Published online: December 30, 2019

IMR Press

Original Research

A novel squaramide compound alleviates cognitive deficits through activation of Akt and Erk1/2 in a rat model of vascular dementia

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DOI:10.31083/j.jin.2019.04.1204

Vascular dementia is the second most common type of dementia, yet no effective treatment for it exists. Akt and Erk1/2 signaling pathways are involved in neuronal survival. It has been reported that bisperoxovanadium (pyridin-2-squaramide), a novel squaramide compound, protects against cerebral ischemia injury via activation of Akt and Erk1/2. Here, the potential neuroprotective effect of bisperoxovanadium is shown for the first time in a model of vascular dementia induced in 6-month-old male Sprague-Dawley rats by two-vessel occlusion injury applied to 6-month-old. Following this lesion, bisperoxovanadium (pyridin-2-squaramide) (1 mg/kg/day) was intragastrically administered for four successive weeks. The Morris water maze test estimated cognitive function. The morphological examination was performed by hematoxylin-eosin staining. Akt and Erk1/2 protein abundance were assessed by Western blot. Results showed that bisperoxovanadium (pyridin-2-squaramide) attenuated not only cognitive dysfunction but also alleviated histopathological changes in rats with vascular dementia. Moreover, bisperoxovanadium (pyridin-2-squaramide) ultimately reduced neuronal apoptosis represented by the Bax/Bcl-2 ratio in the CA1 (cornu ammonis 1) region of the hippocampus. Importantly, the levels of p-Akt(ser^{473}) and p-Erk1/2(Thr^{202}/Tyr^{204}) were increased after treatment with bisperoxovanadium (pyridin-2-squaramide). It is concluded that the novel squaramide compound bisperoxovanadium (pyridin-2-squaramide) might be effective in the treatment of vascular dementia by activation of Akt and Erk1/2.

Keywords

Neuroprotection bisperoxovanadium (pyridin-2-squaramide); vascular dementia; Akt, Erk1/2; rat model

1. Introduction

Vascular dementia (VD) is a type of cognitive disorder caused by cerebral ischemia or cerebral hemorrhage. The main clinical features of VD are progressive memory loss, cognitive decline, and language problems (O'Brien and Thomas, 2015; Wang et al., 2018). The pathophysiological mechanisms involve apoptosis (Ye et al., 2017), oxidative stress (Barus et al., 2019; Ye et al., 2017), neuroinflammation (Barus et al., 2019; Ye et al., 2017), mitochondrial DNA damage (Legge and Hachinski, 2010), and disturbed neurotransmitter release (Barus et al., 2019; Legge and Hachinski, 2010).

PI3K/Akt and Erk1/2 signaling pathways are both associated with neuronal apoptosis and survival (Chan, 2004; Huang et al., 2017; Zhu et al., 2013).Akt, a kind of serine/threonine-protein kinase, can be activated by PI3K and then PIP_2 to PIP_3 (Chang et al., 2007; Di Cristofano and Pandolfi, 2000). Akt activation is well known to exert pro-survival (Chan, 2004; Datta et al., 1999; Luo et al., 2003; Xu et al., 2019; Zhao et al., 2006) and anti-apoptotic (Chan, 2004; Cui et al., 2017; Xie et al., 2017) effects in neuronal injury and neurodegenerative disease.

MAPK/ERK pathway plays a critical role in regulating neuronal survival (Chan, 2004; Hao and Rockwell, 2013; Yuan and Yankner, 2000), plasticity (Chen et al., 2018a; Kim et al., 2005), migration (Segarra et al., 2006) and apoptosis (Chan, 2004; Hao and Rockwell, 2013; Yuan and Yankner, 2000). Erk1/2 is activated by a series of phosphorylation cascades of the MAPK/ERK

Journal of Integrative Neuroscience

pathway. Increasing evidence shows that Erk1/2, to some extent, enhances neuronal survival and reduces neuronal apoptosis in an animal model of VD (Chan, 2004; Chen et al., 2018a; Hu et al., 2017; Lin et al., 2015; Wang et al., 2014; Zhang et al., 2018). Hence, PI3K/Akt and MAPK/ERK pathways may become promising targets for the development of treatments for VD.

