Histone Deacetylase 3 Inhibition Decreases Cerebral Edema and Protects the Blood-Brain Barrier after Stroke

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Research Article

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Abstract

Introduction: We have previously shown that selective inhibition of histone deacetylase 3 (HDAC3) decreases infarct volume and improves long-term functional outcomes after stroke. In this study, we examined the effects of HDAC3 inhibition on cerebral edema and blood-brain barrier (BBB) leakage and explored its underlying mechanisms.

Methods: Adult male Wistar rats were subjected to 2-h middle cerebral artery occlusion (MCAO) and randomly treated i.p. with either vehicle or a selective HDAC3 inhibitor (RGFP966) at 2 and 24 hours after stroke. Modified neurological severity scores (mNSS) were calculated at 2 hours, 1 day, and 3 days. H&E and Evans blue dye (EBD) assay were employed to assess cerebral edema and BBB leakage, respectively. Western Blot for matrix metalloproteinase-9 (MMP9) and immunostaining for HDAC3, GFAP, Iba-1, albumin, aquaporin-4, claudin-5, ZO-1, and NF-kappa were performed.

Results: Early RGFP966 administration decreased cerebral edema ($p=0.002$) and BBB leakage, as measured by EBD assay and albumin extravasation ($p=0.001$). RGFP966 significantly increased the expression of tight junction proteins (claudin-5 and ZO-1) in the peri-infarct area. RGFP966 also significantly decreased HDAC3 expression in GFAP+ astrocytes, which correlated with better mNSS ($r=0.67$, $p=0.03$) and decreased cerebral edema ($r=0.64$, $p=0.04$). RGFP966 decreased the expression of aquaporin-4 in GFAP+ astrocytes ($p=0.002$), as well as, the inflammatory markers Iba-1, NF-kappa, and MMP9 in the ischemic brain ($p<0.05$).

Conclusions: Early HDAC3 inhibition decreases cerebral edema and BBB leakage. BBB protection by RGFP966 is mediated in part by the upregulation of tight junction proteins, downregulation of aquaporin-4 and HDAC3 in astrocytes, and decreased neuroinflammation.

Introduction

Stroke is a major cause of serious disability and a leading cause of death in the United States [1]. Ischemic stroke triggers complex pathophysiological events that result in brain damage and cerebral edema [2]. Malignant cerebral edema is one of the most devastating complications of ischemic stroke, with a mortality rate of up to 80% in untreated patients [3]. Decompressive hemicraniectomy is a lifesaving procedure for patients with malignant brain edema [4]. However, hemicraniectomy carries significant morbidity and mortality and is often considered late in the clinical course of brain edema, as a last resort. Thus, there is an unmet need for new treatment options that can help prevent or slow the development of malignant edema after stroke [5].

The cascades of events after acute ischemic stroke that lead to increased blood-brain barrier (BBB) permeability and cerebral edema formation are diverse [6, 7] and include disruption of tight junction(TJ) proteins [8], upregulation of glial water channel aquaporin-4 (AQP4) [9], and an inflammatory response involving activation of microglia [10] and upregulation of matrix metalloproteinase-9 (MMP-9)[11] and NF-kappa [12].

Emerging evidence suggests that epigenetic regulation plays an important role in cerebral edema [13]. Posttranslational modifications to chromatin (eg, acetylation) have profound effects on gene expression, and thus provide a multifaceted mechanism for regulation. Histone deacetylases (HDACs) are a superfamily of chromatin-modifying enzymes that silence transcription through modification of histones by the removal of acetyl groups [14, 15]. Stroke induces a global reduction in acetylation levels of histones H3 [16–20] and H4 [21] in the ischemic brain. Histone deacetylase 3 (HDAC3) is the most highly expressed class I HDAC in the brain [22, 23]. We have previously shown that selective inhibition of HDAC3 decreases infarct volume and improves long-term functional outcomes after stroke [24]. In this study, we examined the effects of HDAC3 inhibition on cerebral edema and BBB leakage and explored its underlying mechanisms.

Materials And Methods

All experiments were strictly conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center.

