Original Article

Neuroprotection of histone deacetylase inhibitor TMP269 in cerebral ischemia/reperfusion rat

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Abstract: Ischemia stroke is an important clinical problem with few efficient treatments. Histone deacetylase has been considered to be associated with ischemic stroke. Studies have identified a variant in HDAC9 associated with large-vessel ischemic stroke. Therefore, HDAC9 inhibitors may function as therapeutic drugs to prevent neurological ischemia. It was also reported that HDAC9 contributed to brain microvessel endothelial cell injury, through increasing inflammatory responses and reducing the expression of tight-junction proteins including occludin. TMP269 is a selective HDAC inhibitor, but it remains poorly characterized in field of stroke. Therefore, we tested TMP269 in a rat model of transient middle cerebral artery occlusion (tMCAO). Sprague-Dawley rats (male, 250-280 g) were randomly assigned to three groups: ischemia/reperfusion (I/R, n=22), ischemia/reperfusion with TMP269 treatment (TMP269, n=23), and sham surgery (sham, n=18). TMP269 was injected one hour before induction of ischemia. Rats were evaluated for neurological functional outcomes 24 hours after MCAO (n=6/group), and then were sacrificed for HDAC9, and occludin protein quantification by Western blot. Other rats were sacrificed to assess infarct volume and edema rate by 2,3,5-triphenyltetrazolium chloride (TTC) staining (n=6/group), and to investigate histological changes by hematoxylin and eosin (H&E) staining (n=6/group). The results showed that TMP269 improved neurological status and infarct volume, increased occludin protein levels after MCAO, suggesting that TMP269 may provide neuroprotection.

Keywords: Histone deacetylase, inhibitor, TMP269, ischemia/reperfusion, neuroprotection

Introduction

Stroke is a most common disease affecting the quality of life worldwide [1]. Each year, about 15 million people experience a stroke [2]. Any treatment that could decrease the incidence of this disease would have significant economic and patient benefits. The determination of the pathogenesis of cerebral ischemia and the development of better therapeutic targets to prevent ischemia is current challenges in neurology. Increased understanding of epigenetic mechanisms such as histone modifications, DNA methylation, chromatin remodeling, and regulation by non-coding RNAs (ncRNAs) have attracted increasing attention in the pathogenesis of stroke [3]. Of these factors, histone deacetylase (HDACs)-mediated epigenetic mechanisms seem to play an essential regulatory role.

HDACs are a group of enzymes that act in the homoeostasis of histone acetylation and gene transcription [4, 5]. HDACs are categorized into four classes based on structure, function, and similarity to yeast orthologues, including class I HDACs (HDAC1, 2, 3, and 8), class II HDACs (HDAC4, 5, 6, 7, 9, and 10), class III HDACs, and class IV HDAC (HDAC11) [6-9].

Previous studies have showed the regulating effect of HDAC9 on ischemia. HDAC9 is a member of the class IIa HDACs (HDAC4, 5, 7, 9), and is ubiquitously expressed, with high levels of expression in cardiac tissue, muscle, and the brain [10]. HDAC9 is involved in lipid metabolism, glucose metabolism, and angiogenesis [11-13]. A recent Genome-Wide Association study (GWAS) study [14] reported that HDAC9 was associated with large-vessel ischemic stroke [14]. Shi et al observed that HDAC9 was significantly up-regulated in the ischemic brain [15]. This study also demonstrates that HDAC9 contributes to endothelial cell injury and indicates that HDAC9 is a vital component of a signal transduction pathway in ischemic cerebral
injury, suggesting the potential of HDAC9 inhibitors (HDACis) to serve as new therapeutic intervention strategies for ischemic stroke.

Over the past decades, many HDACis related to neurological diseases have been extensively researched. The best-studied HDACis in experimental ischemic stroke are small molecular compounds with high blood brain barrier permeability. Either pretreatment or post injury administration with these compounds has been proved to reduce infarct volume in experimental animal stroke models [16-18]. However, available HDACis may inhibit multiple HDAC proteins. TMP269 [19] is a novel and selective class IIa histone deacetylase inhibitor with measured IC50s of 126/80/36/9 nM for HDAC 4/5/7/9, respectively. Based on this, we predicted that TMP269 should be able to inhibit HDAC9 at low concentrations, but there were no previous reports testing TMP269 in vivo.