Bisperoxovanadium (pyridin-2-squaramide) (bpV(pis)) is a novel squaramide compound. The previous study has shown that bpV (pis) protects rat brain from cerebral ischemic injury (Zhang et al., 2017). This study was aimed at investigating the effect of bpV (pis) and seeking the underlying molecular mechanism in VD rats.

2. Materials and methods

2.1 Source of the compound

High-performance liquid chromatography and mass spectrometry analysis were used to determine the purity (95%) and the molecular mass of BpV(pis) (Fig. 1A) respectively.

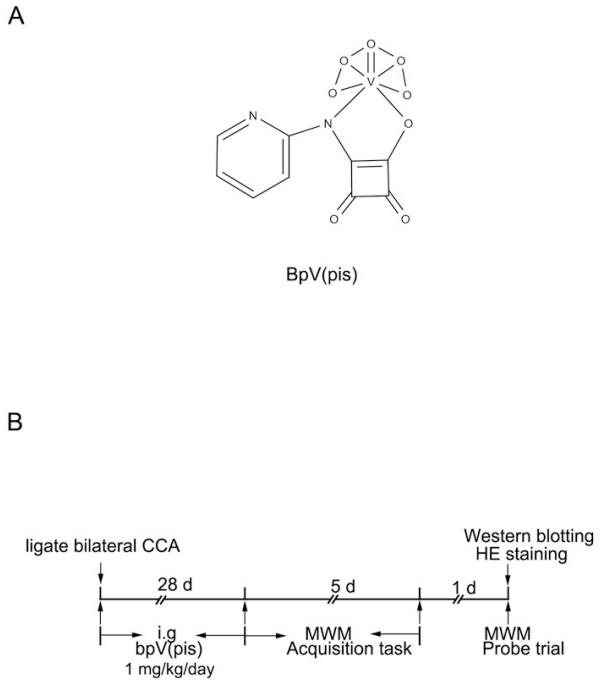

Figure 1. (A) The chemical structure of bisperoxovanadium (pyridin-2-squaramide) [bpV(pis)] (Courtesy Qi Wan Lab, Qingdao University). (B) The diagram of experimental procedures. The model rats were intragastrical administered bpV(pis) for 28 days after the 2-vessel occlusion surgery by ligating two sides of CCA. Then spatial learning and memory functions were detected by MWM including acquisition task and probe trial tests. Finally, morphological examination was performed by HE staining. Akt and Erk1/2 protein abundance were assessed by Western blot.

2.2 Animals and experimental groups

The experimental animals were Sprague-Dawley rats supplied by the experimental animal center of Hubei University of Medicine. All experimental rats are 6 months old and weigh 200-300 g. The rats were kept in an appropriate temperature (23 °C-25 °C) room. The room keeps the light and dark conditions alternate for 12 hours and free access to food and water. This study was approved by the animal care and ethics committee of the Hubei University of Medicine. The experimental groups include sham group (n = 10), sham + bpV(pis) group (n = 10), VD + Vehicle group (n = 10) and VD + bpV(pis) group (n = 10). The VD group underwent 2-vessel occlusion (2VO). In sham group, the same operation procedure was performed except artery ligation. BpV(pis) (1 mg/kg/day, 1 mL, dissolved in ethanol in sterile saline) was administrated by intragastric (i.g) administration. Vehicle rats received intragastric (i.g) administration of the same dosage of saline containing ethanol solution without bpV(pis).

2.3 A model of vascular dementia in rats

2VO technique was performed to induce the model of VD (Hu et al., 2017). After anesthetizing rats with 4% isoflurane in mixture gas of 70% N_2O and 30% O_2, we gently separated out two sides of common carotid arteries of rats by glass dissecting tools. And the arteries were ligated by using 4-0 silk suture in VD group. But there was no need to ligate the common carotid arteries in sham group. After finished the operation, rectal temperature of animal was maintained at 37-38 °C with electric blanket.

