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Protective effects of ex-527 on cerebral ischemia reperfusion injury through necroptosis signaling pathway attenuation

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Abstract

Necroptosis, a novel type of programmed cell death, is involved in ischemia/reperfusion-induced brain injury. Sirtuin1 (Sirt1), as a well-known member of histone deacetylase class III, plays pivotal roles in inflammation, metabolism and neuron loss in cerebral ischemia. We carried out an exploration of the relationship between Sirt1 and necroptosis signaling pathway and its downstream events by administration of ex-527, as selective and potent inhibitor of Sirt1, and necrostatin-1 (nec-1), as necroptosis inhibitor, in an animal model of focal cerebral ischemia. Our data showed different patterns of sirt1 and necroptosis critical regulators including receptor-interacting protein kinase 3 and mixed lineage kinase domain-like protein gene expressions in prefrontal cortex and hippocampus following ischemia/reperfusion. We found that ex-527 microinjection reduces infarction volume of ischemic brains and improves survival rate but not stroke-associated neurological deficits. Additionally, treatment with ex-527 effectively abolished the elevation of the critical regulators of necroptosis while necroptosis inhibition through nec-1 microinjection could not influence Sirt1 expression levels. Our data also demonstrated that the ex-527 relieves ischemia-induced perturbation of necroptosis-associated metabolic enzymes activity in downstream. This work provides a

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new approach to the possible neuroprotective potential of ex-527 orchestrated by necroptosis pathway inhibition to alleviate ischemia/reperfusion brain injury.

Graphical Abstract

Selective and potent inhibitor of Sirt1, ex-527, reduces infarction volume of ischemic brains and improves survival rate.

Treatment with ex-527 effectively abolishes the elevation of the critical regulators of necroptosis.

Ex-527 relieves ischemia-induced perturbation of necroptosis-associated metabolic enzymes activity in downstream.

Keywords: Cerebral ischemia/reperfusion, Necroptosis, Sirt1, Neuroprotection

Introduction

Emerging evidence on Tumor necrosis factor receptor (TNFR) potential to induce both apoptosis and necrosis in 1988 (Laster et al., 1988) paved the way for discovery of necroptosis in 2005 (Degterev et al., 2005). Necroptosis/programmed necrosis exacerbates pathologic conditions in many diseases and disorders such as myocardial ischemia (Luedde et al., 2014), renal injury (Linkermann et al., 2012), liver injury (Roychowdhury et al., 2013), inflammation (Berger et al., 2014) and neurodegenerative diseases (Zhang et al., 2017). Several studies imply that necroptosis is the main event following brain ischemia and its inhibition by necrostatin-1 (nec-1) rescues cells from fatal outcomes of cerebral ischemia (Chen et al., 2017; Yang et al., 2017b). Necroptosis cell death could also be resulted from energy failure, oxidative stress and reactive oxygen species (ROS) production (Vandenabeele et al., 2010). In a vicious cycle, necroptosis aggravates disrupted oxidative metabolism and inflammation dramatically (Pasparakis and Vandenabeele, 2015). On the other side, there is a robust link between cell metabolism catastrophe subsequent to oxidative stress and cerebral ischemia/reperfusion (Narne et al., 2017). Hence, cell metabolism stabilization and oxidative stress inhibition are capable of alleviating brain ischemia-induced necroptosis and lethal conditions that occur following cerebral ischemia/reperfusion.

Histone deacetylase class III (HDAC III), which regulates gene transcription, has seven members in mammalian named Sirtuin 1-7 (Sirt1-7) (Dai and Faller, 2008). Sirt1, as a well-studied member of this family, has pivotal roles in inflammation, metabolism and neurodegeneration (Yamamoto et al., 2007). Sirt1 induction/inhibition as a powerful tool to elucidate precise role of Sirt1, shows converse functions of this molecule in equal situations (Sussmuth et al., 2015; Tulino et al., 2016). Ex-527 is a potent and selective inhibitor of Sirt1 that exhibits different effects in experimental models of diseases and disorders. It has been shown that ex-527 has beneficial effects on acute lung injury (Huang et al., 2017) and depression/anxiety-like behaviors (Kim et al., 2016b). However, since the exact mechanism by which ex-527 exerts its effects is still debated, it is not surprising that administration of this small molecule inhibitor of Sirt1 dreadfully affects

atherosclerosis (Yang et al., 2017a), hepatic ischemia/reperfusion injury (Sun et al., 2017), Parkinson's disease (Guo et al., 2016) and nephrotoxicity (Guo et al., 2016).

