:800; Abcam), rabbit anti-p-Akt (1:1000; Abcam) and mouse anti-gapdh (1:2000; Proteintech). Secondary antibodies were diluted in TBST as follows; goat anti-Mouse IgG (1:5000; Proteintech) and goat anti-Rabbit IgG (1:3000; Proteintech).

ELISA assay
Whole blood was collected by cutting off the right carotid artery 72 h after model establishment. We performed anesthesia first and then cut off the common carotid artery for whole blood collection. Serum was collected after centrifugation of whole blood. Protein levels of TNF-a, IL-6 and IL-1β were detected using an ELISA kit (Meilianl Biotechnology Co., Ltd.), according to the manufacturer’s protocol.

Early behavioral testing
Three physiological reflexes of newborn rats, including the cliff aversion, negative geotaxis, and righting reflexes, were tested 1 day after model establishment (PND8) [13]. The cliff aversion reaction was used to assess the ability of rodents to respond to adverse environments. Rats were placed on the edge of a table 1 m above the ground. The time taken to spontaneously twist the body (90°) and retreat from the edge was recorded. The negative geotaxis reflex evaluates motor coordination and vestibular sensitivity. Neonates were tested by placing them prone on an inclined plane (40° inclination). The time taken for reorientation was recorded. The righting reflex test evaluates the tendency to regain former body position after displacement. Rats were placed supine, and the time taken to move into the prone position with the front and back paws on the ground was recorded. If the time taken for the cliff aversion or negative geotaxis reflexes exceeded 20 s, the results were excluded [13].

Morris water maze test
The Morris water maze (MWM) test was used to assess hippocampal-dependent spatial learning and memory [14]. A circular pool (160 cm in diameter) was filled with opaque water (25.0 ± 1.0°C, covering the platform by a depth of 1 cm). A camera connected to a video tracking system was placed above the pool as a recorder. Rats were weaned 21 days after successfully establishing the model and separated from their mothers (PND8–PND28); they were then fed with fodder for 7 days (PND29–PND35). The MWM was conducted between PND36 and PND40. Improvements in spatial learning and memory were represented by a decrease in path latency to reach the platform. The rats were continuously trained four times a day with 30 s intervals each time for a total of 4 days. The time taken to find the platform from the different quadrants was recorded, which was the incubation period of escape to the platform. The average of the four results was considered as the final result. At PND40, the platform was removed.

Swimming trajectories of rats within 120 s and the number of platform crossings from any entry point were recorded.

Statistical analyses
SPSS 23.0 software was used to analyze the data. GraphPad Prism 7 software was used to generate graphs. Student’s t-test was just used for two-group comparisons. One-way ANOVA was used to evaluate the significant difference among multiple groups, Two-way ANOVA was used to evaluate the significant difference among multiple groups in MWM, and Turkey test was used for test comparisons after ANOVA. P < 0.05 was considered statistically significant.

Results

Hypoxia-ischemia triggered neuroinflammation
Compared to those in the Sham group, cellular arrangement in the left hippocampal CA1 and DG regions in the HIBD group was irregular, cellular outline and morphology were abnormal and nucleoli were obscure with deviation and fragmentation (Fig. 1a,b). In addition, there was a significant increase in the mRNA level of TNF-a, IL-1β and IL-6 in brain tissues in the HIBD group compared to the Sham group (P < 0.05; Fig. 1c), the protein level of TNF-a, IL-1β and IL-6 in blood serum also increased (P < 0.01; Fig. 1d–f). Those results indicate that the HIBD model was established successfully, and inflammatory responses occurred in HIBD rats.

LMP7 expression was increased in the hippocampus of hypoxic-ischemic brain damage rats
As shown in IHC staining, the number of LMP7 immunopositive cells was increased in the left hippocampal CA1 and DG regions in the HIBD group compared to that in the Sham group (Fig. 2a,b). RT-qPCR (Fig. 2c) and western blot (Fig. 2d) showed that compared with the Sham group, LMP7 was highly expressed in the left hippocampus of HIBD rats 6 h after HI, and the expression increased gradually and reached a peak at 72 h (P < 0.05).