2.4 Morris water maze (MWM) test

The spatial cognitive function of rats were detected by MWM (Shi et al., 2017). The main components of the MWM device include a digital video camera, a circular water tank (150 cm diameter, 60 cm height) filling with 28 ± 1 °C water (30 cm depth) and a platform (14 cm diameters). The platform was placed in the middle of the target area and below 1.5 cm of the water surface. The digital video camera was performed to monitor rats' swimming trace and transfer parameters to a behavior softer system. During the five days of acquisition phase, each rat was placed randomly into the pool swimming freely to search the underwater platform. The escape latency that is the time of finding the platform was recorded by the digital video. If rat could not find the platform during 120 s, rat would be guided to the platform to stay 20 s. On the sixth day, the probe trial was used to detect memory function of rats. In this phase, rats swam in the tank for 120 seconds after removing platform. The proportion of swimming time in the target area was recorded.

2.5 Hematoxylin and eosin staining

After perfusion with 4% paraformaldehyde, the brain tissue of rat was rapidly removed from the cranial cavity. The brain tissue was treated with paraffin embedding, and the thickness of the slice was 5 μm. Then H.E was used to stain the brain slices and the light microscopy (OLYMPUS DP73, Japan) was applied to observed morphological changes of neurons.

2.6 Western blot

The proteins expression of hippocampus tissues were analyzed by Western blot (Zhang et al., 2017). PVDF membrane (Millipore, USA) was incubated in 5% skim milk dissolved in TBST buffer for 1 hour. Then primary antibodies p-Akt(ser^{473}), Akt, β-actin, Bcl-2, Bax, p-Erk1/2(Thr^{202}/Tyr^{204}) and Erk1/2 (Santa Cruz, CA) were used to incubate with PVDF membrane at 4 °C for 24 hours.

And the protein bands were marked by secondary antibody conjugation of horseradish peroxidase. The EC3 Imaging System (UVP, USA) was applied to observe PVDF membrane's blot images. And the protein bands density was quantified by Image J software.

2.7 Experimental procedures

The experimental procedures are presented in Fig. 1B. Following the model surgery 2VO, VD model rats were received intragastric (i.g) administration of bpV(pis) (1 mg/kg/day, 1 mL, dissolved in ethanol in sterile saline) for four successive weeks. Vehicle rats received the same dosage of saline containing of ethanol no containing of bpV(pis). On the 29^{th} day, the MWM tests were performed to estimate rat cognitive function for six days. After the MWM tests, rats were anesthetized, perfused with 4% paraformaldehyde, and obtained brain tissue from the cranial cavity. Then Western blot and he H.E staining experiments were carried out.

2.8 Statistical analysis

The data of this study were analyzed by GraphPad Prism 5.0 software. All the data were presented as the mean ± S.E.M. Two-way ANOVA determined the neurobehavioral evaluation. Western blot results were conducted via one-way ANOVA followed Tukey--Kramer tests. Statistical significance was placed at $P < 0.05$.

3. Results

3.1 BpV(pis) attenuates rat learning and memory impairment induced by 2VO

In the acquisition task test, no significant difference in escape latency was found between sham group and sham + bpV(pis) group during the training period, as shown in Fig. 2A ($P > 0.05$). However, the escape latency time for bpV(pis)-treated VD rats was significantly longer than for the sham group starting from the 2^{th} day of the acquisition task period ($P < 0.05$, 64.88 ± 2.047 s vs. 43.02 ± 1.48 s). The escape latency decreased significantly for