Drug Preparation and Properties: RGFP966 (a selective HDAC3 inhibitor) was purchased from Biorbyt (Cambridge, UK). RGFP966 is an N-(o-aminophenyl)carboxamide HDAC inhibitor [25–27]. With systemic administration, the distribution of RGFP966 to the CNS is relatively efficient, with a brain: plasma ratio of 0.45 [28]. A substrate-dependent biochemical assay using recombinant human HDACs found that RGFP966 is a specific inhibitor for HDAC3, with an IC_{50} of 0.08 μM and no effective inhibition of any other HDACs at concentrations up to 15 μM [28]. With systemic administration of 10 mg/kg RGFP966, the maximum drug concentration (Cmax) in the brain is 3.15 μM; thus, RGFP966 at the dose used in this study is a specific inhibitor of HDAC3 in vivo [28].

Animal Middle Cerebral Artery Occlusion (MCAO) Model

As previously described [29], adult male Wistar rats (270–300 g, 2–3 months) were anesthetized with isoflurane, administered via a precision vaporizer in oxygen (3.5-5% for induction, followed by 1.5% for maintenance). The analgesic Buprenorphine SR 1.2 mg/kg was administrated subcutaneously before surgery. Body temperature was maintained at 37 ± 0.5°C throughout the surgical procedure using a heating pad and a feedback-regulated water heating system. A 4 − 0 nylon suture with its tip rounded by heating near a flame was inserted into the external carotid artery (ECA) through a small puncture. The nylon suture, whose length was determined by the animal's weight, was gently advanced from the ECA into the lumen of the internal carotid artery (ICA) until the suture blocked the origin of the middle cerebral artery (MCA). The nylon suture was retained inside the ICA for 2 hours and the neck incision was closed. The animals were moved to their cage to awaken. Animals were re-anesthetized with isoflurane after 2 hours, and restoration of blood flow was performed by the withdrawal of the suture until the tip cleared the lumen of the ECA. The incision was then closed.

Experimental Groups

To examine the effects of HDAC3 inhibition on cerebral edema and BBB leakage, adult male Wistar rats was subjected to 2-h MCAO, and randomly selected animals were treated i.p. with either vehicle (1% Tween 80) or a selective HDAC3 inhibitor (RGFP966, 10 mg/kg) at 2 and 24 h after MCAO. The dose of RGFP966 used in this study (10 mg/kg) was selected based on our previous study [24] and other studies demonstrating proper concentrations in the brain [28, 30]. Before each administration, RGFP966 was freshly dissolved in 1% Tween 80. Rats were sacrificed 3 days after 2-h MCAO for histological and immunohistochemistry analysis (n = 6/group) and at 48 hours for EBD and Western blot assays (n = 4/group).

Modified Neurological Severity Score (mNSS) and Inclusion and Exclusion Criteria: mNSS is a composite of motor, sensory, balance, and reflex tests. mNSS is graded on a scale of 0 to 18 (normal score: 0; maximal deficit score: 18). One point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe is the injury [31, 32].

mNSS was employed as a prespecified severity inclusion and exclusion criteria [33]. Specifically, if the animal's score was mNSS 5 or more after MCAO surgery, it was included in the randomization. Animals that scored less than 5 were excluded. For each experimental animal, mNSS was performed before MCAO, at 2 hours, 1 day, and 3 days after MCAO.

Tissue Preparation for Immunohistochemistry

Animals were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) via i.p. injection. The animals were then subjected to cardiac puncture and perfused with saline followed by 4% paraformaldehyde (4% PFA) via a needle inserted into the left ventricle of the heart. The brains were removed, fixed in 4% PFA overnight, and then embedded in 20% sucrose for 2 days. Using a rat brain matrix (Braintree Scientific, MA), each forebrain was cut into 2 mm thick coronal blocks for a total of seven blocks per animal.

Quantification of Cerebral Edema

A series of 10 μm thick sections was cut from each block and stained with hematoxylin and eosin (H&E) for calculation of the cerebral edema for each group, as previously described [33, 34]. Each H&E–stained coronal section was digitized under 2.5 x objective of Celestron Digital Microscope Pro. and analyzed using NIH ImageJ software. Cerebral edema was calculated by subtracting the volume (mm^3) of the contralateral hemisphere from the ipsilateral hemisphere [33, 34].