Although both Sirt1 and necroptosis pathway have been notified to be involved in a wide range of diseases, there is a dearth of studies investigating counteraction of Sirt1 and necroptosis cell death. Here, we carried out an exploration of the relationship between Sirt1 and necroptosis by ex-527 and nec-1 administrations in an animal model of focal cerebral ischemia. Our findings represent a novel perspective of how Sirt1 activity is regulated in relationship with necroptosis pathway. Our findings also provide potential therapeutic use of ex-527 to abolish mortality rate in brain stroke.

Material and Methods:

Animals and Experimental design

All animal procedures were approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences in accordance with the international guidelines for animal experiments (No. 80–23, revised 1996). The study received written approval from the Neuroscience Research Center Ethics Board (code IR.SBMU.PHNS.REC.1394.35). Adult male Wistar rats (weighing from 230 to 280 g) obtained from our breeding colony (Neuroscience Research Center). Animals were maintained in an air-conditioned animal room with controlled temperature (23 ± 2 °C) and humidity, 12-h light/dark cycle. Rats were given *ad libitum* access to food and water. All efforts were made to reduce animal suffering and to minimize the number of animals used.

In the first step and in order to establish time profile of gene expression of sirt1 and necroptosis pathway critical regulators including receptor interacting protein kinase 3 (rip3) and mixed lineage kinase domain-like protein (mlkl), animals were randomized into three groups in accordance with reperfusion time 6, 12 and 24 hours following 60 min of focal ischemia. Following defined reperfusion time points, purposed tissues, prefrontal cortex and hippocampus, were collected for subsequent analyses. Moreover, sham groups have been assumed at each time point (fig 1a).

In the next step, rats were randomly assigned into the following four experimental groups: (I) sham-vehicle group, (II) MCAO-vehicle group, (III) MCAO+ex-527, and (IV) MCAO+nec-1. Experimental animals in two last groups received intracerebroventricular (i.c.v) injections of 10 µg ex-527 (Sigma-Aldrich, E7034, St. Louis, MO, USA) as Sirt1 inhibitor (Yan et al., 2013) or 1 µg nec-1 (Sigma-Aldrich, N9037, St. Louis, MO, USA) as necroptosis inhibitor (Xu et al., 2016) dissolved in 5 µl mixture of dimethyl sulfoxide (DMSO) and normal saline 1 hour before MCAO procedures. Animals were sacrificed 24 hours after reperfusion (fig 1b).

Stereotaxic Surgery

The animals were anesthetized with 1/3 subcutaneous and 2/3 intraperitoneal injections of chloral hydrate (400 mg/kg). They were then injected slowly over a period of 10 minutes using a Hamilton syringe into right cerebral ventricle with stereotaxic coordinate of AP -0.5 mm, L 1.5 mm, and DV 4 mm (from the skull surface) according to the rat brain atlas of Paxinos and Watson (Paxinos G, 2007).

Middle cerebral artery occlusion (MCAO) model

After making a neck midline incision, the right common carotid artery and its bifurcations were exposed, and then an intraluminal silicone-coated suture (403934PK, Docol, Sharon, MA, USA) was inserted into the internal carotid artery to occlude MCA. Reperfusion was launched by gently withdrawing the filament at 60 min following occlusion (Longa et al., 1989). For the sham groups, following vehicle microinjection, the arteries were surgically prepared for insertion of the filament, but no filament was inserted. Neurological deficits were evaluated following 24 hours of reperfusion using Bederson's scoring system (0=no deficit, 1=forelimb flexion, 2=decreased resistance to lateral push, 3=circling, 4=no spontaneous movement) (Bederson et al., 1986). To determine the infarction volume, immediately after the examination, rats were sacrificed by CO₂ asphyxiation. Brains were removed, coronal slices were made and sections were immersed in triphenyltetrazolium chloride (TTC) (Merck, Germany) 2% in normal saline at 37° C for 30 min. Stained sections were scanned and processed by an ImageJ Analyzer

(V1.41, National Institutes of Health, Bethesda, MD, USA) to calculate corrected infarct volume. The presence or absence of infarction was determined in all rats by examining TTC-stained.