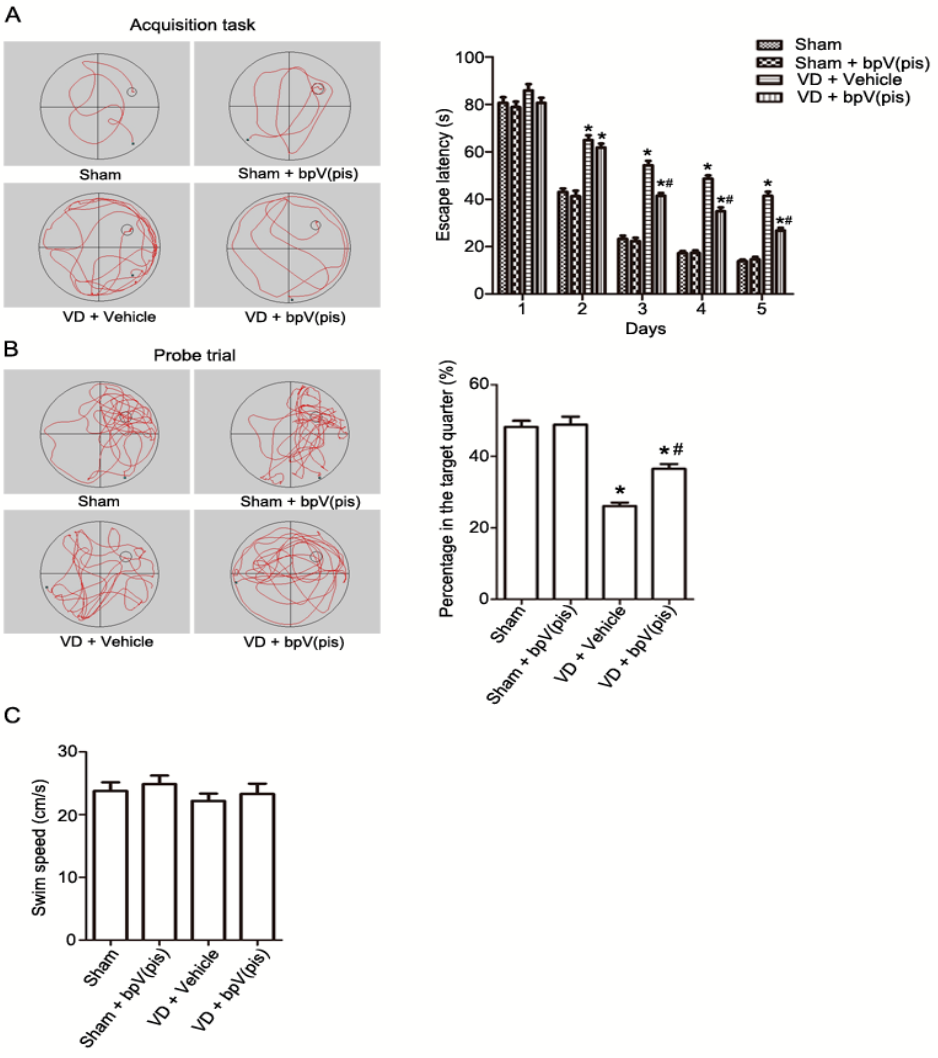

Figure 2. Effect of bpV(pis) on the spatial learning and memory functions of model rats induced by 2VO injury. The data were presented as means ± SEM. (A) Left: The sample tracks in the acquisition task test on day 3. (A) Right: The daily escape latencies of the acquisition task test (n = 10; $^{*}P < 0.05$ compared with sham; $^{\#}P < 0.05$ compared with VD + Vehicle group). (B) Left: The sample tracks in the probe test. (B) Right: The proportion of swimming time in the target area in the probe trial (n = 10; $^{*}P < 0.05$ compared with sham group; $^{\#}P < 0.05$ compared with VD + Vehicle group). (C) Swim speed among all four groups.

the VD - bpV(pis) group when compared with the VD + Vehicle group on day three (F = 107.00, $P < 0.05$, 41.45 ± 1.17 s vs. 54.28 ± 1.92 s), on day four ($P < 0.05$, 34.91 ± 1.58 s vs. 48.66 ± 1.34 s) and day five($P < 0.05$, 26.80 ± 1.10 s vs. 41.43 ± 1.71 s).

Results of the probe trial were shown Fig. 2B. The proportion of swimming time decreased significantly for the VD - Vehicle group when compared with the sham group (F = 44.78, $P < 0.05$, 48.19% ± 1.70% vs. 26.06% ± 0.96%). The bpV(pis)-treated VD rats performed better than the vehicle-treated VD rats ($P <$ 0.05) ($P < 0.05$, 36.49% ± 1.29% vs. 26.06% ± 0.96%). And there were no significant difference in swim speed among all the groups (Fig. 2C).