Immunohistochemistry: A series of coronal sections (10 μm thick) were obtained from the center of the lesion (Bregma − 1 mm to + 1 mm) and mounted on slides for analysis. For immunohistochemistry, the following primary antibodies were employed: anti-HDAC3 (Abcam, ab32369, 1:500), anti-GFAP (Millipore, MAB3026, 1:600), anti–Iba-1 (Novus Biologicals, NB100-1028ss, 1:500), anti-albumin (Invitrogen, MAB1455, 1:400), anti-AQP4 (Milipore Sigma, AB3594, 1:200), anti–claudin-5 (Santa Cruz, sc-374221,

1:400) and anti-ZO-1 (Invitrogen, 61-7300, 1:400). Negative controls were performed by omitting the primary antibody. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI).

Image Acquisition and Quantitation

For quantitative measurements, immunostained coronal sections were digitized using a 20–40 x objective epifluorescence (Nikon Eclipse E600) microscope. Four-six fields of view were acquired from the peri-infarct cortex and the total number of immunoreactive cells were counted using NIH ImageJ software. The total number of positive cells per mm^2 area is presented.

Evans Blue Dye (EBD) Assay

As previously described [35], 2% solution of EBD (Sigma, St. Louis, MO) was prepared in 0.9% saline and injected to the tail vein. After 2–4 hours of EBD injection, the rats were sacrificed and perfused transcardially with 0.9% of saline. The cerebral hemispheres were separated and homogenized in N,N-dimethylformamide (Sigma, MO) and then incubated for 72 h in a water bath at 55°C. The samples were centrifuged at 1,500 g for 20 min. The extracted EBD in the supernatant was quantified by absorbance at 620 nm. Results were expressed as µg of Evans blue per gram of brain hemisphere by comparison with solution standards and reported as fold change compared to the contralateral hemisphere.

Western Blot: Animals were sacrificed and brain tissues were harvested and then snap-frozen in liquid nitrogen and stored at − 80°C. Tissues were thawed, washed in ice-cold PBS, and lysed in RIPA buffer containing protease inhibitors (Sigma). Samples were then sonicated, incubated on ice for 30 minutes, and centrifuged at 10,000 g for 20 min at 4°C. Protein concentration in the supernatant was determined by Pierce BCA Protein Assay Kit (Life Technologies). Equal amounts of protein (20 µg) were combined with loading buffer, boiled for 5 min, and loaded onto 4–20% precast polyacrylamide gel (Bio-Rad Laboratories). Separated proteins were transferred onto nitrocellulose membranes, blocked with casein-based blocking reagent (I-Block, Life Technologies) for 60 minutes at room temperature, and then incubated overnight at 4°C with the following primary antibodies: MMP-9 (Cell signaling, #13667, 1:1000) and acetyl NF-kappa(Cell signaling, S2S3J, 1:1000). Secondary antibodies used were HRP-linked specific for rabbit (1:2000, Cell Signaling) and mouse (1:2000, Cell Signaling). After incubation, membranes were washed with PBS-T and exposed to the appropriate horseradish peroxidase-linked secondary antibody. Blots were developed with Clarity Western ECL Substrate (Bio-Rad Laboratories) and detected using a BioRad ChemiDoc Touch Imaging System (BioRad Laboratories). Data were analyzed using ImageJ software. Total abundance of target protein was normalized to appropriate endogenous control and reported as fold change.

Blinded Assessment of Outcomes

All measurements (mNSS testing, cerebral edema calculation, and immunohistochemical measurements) were performed by an investigator who had no knowledge of the experimental groups and to which an animal belongs in line with STAIR criteria [36, 37].

Statistical Analysis

An unpaired student t-test was used to test differences in histological measures among the treatment groups. Repeated measures two-way ANOVAs were performed for functional tests. Spearman or Pearson correlation coefficients were calculated among the immunostaining evaluation measurements and their correlation with functional outcome and cerebral edema. Statistical significance was set at p-value < 0.05.

Results

Selective inhibition of HDAC3 with RGFP966 improves functional outcomes and decreases cerebral edema and BBB leakage

To investigate whether selective inhibition of HDAC3 improves neurological outcome, another set of rats were subjected to two hours of MCAO, and randomly selected animals were treated intraperitoneally with two doses of either vehicle (1% Tween 80) or a selective HDAC3 inhibitor (RGFP966, 10 mg/kg) at 2 and 24 h after MCAO. Behavioral tests were performed at 2 hours, 1 day, and 3 days after MCAO. Repeated measure ANOVA showed that at 1 and 3 days after MCAO, RGFP966 significantly improved mNSS compared to the control group (Fig. 1A).