RNA extraction and real-time quantitative PCR (real time-qPCR)

Total RNA was extracted from frozen purposed tissues including prefrontal cortex and hippocampus using YTzol (Yektatajhiz azma, Tehran, Iran) according to the manufacturer's instructions then 500 ng of total RNA samples were reverse transcribed into cDNA using the PrimeScript RT reagent Kit (Takara, Shiga, Japan). Sample cDNA was amplified and quantified using ABI System (USA) with SYBR Green Real-Time PCR Master Mix (Ampliqon, Denmark) reagent. The threshold cycles (Ct) were used to quantify the mRNA levels of the target genes. All genes were normalized to β -actin levels. Relative gene expressions were calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The primer pairs for each gene target are summarized in Table 1.

Western blotting

Frozen brain tissue samples were homogenized in RIPA buffer with protease inhibitors and protein concentration was determined with Bradford method (Bradford, 1976). Sixty μ g of total protein per sample separated by SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, USA). For immunodetection, membranes were incubated with following primary antibodies: anti-RIP3 (1:200, sc-135170, Santa Cruz Biotechnology, USA), anti-MLKL (1:1000, ab194699, Abcam, UK) anti-Sirt1 (1:1000, #9475, Cell Signaling Technology, USA), and anti- β -actin as housekeeping protein (1:1000, #4970, Cell Signaling Technology, USA). The attached antibodies were developed with an HRP-linked secondary antibody (1:3000, #7074, Cell Signaling Technology, USA). Signals generated with an enhanced chemiluminescence (ECL) kit (Amersham Bioscience, USA) were quantified using ImageJ software.

Necroptosis enzymes activities

Glutamate dehydrogenase enzyme activity (GLUD) was measured by the method described by Doherty. In this assay, oxidation of the reduced co-enzyme NADH was measured spectrophotometrically during 600 (s) at 340 nm in the presence of reaction mixture consisted of 1M potassium phosphate buffer (pH 7.0), 1M NH₄Cl, 0.1M 2-Oxo-glutarate, 60µg NADH and 100µg protein after 30 min incubation in 37 °C (Doherty, 1970).

Glycogen phosphorylase (GP) enzyme activity was measured by phosphoglucomutase/glucose-6-phosphate dehydrogenase coupled assay. Briefly, reaction buffer contains 50mM KH₂PO₄ (pH 6.8), 1mM MgCl₂, 500µM NADP, 1 U/reaction of phosphoglucomutase and glucose-6-phosphate dehydrogenase and 1 mg/reaction glycogen was mixed with 100µg protein. Rate of enzyme activity was the formation of NADPH by recording absorbance at 340nm during 600 (s). Total GP activity form was measured after addition of 2mM AMP into reaction buffer (Maddaiah and Madsen, 1966).

Immunohistochemistry

Briefly, anesthetized rats were trans-cardially perfused with ice-cold PBS and then 4% paraformaldehyde to fix the cerebral parenchyma. The harvested cerebral hemispheres were post-fixed in 4% paraformaldehyde and paraffin embedded with the aid of tissue processor. Paraffin sections were dewaxed and rehydrated. Antigenic sites were exposed by incubating sections in citrate antigen retrieval buffer (pH 6.0) for 20 min at 100 °C using a water bath. Following antigen retrieval, slides were cooled for an additional 20 min. Endogenous peroxidase activity was quenched by treating sections with a 0.3% solution of hydrogen peroxide. Sections were permeabilized by 0.2% triton-100x. Then, non-specific binding was blocked by 30 min incubation in 10% normal goat serum. The sections were then incubated overnight at 4 °C with rabbit polyclonal anti-MLKL (1:100) and rabbit monoclonal anti-Sirt1 (1:100) antibodies. Immunoreactivities were detected by horse radish peroxidase (HRP) conjugated secondary antibody, and visualized by

incubation in liquid 3, 3'-Diamino benzidine tetrahydrochloride (DAB) (DAKO, Denmark).

Statistical analysis

All data are presented in the form mean $\pm$ SEM. We use t-test to compare statistical significance within two groups or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for multiple groups comparison. All statistical analysis was performed with GraphPad Prism (V5, GraphPad Software, USA). Differences between groups were considered to be significant when $P < 0.05$.