3.2 BpV(pis) alleviated pathological morphological damage of hippocampal neurons in VD model rats

As shown in Fig. 3, H.E. staining results showed that the CA1 (cornu ammonis 1) region neurons of hippocampus both sham and sham + bpV(pis) groups were well stained and normal microstructure, and exhibit clear boundaries, distinct layers and complete and orderly overall structure. However, in the VD + Vehicle group, obvious pathological changes were obvious, such as cytoplasmic hyperchromatism, neuronal shrinkage, loose arrangement, and irregular overall structure. Administration of bpV(pis) to VD rats, alleviated these morphological changes.

3.3 Treatment with bpV(pis) suppresses apoptotic signaling in VD model rats

As shown in Fig. 4, Western blot was applied to test the protein abundance of Bax and Bcl-2. And the ratio of Bax/Bcl-2 was used to characterize the level of apoptosis within each group. No significant difference was found in the Bax/Bcl-2 ratio between sham and sham + bpV(pis) groups. Compared with the sham group, the VD + Vehicle group exhibited a significantly increased ratio (F = 145.50, $P < 0.05$, 100.00% ± 0.00% vs. 388.40% ± 18.99%). However, the increase of the Bax/Bcl-2 ratio induced by 2VO was suppressed when the VD model rats treated with bpV(pis) ($P <$ 0.05, 388.40% ± 18.99% vs. 181.20% ± 10.91%).

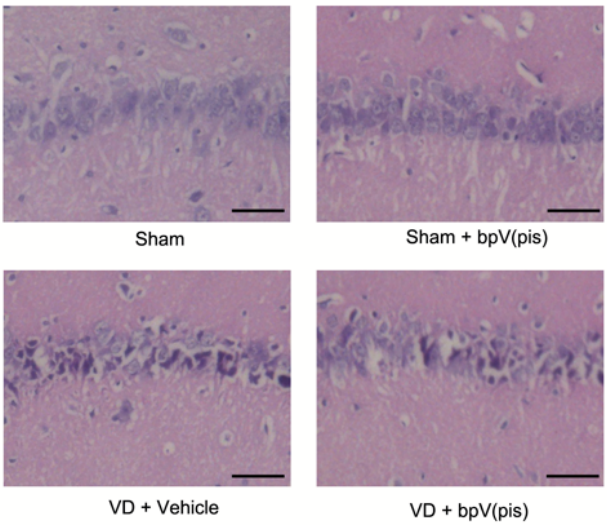

Figure 3. Representative images of HE staining to reveal the effects of bpV(pis) on the morphological changes of the CA1 (cornu ammonis 1) region neurons of hippocampus in the VD model rats. The characteristics of normal cells include clear boundaries, distinct layers and complete and orderly overall structure. However, the damaged neurons are characterized by cytoplasmic hyperchromatism, neuronal shrinkage, loose arrangement, and irregular overall structure. Scale bar = 20 μm (n = 4 for each group).

3.4 BpV(pis) reverses decreased p-Akt(ser473) in hippocampus neurons in following 2VO injury

The level of p-Aktser^{473} was measured by Western blot assay (Fig. 5A-B). Compared with the sham group, no significant difference was observed in the sham + bpV(pis) group. However, results showed no significant difference between the sham group and sham + bpV(pis) groups, whereas vehicle-treated VD rats showed a decreased level of p-Akt (ser473) when compared with sham rats (F = 82.07, $P < 0.05$, 37.90% ± 2.56% vs. 100.00% ± 0.00%). In comparison, a decreased level of p-Akt(ser473) induced by 2VO was reversed when VD rats were treated with bpV(pis) (75.40% ± 3.34%).

3.5 Treatment with bpV(pis) attenuates Erk1/2 inactivation of hippocampus neurons in VD model rat

Western blot assay was applied to assess the level of p-Erk1/2$^{Thr202/Tyr204}$ in a Western blot assay (Fig. 5C-D). No significant difference was observed between the sham group and sham + bpV(pis) groups. 2VO treatment suppressed the expres-