LMP7 inhibition with ONX-0914 improved hypoxic-ischemic brain damage by reducing inflammation
LMP7 and proinflammatory cytokine expression were considerably decreased in the ONX-0914-treated group compared to that in the DMSO-treated group. The lowest expression was observed with an ONX-0914 dose of 20 mg/kg (Fig. 3). The cellular arrangement in the left hippocampal CA1 and DG regions was regular in the ONX-0914-treated group compared to that in the DMSO group, the number of normal cells was increased, and the nucleoli was clear without deviation (Fig. 4a,b). IHC staining (Fig. 4c,d) revealed the loss of NeuN-positive (NeuN+) cells was decreased after ONX-0914 treatment. HIBD rats exhibited prolonged undefined cliff aversion, geotaxis and righting reflexes compared to the Sham group. The reflection time was shorter in the
ONX-0914-treated group than in the DMSO-treatment group ($P < 0.05$; Fig. 5a). The MWM results revealed that the latency to find the platform was longer in the HIBD group than the Sham group, and it was shorter in the ONX-0914 group than the DMSO group ($P < 0.05$; Fig. 5b). The swimming trajectory of HIBD rats was disordered compared to the Sham group, and rats in the ONX-0914-treated group exhibited a more disordered swimming trajectory than the DMSO group (Fig. 5c). After HIBD, the number of times the rats crossed the platform within 120 s was reduced, while it was increased when rats were treated with ONX-0914 ($P < 0.01$; Fig. 5d).

Inflammatory responses in HIBD rats. Compared to those in the Sham group, cellular arrangement was irregular, cellular outline and morphology were abnormal, and nucleoli were obscure with deviation and fragmentation in CA1 (a) and DG (b) regions of rats in the HIBD group (H&E staining; 200× and 400× magnification; $N = 6$ slices per group, 1 slice per rat × 6 rats = 6 slices total). There was a significant increase in TNF-α, IL-1β and IL-6 mRNA (c) and protein (d–f) in the HIBD group compared to the Sham group ($N = 6$ per group, data are presented as mean ± SD, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. the Sham group). H&E, hematoxylin and eosin; HIBD, hypoxic-ischemic brain damage.
ONX-0914 improved hypoxic-ischemic brain damage possibly via PI3K/Akt signaling

Western blot analysis and RT-qPCR revealed that p-Akt expression in the left hippocampus of the HIBD group was higher than that in the Sham group; treatment with ONX-0914 further increased p-Akt expression, while reduced by LY294002 inhibited PI3K/Akt signaling (Fig. 6a). As shown in Fig. 6b, the time of undefined cliff aversion, geotaxis, and righting reflexes in ONX-0914 group rats was prolonged by LY294002. Furthermore, the expression of proinflammatory cytokines in the ONX-0914 group was increased by LY294002 (P < 0.05; Fig. 6c–e).

Discussion

This study demonstrated that LMP7 inhibition by ONX-0914 prevented inflammation in HIBD neonatal rats and improved neurological recovery and indicated that the PI3K/Akt pathway exerts these effects. This is the first study to report that LMP7 inhibition with ONX-0914 ameliorates pathological damage, prevents inflammatory responses, and improves neurological function in HIBD rats, possibly via the PI3K/Akt pathway. Our findings highlight the potential therapeutic effect of LMP7 in neonatal HIBD.

The hippocampus is vulnerable to HI damage, especially in the CA1 and DG regions [15]. In this study, we observed that cells in the ipsilateral hippocampal CA1 and DG regions of HIBD rats were damaged, and the mRNA expression in brain tissues and the protein expression in blood serum of proinflammatory cytokines (TNF-α, IL-1β and IL-6) was significantly increased. Previous studies have reported that microglia in the brains of HIBD rats are rapidly activated to generate proinflammatory cytokines, which exacerbate HI injury by inducing neuronal apoptosis, increasing toxic nitric oxide levels, augmenting blood–brain barrier permeability, and inhibiting neuronal cell regeneration [16,17]. These results demonstrate that the HIBD rat model was established successfully and that HI triggers neuroinflammation. Studies have shown that HI causes acute neuronal injury in the brain, releasing toxic signaling molecules, such as LMP7 involvement in HIBD pathogenesis. The number of LMP7 immunopositive cells was increased in the left hippocampal CA1 (a) and DG (b) regions in the HIBD group compared to that in the Sham group (IHC staining; 200× and 400× magnification; N = 6 slices per group, 1 slice per rat × 6 rats = 6 slices total; the arrows pointing to brown staining indicate LMP7-positive cells). Compared with the Sham group, LMP7 mRNA (c) (N = 6 per group) and protein (d) (N = 6 per group) were highly expressed in the left hippocampus of HIBD rats 6 h after HI, and the expression increased gradually and reached a peak at 72 h (data are presented as mean ± SD, P < 0.05 vs. the Sham group). HIBD, hypoxic-ischemic brain damage; IHC, immunohistochemical.
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Interleukin-33, high mobility protein B1 and ATP, activating pattern recognition receptors on the surface of microglia, astrocytes, vascular endothelial cells and perivascular macrophages, and initiating downstream proinflammatory cascade. Proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, were released into the blood [4,18,19], which is consistent with our study’s results.