Results

Time profile of rip3, mlkl and sirt1 gene expressions in ischemic prefrontal cortex and hippocampus of rat brains

RIP3 has been confirmed as prominent regulator of necroptosis pathway which stimulates MLKL activation and triggers necroptosis downstream events such as metabolism alteration, organelle dysfunction and cellular membrane rupture (Moriwaki and Chan, 2013). In the present study, we explored the relative gene expression of rip3, mlkl and sirt1 in rat brains subjected to different time points of reperfusion including 6, 12 and 24 hours. In prefrontal cortex, our data showed rip3 and mlkl gene expressions increased at 12 and 24 hours of reperfusion in comparison with sham groups ($p < 0.001$) (fig. 2a and b) while sirt1 gene expression amplified earlier than necroptosis markers at 6 and 12 hours of reperfusion ($p < 0.05$) (fig. 2c). There is no significant difference between sirt1 gene expression at 24 hours after reperfusion and its sham group suggesting its reduction back to the baseline. In hippocampus, rip3 and mlkl showed increasing trends during 24 hours following reperfusion ($p < 0.001$) (fig. 2d and e). Elevation of sirt1 gene expression was observed at 6 ($p < 0.05$) and 12 ($p < 0.01$) hours of reperfusion (fig. 2f). Similar to prefrontal cortex, sirt1 went back to the baseline at 24 hours following reperfusion.

Ex-527 improved rat survival and reduced cerebral infarction volume while could not influence neurological deficits

Compared with the sham group, animals in MCAO group showed approximately one hemisphere of infarction. Ischemia severity was decreased with ex-527, as Sirt1 inhibitor, and nec-1, as necroptosis inhibitor, microinjections prior 60 min of occlusion ($p < 0.001$) (fig. 3a and a'). In the group with 60 min of ischemia, the survival rate was 54%, while the survival rates were 68% and 75% in MCAO+ex-527 and MCAO+nec-1 groups respectively (fig. 3b). Neurological deficit scores were decreased significantly in the MCAO+nec-1 group and animals had an apparent improvement compared to the other groups including MCAO and MCAO+ex-527 ($p < 0.05$ and $p < 0.01$) while no significant difference was seen between the MCAO and MCAO+ ex-527 groups (fig. 3c).

Gene expression of rip3 and mlkl was decreased in prefrontal cortex and hippocampus after ex-527 microinjection in ischemic rat brains

To determine whether inhibition of Sirt1 influences ischemia-induced necroptosis in rat brains, we used ex-527 as a potent and selective inhibitor of Sirt1. In prefrontal cortex, our data showed that nec-1 as highly selective inhibitor of necroptosis reduced rip3 gene expression compared to MCAO group ($p < 0.01$). Although significant difference was not observed in rip3 gene expression between animals received ex-527 and MCAO group, there is no significant difference between MCAO+ex-527 and MCAO+nec-1 groups (fig. 4a). Following ex-527 ($p < 0.05$) and nec-1 ($p < 0.001$) microinjection mlkl gene expression decreased at 24 hours after reperfusion (fig. 4b). In hippocampus, rip3 and mlkl gene expressions were decreased by ex-527 administration compared to MCAO group ($p < 0.05$) and ($p < 0.01$) respectively (fig. 4c and d). Statistical differences were not found between ex-527 and nec-1 function to inhibit necroptosis critical gene expression in ischemic rat brains.

Ex-527 reduced necroptosis pathway critical regulators, RIP3 and MLKL, whereas nec-1 could not influence Sirt1 in prefrontal cortex and hippocampus of ischemic rat brains

As our results showed in prefrontal cortex, ex-527 as Sirt1 inhibitor exerts its effect on necroptosis pathway through the reduction of RIP3 and MLKL protein levels compared to MCAO group ($p < 0.01$). Significant differences for critical proteins in necroptosis pathway were not observed between MCAO+ex-527 and MCAO+nec-1 groups (fig. 5b and c). On the other hand, necroptosis inhibition could not persuade Sirt1 protein level (fig 5c). In hippocampus, we found that RIP3 and MLKL protein levels were decreased following ex-527 administration as pretreatment in ischemic rat brains compared to MCAO group, ($p < 0.001$) and ($p < 0.01$) respectively, (fig 5f and g) while necroptosis inhibition could not affect Sirt1 protein level compared to MCAO group (fig. 5h).