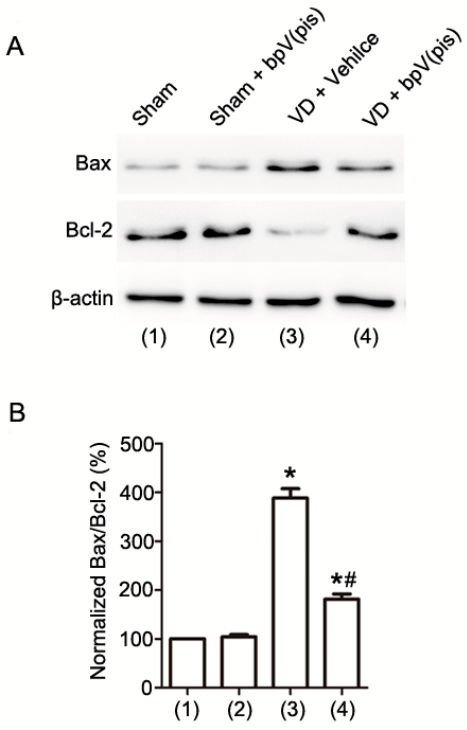

Figure 4. Effect of bpV(pis) on the protein abundance of Bax and Bcl-2 of neurons in the hippocampus. (A) Representative immunoblot of Bax and Bcl-2 from Western blot assay. (B) Bar graph showing the Bax/Bcl-2 ratio (n = 6; $^{*}P < 0.05$ compared with Sham group; $^{\#}P < 0.05$ compared with VD + Vehicle group).

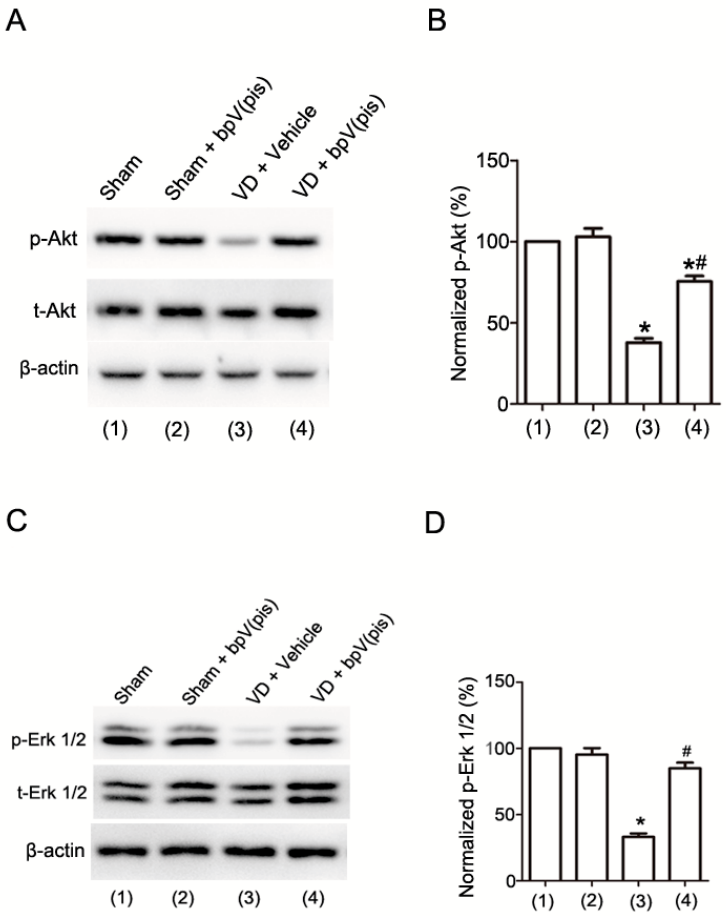

Figure 5. Effect of bpV(pis) on the protein abundance of p-Akt and p-Erk1/2 in 2VO-induced injury. (A) Representative immunoblot of p-Akt and t-Akt from Western blot assay in the hippocampus. (B) Bar graph showing protein level of p-Akt and t-Akt. (n = 6; $^{*}P < 0.05$ compared with sham; $^{\#}P < 0.05$ compared with VD + Vehicle). (C) Representative immunoblot of p-Erk1/2t and t-Erk1/2 from Western blot assay in the hippocampus. (D) Bar graph showing protein level of p-Erk1/2t and t-Erk1/2 (n = 6; $^{*}P < 0.05$ compared with sham; $^{\#}P < 0.05$ compared with VD + Vehicle).

sion of p-Erk1/2$^{Thr202/Tyr204}$ in the hippocampus when compared to the sham group (F = 74.04, $P < 0.05$, 33.10% ± 2.68% vs. 100.00% ± 0.00%). However, bpV(pis) treatment (84.80% ± 4.38%) markedly attenuated the 2VO-induced inactivation of Erk1/2.