We further investigated whether RGFP966 decrease cerebral edema using H&E staining and found that RGFP966 significantly decreased cerebral edema compared to the control group (Fig. 1C). Next, we used EBD assay to measure the degree of BBB leakage and whether selective HDAC3 inhibition decreases BBB. Out data showed that selective inhibition of HDAC3 significantly decreased the extravasation of EBD compared to the control group (Fig. 1B). Immunostaining also confirmed that RGFP966 decreased the extravasation of albumin (Fig. 2A).

RGFP966 increases the expression of tight junction proteins and decreases the expression of AQP4 in GFAP positive cells

To investigate the underlying mechanism of HDAC3 regulation of BBB leakage, we used immunohistochemistry to measure the expression of tight junction proteins in the peri-infarct cortex. Our data showed that RGFP966 increased the expression of ZO-1 and claudin-5 (Fig. 2B **and C**, respectively). RGFP966 also decreased the expression of total AQP4 as well as the expression of AQP4 in GFAP-positive cells (Fig. 3E).

Rgfp966 Decreases The Expression Of Hdac3 In Gfap-positive Cells

Double immunohistochemistry showed that RGFP966 decreased the expression of HDAC3 in GFAP-positive cells (Fig. 3A) and this reduction was correlated with better neurological scores (Fig. 3B) and

decreased cerebral edema (Fig. 3C). A significant correlation was observed between the expression of HDAC3 and AQP4 in GFAP-positive cells (Fig. 3D).

Rgfp966 Decreases Microglia Expression And Inflammatory Markers In The Peri-infarct Cortex

To further investigate whether RGFP966 treatment regulates neuroinflammation after experimental stroke, we measured the expression of activated microglia (Iba-1) and NF-kappa in the peri-infarct cortex using immunostaining. As shown in Fig. 4A-B, selective inhibition of HDAC3 significantly decreased the expression of Iba-1 and NF-kappa–positive cells. Western blot showed that RGFP966 decreased the expression of MMP-9 (Fig. 4C) in the ischemic brain but did not alter the expression of acetylated NF-kappa (Fig. 4D).

Discussion

In the present study, we showed that selective inhibition of HDAC3 with RGFP966 decreases cerebral edema and BBB leakage in vivo and improves functional outcomes. The BBB protection mediated by RGFP966 is associated with the upregulation of tight junction proteins and downregulation of AQP-4 and neuroinflammation. These findings suggest that HDAC3 inhibition represents a promising new therapeutic option to target the underlying mechanisms of cerebral edema after stroke and slow its progression.

Cerebral edema is a devastating complication of stroke [5]. BBB dysfunction after ischemia contributes to cerebral edema [6, 38]. The BBB is a specialized multicellular structure that selectively separates the brain interstitium from the contents of the blood vessels [39, 40]. The BBB is a dynamic barrier, with active communication between cells of the BBB and the brain parenchymal cells [6]. The innermost layer of the BBB is comprised of endothelial cells and the BBB regulates the paracellular permeability of endothelial cells through junction proteins including tight junction (TJ) proteins [41]. Degradation of TJ proteins, such as claudin-5 and ZO-1, leads to increased permeability of BBB [8]. Emerging evidence suggests that epigenetic regulation plays an important role in BBB injury and recovery after stroke [13, 42]. Nonspecific HDAC inhibitors (eg, valproic acid) have been shown to reverse the downregulation of ZO-1 and claudin-5 after MCAO [18]. Using in vitro model of ischemia Zhao et al showed that pretreatment with RGFP966 attenuated transendothelial cell permeability and upregulated claudin-5 [43]. In line with these reports, our data showed that selective inhibition of HDAC3 in vivo increases the expression of ZO-1 and claudin-5.

Aquaporin channels have been shown to mediate the plasmalemmal water flux in cerebral edema [6]. In the brain, AQP4 is mainly expressed in the astrocytes [44], and it has been shown to facilitate astrocytic swelling [45–47]. Overexpression of AQP4 enhances ionic edema formation [45], and the deletion of AQP4 has been shown to impair cell water uptake in several brain injury models [46, 47]. Our data showed that selective inhibition of HDAC3 decreased the expression of total AQP4 as well as the expression of

AQP4 in GFAP-positive cells. We also found a significant correlation between the expression of HDAC3 and AQP4 in GFAP-positive cells and the decreased expression of HDAC3 in GFAP-positive cells was correlated with better neurological scores and decreased cerebral edema This data support the role of histone deacetylases such as HDAC3 in epigenetic regulation of AQP4 and cerebral edema after ischemic stroke.