Ex-527 alleviated disturbed metabolic pathways through decrease of glycogen phosphorylase and glutamate dehydrogenase activities

Glycogenolysis and glutaminolysis provide substrate for Krebs cycle and ATP production. Throughout necroptosis, RIP3 activates some metabolic enzymes including glycogen phosphorylase and glutamate dehydrogenase, and governs metabolic state of damaged cells (Zhang et al., 2009). In the present study, augmented glycogen phosphorylase and glutamate dehydrogenase activities were decreased by administration of ex-527 one hour before ischemia/reperfusion compared to MCAO group in prefrontal cortex, ($p < 0.01$) and ($p < 0.001$) respectively (fig. 6a and b). Moreover, ex-527 functioned as effective as nec-1 to reduce critical metabolic enzymes activity of necroptosis pathway in hippocampus (fig. 6c and d).

Discussion

Our results first revealed that rip3 and mlkl continually increase during 24 hours after ischemia-reperfusion while sirt1 transiently increases at early stages after reperfusion in prefrontal cortex and hippocampus in rat brains. In the next step, we found that Sirt1 inhibition by ex-527 reduces infarction volume of ischemic brains and improves

survival rate but not stroke-associated neurological deficits. Contrary to our initial hypothesis, treatment with the selective Sirt1 inhibitor, ex-527, effectively abolished the elevation of RIP3 and MLKL. On the other hand, necroptosis inhibition through nec-1 microinjection could not influence Sirt1 expression levels. Additionally, the numbers of MLKL immunoreactive neurons obviously increased in prefrontal cortex and hippocampal CA1 following 60 min of middle cerebral artery occlusion and 24 hours of reperfusion. These increased levels of MLKL were reduced by ex-527 and nec-1 pretreatments, whereas Sirt1-positive neurons were not decreased by nec-1. Our data also demonstrated that the ex-527 relieves ischemia-induced perturbation of necroptosis-associated metabolic enzyme activity as effective as nec-1. Our findings provide an insight into communication of Sirt1 signaling and necroptosis pathway, and also the neuroprotective effects of ex-527 to avert necroptosis regulators.

Sirt1 is a celebrated member of sirtuins/HDAC III family. Considering critical role of Sirt1 in physiologic and pathologic conditions, it has been shown that treatment with its activators or overexpression of Sirt1 alleviates many diseases and disorders (Huang et al., 2012; Li et al., 2018). Sirt1 is expressed predominantly in many regions of the brain. Due to its expression pattern in susceptible brain areas, this protein has attracted considerable interest as a new treatment for neurodegenerative diseases (Ng et al., 2015). Accumulating evidence shows that Sirt1 exerts neuroprotective effects and cognitive enhancements by activation of neurotrophic factors in 3xTg-AD a triple-transgenic mouse model of Alzheimer's disease (Corpas et al., 2017). Sirt1 also reduces motor neuron degeneration in an animal model of amyotrophic lateral sclerosis (Watanabe et al., 2014). Although in most of studies Sirt1 plays a neuroprotective role, our results demonstrate a neuroprotective potential for Sirt1 inhibition. In line with our data, other studies support the hypothesis of Sirt1 inhibition as a protective strategy in various neurodegenerative diseases (Green et al., 2008; Li et al., 2008). Additionally, Sirt1 exhibits dual functions, protective and destructive, in Huntington's disease (Jiang et al., 2011; Sussmuth et al., 2015). Thus, role of Sirt1 in a wide range of diseases is still a matter of controversy.