In eukaryotes, proteins are degraded mainly by UPS, and about 80% of them are cleared by its recognition. In UPS, proteasome subunit β1, β2 and β5 determine the protein degradation efficiency. Under certain special stimulation, the immune subunit βi, β2i and β5i (low molecular weight Polypeptide 7, LMP7) replace the original β1, β2 and β5 subunits and assemble into a complex called

ONX-0914 reduces inflammatory responses. ONX-0914-treatment considerably decreased the expression of LMP7 protein (a) and mRNA (b) in the DMSO-treated group, also decreased the expression of proinflammatory cytokine (TNF-α, IL-1β and IL-6) mRNA (c–e) and protein (f,g), the lowest expression was observed with an ONX-0914 dose of 20 mg/kg (N = 6 per group, data are presented as mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.005 vs. the Sham group; *P < 0.05, **P < 0.01 vs. the DMSO group).

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immunoproteasome. Among them, LMP7 has a unique function and the strongest activity [20,21]. LMP7 inhibition is thought to be an effective method for alleviating neuroinflammation, such as that seen in autoimmune neuritis [7] and Alzheimer's disease [8]. In this study, LMP7 expression was visibly upregulated in the left hippocampus of HIBD model rats, and the number of LMP7-positive cells was also increased, which further supports the idea that LMP7 plays a role in HIBD inflammation and progression. ONX-0914 is the first proteasome inhibitor described as selective for the chymotrypsin-like subunit of the immunoproteasome. Exposure to ONX-0914 in vitro and in vivo blocked the presentation of LMP7-specific epitopes (UTY246-254 and GP33) and downregulated the expression of LMP7 [22]. It has been found to play a neuroprotective role in various experimental models [6,23]. In this study, ONX-0914 ameliorated pathological damage in the hippocampus downregulated
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The expression of mRNA expression in brain tissues and the protein expression in blood serum of proinflammatory cytokines and prevented inflammatory responses. To evaluate the influence on neurofunction, an early behavioral test was done 1 day after the model was established. It is a specific method to evaluate the ability of rodents to respond to adverse environments, motor coordination and vestibular sensitivity, and the tendency to regain former body position after displacement. We also conducted MWM between PND36 and PND40 to assess hippocampal-dependent spatial learning and memory. As shown in our experiment, the time of cliff aversion, negative geotaxis and righting reflexes were shortened by ONX-0914-treatment, the ability of learning and memory also improved by ONX-0914-treatment. All these results prompted that ONX-0914 can enhance behavioral performance in neonatal rats with HIBD. Our findings indicate that LMP7 inhibition, such as that induced by exogenous ONX-0914 treatment, inhibits the production of LMP7, downregulates its expression, is a promising strategy for improving HIBD sequelae.

Accumulating studies have indicated that the PI3K/Akt pathway is linked to the development of CNS diseases, such as subarachnoid hemorrhage brain injury [24] and ischemia/reperfusion injury [25]. In our study, the PI3K/Akt signaling pathway inhibitor LY294002 was used in neonatal rats with HIBD to validate the neuroprotective mechanisms of LMP7. We found that ONX-0914 activated the PI3K/Akt signaling pathway. However, inhibiting the PI3K/Akt pathway with LY294002 abolished the protective effects of ONX-0914, which manifested as increased proinflammatory cytokine levels and prolonged time of early behavioral testing. Previous studies have demonstrated that activation of the PI3K/Akt signaling pathway attenuates inflammatory diseases, such as rheumatoid arthritis [26] and mesangial proliferative glomerulonephritis [27]. This evidence suggests that ONX-0914 improves HIBD, possibly via PI3K/Akt signaling.

In summary, our findings indicate that LMP7 aggravates brain injury by triggering inflammatory responses in HIBD rats, and LMP7 inhibition by ONX-0914 exerts...
LY294002 attenuates the protective effect of ONX-0914 on the brain of HIBD rats. ONX-0914 improves HIBD possibly via the PI3K/Akt signaling pathway. The expression levels of p-Akt protein in the left hippocampus of rats were increased after HI, treatment with ONX-0914 further increased p-Akt expression, while reduced by LY294002 inhibited PI3K/Akt signaling (a). The time of undefined cliff aversion, geotaxis, and righting reflexes in ONX-0914 group rats was prolonged by LY294002. The expression of proinflammatory cytokines in ONX-0914 group was increased by LY294002 (N = 6 per group; data are presented as mean ± SD. *P < 0.05, **P < 0.01 vs. the Sham group; †P < 0.05, ††P < 0.01 vs. the DMSO group). HIBD, hypoxic-ischemic brain damage.

Acknowledgements
We would like to thank Editage (www.editage.cn) for English language editing.

This work was supported by the Applied Basic Research Programs of Science and Technology Department of Sichuan Province [grant number 2018]JY0301], the Foundation of Chengdu Medical College [grant numbers CYTD18-06], and the Foundation of Development and Regeneration Laboratory [grant numbers SYS19-05].

Conflicts of interest
There are no conflicts of interest.

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