Due to BBB breakdown after ischemic stroke, leukocytes infiltrate the brain tissue and cause inflammatory response resulting in secondary BBB disruption and worsening edema [48, 49]. Activated microglia outnumber the peripheral blood monocytes infiltrating the ischemic brain [50]; therefore, resident microglia are the main damaging inflammatory cells to BBB [7, 10]. MMP-9 is mainly derived from neutrophils and has been shown to play an important role in BBB disruption [51]. NF-kappa is a central regulator of the inflammatory response [12], and it has been proposed that modulating the activity of NF-kappa could attenuate inflammation after ischemic stroke [12, 52, 53].

Emerging evidence indicates that activation of immune cells after injury requires transcriptional changes that are highly regulated by epigenetic mechanisms [54, 55]. HDAC3 has been implicated in the regulation of inflammatory gene expression [56], and it has been shown to function as a brake of the alternative/anti-inflammatory activation [57]. Our data support that selective inhibition of HDAC3 regulates neuroinflammation after experimental stroke. We found that RGFP966 significantly decreases the expression of activated microglia and NF-kappa. RGFP966 treatment also decreases the expression of MMP-9 in the ischemic brain.

The current study provides evidence that early administration of a selective HDAC3 inhibitor (RGFP966) in vivo decreases cerebral edema and BBB leakage after ischemic stroke. The BBB protection by RGFP966 is mediated in part by the upregulation of tight junction proteins, downregulation of AQP4 and HDAC3 in astrocytes, as well as the attenuation of neuroinflammation. These data suggest that targeting HDAC3 might be a novel therapeutic approach for the treatment of cerebral edema after stroke.

Declarations

Author Declarations:

- Ethics approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experiments were were approved by the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center.
- Consent to participate: Not applicable
- Consent for publication: Not applicable
- Availability of data and materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
- Competing interests: The authors have no relevant financial or non-financial interests to disclose.

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- Authors' contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hui Lu, Chin I Lin,Victoria Clendaniel, Rudy Matheson and Amjad Shehadah. The first draft of the manuscript was written by Amjad Shehadah and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.
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Compliance with Ethical Standards:

- Disclosure of potential conflicts of interest: The authors declare that they have no conflict of interest.
- Research involving Human Participants and/or Animals: Animals
- Informed consent: Not applicable

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Figures

Figure 1

RGFP966 improves neurological outcome, decreases cerebral edema and BBB leakage

Panel **A** shows that RGFP966 improves neurological severity scores at 1 and 3 days after MCAO compared to the control group. Panel **B** shows that RGFP966 decreases BBB leakage, as measured by Evans blue dye extravasation, compared to the control group Panel **C** shows that RGFP966 decreases cerebral edema, as measured by H&E, compared to the control group. SD = standard deviation.

Figure 2

RGFP966 decreases the expression of albumin and increases the expression of ZO-1 and claudin-5 in the peri-infarct cortex

Panel **A** shows that RGFP966 significantly decreases the expression of albumin in the peri-infarct cortex at 3 days compared to the control group. RGFP966 also increases the expression of ZO-1 (Panel **B**) and claudin-5 (Panel **C**) in the peri-infarct cortex. SD = standard deviation.

Figure 3

RGFP966 reduces the expression of HDAC3 and AQP4 in GFAP-positive cells

Panel **A** shows that RGFP966 significantly decreases the expression of HDAC3 in GFAP-positive cells. This reduction was correlated with reduced neurological severity scores (Panel **B**) and decreased cerebral edema (Panel **C**). Panel **D** shows that HDAC3 expression correlates with AQP4 expression in GFAP-positive cells. Panel **E** shows that RGFP966 decreases the expression of total AQP4 and AQP4 in GFAP-positive cells. SD = standard deviation.

Figure 4

RGFP966 decreases inflammatory markers in the ischemic brain

Panel **A-B** shows that RGFP966 decreases the immunoreactivity of Iba-1 (**A**) and NF-kappa (**B**) in the peri-infarct cortex. **Panel C** shows that RGFP966 decreases the expression of MMP-9 as measured by Western Blot. There was no difference in the expression of acetyl NF-kappa between the groups (**D**). SD = standard deviation.