Our results showed that focal cerebral ischemia and then reperfusion induce upregulation of critical necroptosis regulators, rip3 and mlkl, in prefrontal cortex and hippocampus in rat brains during 24 hours. This data has been proven by similar findings in neuroinflammation context and global cerebral ischemia-reperfusion in our laboratory (Nikseresht et al., 2017; Ryan et al., 2018). We also found increase of sirt1 gene expression in early stages of reperfusion. In agreement with our data, there is some evidence indicating amplification of sirt1 gene expression in permanent and transient models of cerebral ischemia (Fu et al., 2014; Meng et al., 2015). In the next part of the study, surprisingly our results demonstrate protective effects of ex-527 as a potent Sirt1 inhibitor, including decline in mortality rate and infarction volume in rats with ischemic brain. Although it has been shown that Sirt1 activation is a promising candidate to treat or prevent several diseases and disorders, considerable amount of studies suggest that Sirt1 inhibition may have beneficial effects on some pathologic conditions. For instance, reduction of Huntington's disease pathology is observed in mouse and cellular models of this neurodegenerative disease by ex-527 administration which eradicates by genetic elimination of Sirt1 (Smith et al., 2014). In addition, increased levels of Sirt1 in the nucleus accumbens bring about depression- and anxiety-like behaviors in open-field, elevated-plus-maze, and forced swim tests which are reversed by ex-527 (Kim et al., 2016b). Moreover, some neurogenesis properties are reported for ex-527. This Sirt1 inhibitor accelerates differentiation of pluripotent P19 cells into active neurons with voltage-dependent sodium currents and depolarization-induced action potentials (Kim et al., 2016a). It is also noticeable that ex-527 has ROS scavenging and antioxidant capacities. As Li and colleagues demonstrated, ex-527 protects obviously endothelial cells against H₂O₂ through the decrease of the ROS and malondialdehyde levels and increase of superoxide dismutase and glutathione peroxidase levels resulting in cell viability improvement (Li et al., 2014). Although our data showed many parallels between ex-527 and nec-1 administrations in necroptosis pathway inhibition, ex-527 could not reach nec-1 in survival rate acceleration and infarction volume reduction. It might have happened by anti-apoptotic (Wang et al., 2012) and anti-inflammatory (Zhang et al., 2016) effects of nec-1 which ex-527 may have none of these special features, but further studies are needed to prove or disprove this hypothesis. Despite ex-527 ability to

improve survival rate, this inhibitor could not recover neurological deficits in rats with ischemia/reperfusion. Our results also revealed that ex-527 stabilizes disturbed energy metabolism and relieves activation of necroptosis-related metabolic enzymes including glycogen phosphorylase and glutamate dehydrogenase. Many studies demonstrate that attenuation of critical regulators of necroptosis pathway can decrease necroptosis metabolic enzymes activity in downstream (Fakharnia et al., 2017; Nikseresht et al., 2015; Zhang et al., 2009). Depletion of intracellular nicotinamide adenine dinucleotide (NAD^+) and ATP levels can result in necroptotic response in injured cells (Pasparakis and Vandenabeele, 2015). NAD^+ is an important energy substrate, and necroptosis inhibition by nec-1 prevents RIP3 upregulation, NAD^+ depletion and CA1 neuronal death after global cerebral ischemia (Yin et al., 2015). Likewise, Sirt1 is a key metabolic/energy sensor, so Sirt1 manipulation influences cellular metabolism (Chang and Guarente, 2014). Some researchers believe that Sirt1 deficiency abolishes the neuroprotection of NAD^+ in ischemic stroke (Hu et al., 2017; Wang et al., 2011). Conversely, Liu and colleagues demonstrated that bioenergetic interventions by Sirt1 inhibitors can conserve cellular NAD^+ levels and protect neurons against ischemic damage leading to bioenergetic defects (Yamamoto et al., 2014).

Our results demonstrated protective effects of Sirt1 inhibition in a focal model of cerebral ischemia/reperfusion, but transient amplification of sirt1 at 6 and 12 hours following reperfusion and its reduction to baseline at 24 hours bring to mind behavior of protective factors which are activated for a limited period of time following frustrating conditions. Since increased Sirt1 levels could be a therapeutic strategy to rescue cells from ischemia damage (He et al., 2017; Pantazi et al., 2014), it is possible that in our study Sirt1 tried to protect cells in early hours after reperfusion. So it is unclear if some of the protective effects of ex-527 are not due to inhibition of Sirt1. In agreement with this theory, Valle and colleagues reported neuroprotective effects of ex-527 administration in a mouse model of amyotrophic lateral sclerosis whereas other efficient Sirt1 inhibitors are not capable to protect against degeneration of motor neurons (Valle et al., 2014). One of the main limitations of this study is that we cannot conclude whether the observed neuroprotective effects result from Sirt1 inhibition or are totally independent of ex-527 potential to inhibit Sirt1. It should be noted that the results of the present study have been

observed in an ischemic neural tissue context which may not be detected in other contexts and conditions. For instance, it has been shown that in an animal model of compression-induced skeletal muscle injury, ghrelin could inhibit programmed cell death while intraperitoneal co-treatment of ghrelin and ex-527 (1mg/kg) for 2 days reversed the protective effects of ghrelin. Indeed, ex-527 increased necroptosis proteins including RIP1 and RIP3 and augmented the elevation of apoptotic indicators including Bax protein and DNA fragmentation in the compressed muscles (Ugwu et al., 2017).