In the present study, we tested TMP269 in a rat model of middle cerebral artery occlusion. We hypothesized that TMP269 would reduce neurological damage during cerebral ischemia.

Materials and methods

Animals and experimental groups

The animal experimental protocols were based on the guidelines of the Committee for the Care and Use of Laboratory Animals at Fudan University. Healthy male Sprague-Dawley rats (Jiesijie Lab Animal Ltd, Shanghai, China) were used, weighing 250-280 g. They were housed at 24°C with free access to water and standard rat chow. Animals were randomly assigned to 3 experimental groups: sham surgery, ischemia/reperfusion (I/R), or ischemia/reperfusion with TMP269 treatment (TMP269). TMP269 (4 mg/kg, Selleck (China)) was administered by intra-peritoneal injection 1 hour before ischemia.

Middle cerebral artery occlusion (MCAO)

Ischemia was induced by left middle cerebral artery occlusion (MCAO), as previously described [20]. In brief, rats were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.), and body temperature was maintained at 37°C by using a homoeothermic blanket. A nylon monofilament with a silicone tip (Beijing Cinontech Co.LTD) was advanced from the external carotid artery into the lumen of the internal carotid artery. After 90 minutes of cerebral ischemia, the monofilament was withdrawn to allow reperfusion. After 24 hours of reperfusion, the rats were anaesthetized again and then sacrificed. In the sham group, rats underwent the same surgical procedure but without MCAO. Once animals recovered from anesthesia, they were scored based on a four-point scale [21] as follows: 0, no neurological symptoms; 1, unable to completely extend the front contralateral paw; 2, rotating while crawling and falling to the contralateral side; 3, unable to walk without assistance; and 4, unconsciousness (did not recover). Rats with scores of 1-3 points were considered successful models and were included in the study. When the procedures were finished, the animals were returned to their cages and again allowed free access to food and water. Overall, the number of mice used in this study for data collection and analysis was six per experimental treatment.

Evaluation of neurological status

The final neurological status was assessed in rats 24 h after MCAO in a blinded fashion by using a seven-point scale [22] as follows: 0, no deficit; 1, failure to extend right forepaw fully; 2, decreased grip of the right forelimb when held by tail; 3, spontaneous movement in all directions, but torso turning to right when held by tail; 4, circling or walking to the right; 5, walking only in response to tactile stimulation; 6, no spontaneous activity; and 7, dead. The person who made the evaluation was blinded to group assignment.

Beam-walking test

We used the beam-walking test to assess coordination and integration of motor movement in rats after ischemia. The rats were trained for 3 days to traverse the beam before ischemia induction and were tested the day before surgery to establish a baseline measure. The animals were next tested 24 h after ischemia induction. The beam-walking apparatus consisted of a square beam (2.5 cm × 140 cm × 42 cm) connected to a box (40 cm × 25 cm × 25 cm). The performance of the beam-walking task was video recorded and 3 trials were recorded for each analysis. Performance was rated as follows [23]: the rat was not able to stay on the beam, 0 points; the rat did not move, but was able to stay on the beam, 1

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point; the rat tried to traverse the beam, but fell, 2 points; the rat traversed the beam with more than 50% footslips of the affected hindlimb, 3 points; the rat traversed the beam with more than one footslip, but less than 50%, 4 points; the rat had only one slip of the hindlimb, 5 points; the rat traversed the beam without any slips of the hindlimb, 6 points. The person who made the evaluation was blinded to group assignment.

Quantification of lesions

Cerebral infarction volumes were measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Rats (n=18, 6 per group) were anesthetized with 10% chloral hydrate, and the brains were dissected, washed in phosphate buffered saline (PBS), incubated at -20°C for 5 minutes, then sliced into 6 coronal sections of 2 mm thickness. Sections were immediately placed into 2% TTC (Sigma Co.Ltd.) in PBS at 37°C for 30 minutes. After staining, sections were photographed using a digital camera (DSC-TX10, Sony, Japan), and infarct size was calculated based on the photographs. To minimize the effect introduced by brain edema, an indirect method was used to calculate infarct volumes [24]: ipsilateral infarct volume/volume of the contralateral hemisphere × 100%. Cerebral edema was counted from sections stained by TTC according to the formula [25]: [(ipsilateral volume-contralateral volume)/contralateral volume] × 100%.

Hematoxylin and eosin staining

After 24 h of reperfusion, the brains were removed and fixed in 4% paraformaldehyde overnight at 4°C. After paraffin embedding of coronal sections (4 μm in thickness), the sections were stained with hematoxylin and eosin (H&E). Morphological features of the brain damage under microscope, comparing with changes in the gross specimen were observed through a light microscope (Olympus) at a magnification of × 200 and then documented by digital photography.

Western blotting

Ipsilateral cortical tissue surrounding the ischemic zone was harvested after the animals were sacrificed. Cortical protein extracts and Western blotting analysis were performed as previously described [26]. Protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filter (NC) membranes. After blocking for 2 hours, the membranes were incubated overnight with primary antibody in 5% skim milk powder. Membranes were then washed and incubated with secondary antibodies for 1 hour at room temperature. Finally, an Electro-Chemi-Luminescence (ECL) kit was used to treat the membranes and develop the signal, which was detected using Alpha Innotech (Bio-Rad). The following primary antibodies were purchased: rabbit polyclonal IgG anti HDAC9 antibody (Abcam), mouse monoclonal anti-β-actin antibody (Abcam), mouse polyclonal IgG anti occludin antibody (Invitrogen Zymed).

Statistical analysis

Statistical analyses were performed using SPSS 17.0 statistical software, (SPSS Company, USA). Group differences were analyzed using a one-way analysis of variance (ANOVA) followed by post hoc tests. Statistical significance of the physiological variables was determined with an independent sample t-test. P
values < 0.05 were considered statistically significant differences.

**Result**

*Expression of the HDAC9 protein*

The protein expression of HDAC9 was measured by western blot. As shown in Figure 1, compared with the sham group, the expression of HDAC9 was increased significantly in both the I/R group (p < 0.05, n=6) and the TMP269 group (p < 0.05, n=6). However, the level of HDAC9 in the tissue from the TMP269 group was lower than the samples from the I/R group (P < 0.05, n=6). This indicated that TMP269 treatment could suppress the increased expression of HDAC9 induced by ischemia-reperfusion injury.

**Neurological status**

24 h after MCAO, the neurological status of the rats was assessed using a 7-point scale. As shown in Figure 2A, the neurological status scores at 24 h after reperfusion in the sham group were about zero, indicating that there was no neurological deficit. After undergoing cerebral ischemia and reperfusion, the rats were impaired (I/R group, 4.33±0.34), and rats in the TMP269 group showed significant improvement (3±0.26) relative to rats in I/R group (P < 0.05, n=6). In addition to the 7-point assessment, to investigate the neurological
outcomes, we used a beam-walking test to assess the deficits in coordination and integration of motor movement in the rats after ischemia. The results of the test are presented in Figure 2B. The beam-walking test showed significant impairment for both the TMP269-treated rats and I/R-treated rats when compared to the sham-treated rats (5.67±0.21) (P < 0.05, n=6). Treatment with TMP269 (1.00±0.37) improved performance for the beam-walking test when compared to the I/R-treated rats (2.17±0.31) at 24 h after ischemia (P < 0.05, n=6).

Correlation of HDAC9 with neurological deficits

The possible correlation of the relative expression of HDAC9 and neurological deficits was assessed by Pearson's correlation coefficient (R). The expression of HDAC9 was positively correlated with the scores of neurological deficits (Figure 2C). These results implied that there might be an association or even a positive correlation between the expression of HDAC9 and neurological deficits.

Cerebral infarct volume and edema rate

Rats were sacrificed to determine the infarct volume by 2,3,5-triphenyltetrazolium chloride (TTC) staining (Figure 3). The white zone in brain tissues represented damaged brain areas resulting from ischemia, and the red zone represented normal brain tissue (Figure 3A). Sham-operated rats exhibited no ischemia (not shown). The I/R group exhibited cerebral infarct volume of about 47.70±4.36%, but the TMP269 treatment group had decreased infarct volume, 35.00±1.38% (P < 0.05; n=6; Figure 3B). Compared with the I/R group, the brain edema rate was significantly reduced in the TMP269 group (P < 0.05, n=6; Figure 3C).