In summary, our study demonstrates that ex-527 administration exerts protection against cerebral ischemia/reperfusion by means of necroptosis pathway regulators and their downstream events inhibition. Additional studies are needed to strengthen current results and elucidate the underlying mechanisms of neuroprotective properties of ex-527.

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Conflict of Interest

The authors have no any conflict of interest to declare.

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Figure 1 Time scales of experimental design

Figure 2 Time profile of rip3, mlkl and sirt1 gene expressions in ischemic prefrontal cortex (PFC) and hippocampus (HIP). Rats were subjected to different reperfusion time points including 6, 12 and 24 hours following 60 min of middle cerebral artery occlusion. (A, B, D and E) increasing gene expression pattern of rip3 and mlkl as necroptosis critical regulators was observed in both prefrontal cortex and hippocampus compared to the sham groups during 24 hours following reperfusion. (C and F) sirt1 gene expression increased at 6 and 12 hours and then reduced to the baseline at 24 hours following reperfusion in purposed tissues. Statistical analyses were evaluated by Student's T-test between experiment and sham groups. (n=4-6) (*P<0.05, **P<0.01 and *** P<0.001)

PFC

HIP

Figure 3 ex-527 microinjection effects on infarction volume, survival rate and neurological deficits in MCAO model of cerebral ischemia. (A and A') ex-527 decreased infarct volume stained by TTC in comparison with MCAO group (60 min of occlusion and 24 hours of reperfusion) while this small molecule inhibitor of Sirt1 could not reach nec-1 potency in attenuating infarct size. Infarct area appeared white, whereas viable noninfarcted tissue stained rose-pink. (n=4) (B) The rate of mortality of rats was decreased 24 hours after reperfusion in animals with ex-527 and nec-1 microinjections. (n=15, 22, 22 and 20/group respectively) (C) Neurological scores were examined based on Bederson's scoring system after 24 hours of reperfusion. (n=12, 15 and 15/group respectively) (*P<0.05, **P<0.01 and *** P<0.001)

Figure 4 Quantitative analysis of the expression of rip3 and mlkl genes at 24 hours of reperfusion in prefrontal cortex and hippocampus following ex-527 and nec-1 microinjections prior to 60 min of cerebral ischemia. (A and B) e-527 decreased rip3 and mlkl gene expressions in prefrontal cortex. (C and D) ex-527 decreased rip3 and mlkl gene expressions in hippocampus. Normal is purpose tissues obtained from intact animals. (n=4-6) (*P<0.05, **P<0.01 and *** P<0.001)

PFC

HIP

Figure 5. RIP3, MLKL and Sirt1 protein levels 24 hours following reperfusion in animals received ex-527 and nec-1 microinjections as pretreatment. (A and E) RIP3, MLKL and sirt1 immunoblottings in prefrontal and hippocampus. (B and C) RIP3 and MLKL protein levels were decreased following ex-527 pretreatment in prefrontal cortex. (D) Sirt1 protein level could not be influenced by nec-1 administration in prefrontal cortex. (F and G) RIP3 and MLKL protein levels were diminished following ex-527 pretreatment in ischemic hippocampus. (H) Sirt1 protein level could not be influenced by nec-1 administration in hippocampus of ischemic rat brains. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) (n=4) (I) MLKL and sirt1 immunoreactivities in prefrontal and CA1 region of hippocampus in ischemic rat brains. Insets on the corners show target cells with higher magnification determined by arrows. (scale bar=100 μm) (n=3)

Figure 6 ex-527 and nec-1 impacts on glycogen phosphorylase and glutamate dehydrogenase metabolic enzymes activity after cerebral ischemia. (A and B) ex-527 administration decreased glycogen phosphorylase and glutamate dehydrogenase activity elevations at 24 hours following reperfusion in prefrontal. (C and D) Similar effects of ex-527 and nec-1 were found on glycogen phosphorylase and glutamate dehydrogenase activities in hippocampus. (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$) (n=4)

PFC

A

B

HIP

C

D

Table 1. Primer sequences used for qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
rip3	TGGTGACAGGATTCATGGAGA	CAGGCGACAGAGGAGAGG
mlkl	TTCTCCCAACATCCTGCGTA	TCAGGGTTCCGAGTTCACAG
sirt1	ACCTCCTCATTGTTATTGGGTCT	GCATACTCGCCACCTAACCT
β-actin	TCTATCCTGGCCTCACTGTC	AACGCAGCTCAGTAACACTCC

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