Hematoxylin and eosin staining

After H&E staining, the cortex was observed by light microscope (Figure 4). The brains of the sham group showed no evident morphological changes (Figure 4A). Obvious deformation and swelling of the nerve cells, interstitial edema, and tissue damage occurred in the cortex region of I/R group (Figure 4B), but TMP269 administration ameliorated the damage caused by reperfusion injury (Figure 4C).

Expression of the occludin protein

The protein expression of occludin was measured by western blot. As shown in Figure 5, compared with the sham group, the expression of occludin was decreased significantly in the I/R group (p < 0.05, n=6). However, the level of occludin from the TMP269 group was higher than the samples from the I/R group (P < 0.05, n=6).

Discussion

Currently, ischemia induced by MCAO is widely used to investigate the effects of pre- and post-
Protective effect of TMP269 in I/R conditioning on focal cerebral ischemia in experimental animals. As a pre-conditioning procedure, TMP269 was administered an hour before MCAO. In TMP269 treated rats, this treatment provided neuro-protection against brain ischemic injury as indicated by neurological status scores and beam-walking test. TTC staining revealed that TMP269 significantly reduced the cerebral infarct volume and alleviated brain edema. HE staining also revealed the protective effect of TMP269.

HDAC9, a member of the class IIA HDAC proteins, was highly expressed in the brain and skeletal muscle. Emerging evidence has linked HDAC9 to neuronal physiology and pathology [27-29]. Western blot analysis demonstrated that HDAC9 was up-regulated in the ischemic cerebral hemisphere after cerebral I/R injury in rats, in agreement with the previous experiment [15]. Moreover, we found that positively correlated expression of HDAC9 with the neurological deficits. Higher expression of HDAC9 corresponded to more severe neurological deficits. Thus, excessive HDAC9 after stroke is deleterious, and treatments that suppress this effect could present neuroprotective effects against brain injury after MCAO.

Interestingly, we also found that the administration of TMP269 before ischemia injury inhibited the expression level of HDAC9 compared to the I/R group (Figure 3). One potential mechanism for this inhibition is that TMP269 may increase the acetylation level of HDAC9, making it more easily degradable, resulting in decreased levels in the TMP269 group. However, the detailed mechanism of TMP269 action was not determined in this study.

Evidence demonstrates that HDAC9 contributes to brain microvessel endothelial cell injury and the integrity of the blood brain barrier (BBB) [15]. Our previous study showed that HDAC9 regulates ox-LDL-induced endothelial cell apoptosis by participating in inflammatory reactions [30]. Those results indicate that HDAC9 plays an important role in endothelial function. In our study, we found that the administration of TMP269 before ischemia injury elevated the expression level of occludin compared to the I/R group. Occludin was key structural protein sealing the blood brain barrier (BBB) and forming tight junctions [31, 32] and they are highly enriched in brain capillaries.

Figure 4. Pathological brain tissue changes after cerebral I/R. H&E staining showed that neurons had normal morphological features in the sham-operated group. The images of the I/R group showed multiple vacuolated interspaces (arrow). TMP269 administration ameliorated the damage caused by reperfusion injury.

Figure 5. Western blot analysis of occludin in the cortex ipsilateral to MCAO. Compared with the sham group, the expression of occludin was decreased significantly in the I/R and groups. The level of occludin in the TMP269 group was higher than the level in the I/R group. Data represent the mean ± SEM (n=6). *P < 0.05 versus I/R group.
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When BBB permeability increased, expression levels of occludin may decreased. Thus, our result suggested that TMP269, a HDAC inhibitor, may protect brain function by decreasing the permeability of BBB. However, further study is needed to verify this point of view.

This study is the first report of the use of TMP269 in animals. Here, we did not verify whether the acetylation level was decreased after using TMP269. However, the rats' behavioral outcomes, infarct volume, and brain edema were better when treated with TMP269. Importantly, the mobility of rats in the TMP269 group indicated little drug toxicity when used as treatment before ischemia/reperfusion injury. Thus, we considered that TMP269 confers a neuroprotective effect for ischemia/reperfusion injury in rats.

In conclusion, this study demonstrated that administration of TMP269 one hour prior to ischemia resulted in better outcomes in rats.

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Disclosure of conflict of interest

None.

Abbreviations

HDAC9, Histone deacetylase 9; tMCAO, transient middle cerebral artery occlusion; I/R, ischemia/reperfusion; TTC, 2,3,5-triphenyltetrazolium chloride; H&E, hematoxylin and eosin.

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