4. Discussion

Squaramide is a kind of common bioactive group. Some compounds derivatized from squaramide are known to be characteristically highly activated, exhibit strong stability, low cost and easy synthesis. Additionally, it has been reported that phosphatase and tension homolog deleted on chromosome ten (PTEN) inhibition induces neuroprotection in ischemic stroke (Liu et al., 2010, 2018; Zhang et al., 2017) and bisperoxovanadium compounds not only inhibit phosphatase activity of PTEN (Schmid et al., 2004), but also increases the protein kinase activity of Akt (Stambolic et al., 1998). Hence, the novel squaramide compound, bpV(pis), was designed and synthesized including the two compounds of pyridine-2-squaramide and V_2O_5. Moreover, it has previously been reported that bpV(pis) confers neuroprotection in cerebral ischemia injury (Zhang et al., 2017).

The effect of bpV(pis) was tested for the first time in the vascular dementia rats induced by 2VO. Consistent with previous findings, animal spatial cognitive function and memory functions and morphological structure in hippocampus tissue were impaired in the VD model (Chen et al., 2018b; Hu et al., 2017; Li et al., 2017; Ma et al., 2018), whereas bpV(pis) treatment significantly alleviated cognitive impairment and the morphological changes induced by 2VO.

Apoptosis plays an important role in the pathophysiological mechanisms of VD (Ye et al., 2017). In agreement with previous findings, it was found that Bcl-2/Bax ratio was decreased in the hippocampus of VD animals (Chen et al., 2018b; Jiang et al., 2019) and bpV(pis) reversed this decrease of the Bcl-2/Bax expression ratio that was induced by 2VO in VD model rats.

Further, it was found that the protein level of both p-Akt(ser^{473}) and p-Erk1/2(Thr^{202}/Tyr^{204}) were decreased in VD model rats and bpV(pis) treatment significantly reduced this decrease induced by 2VO. Previous studies have demonstrated that PI3K/Akt and MAPK/ERK signaling pathways are both associated with neuronal

apoptosis. Activation of PI3K/Akt and Erk1/2 signaling pathways inhibit apoptotic pathways by phosphorylating many target molecules. Akt phosphorylates the apoptotic molecule Bad contributing to reduced cytochrome *c* release (Chan, 2004; White et al., 2000) and phosphorylates caspases-9 on Ser-196 to inhibit its proteolytic activity (Cardone et al., 1998; Chan, 2004). Erk1/2 inhibits the activation of Bad by phosphorylation of 90-kDa ribosomal S6 kinases (Chan, 2004; Frodin and Gammeltoft, 1999). Erk1/2 phosphorylation of Bad on Ser-112 mediates the inhibitory effects of transforming growth factor-β1 on Bad activity (Chan, 2004; Zhu et al., 2002). Therefore, it is probable that bpV(pis) inhibits neuronal apoptosis activation of Akt and Erk1/2 kinase, thus leading to enhanced neuronal survival.

In addition to inhibiting neuronal apoptosis by activating Akt and Erk1/2, previous studies have shown that activating Akt due to PTEN inhibition of the phosphatase activity of PTEN promotes neuronal survival by inhibiting a GluN2B-containing NMDA receptor (Ning et al., 2004), enhancing the expression and function of the GABAA receptor (Liu et al., 2010) and increasing TDP-43 nuclear translocation (Zheng et al., 2012). Other studies have demonstrated that up-regulated Erk1/2 activity mediates neuroprotective effects induced by some growth factors in an animal model of stroke (Buisson et al., 2003; Lin et al., 2015; Sansone et al., 2013; Zhu et al., 2002). Therefore, it is speculated here that the beneficial role induced by bpV(pis) may in part be due to inhibition of apoptosis by activation of Akt and Erk1/2 and partly due to activation of other pro-survival signaling pathways mediated by PI3K/Akt and MAPK/ERK pathways (Fig. 6). The detailed mechanisms remain to be further explored.

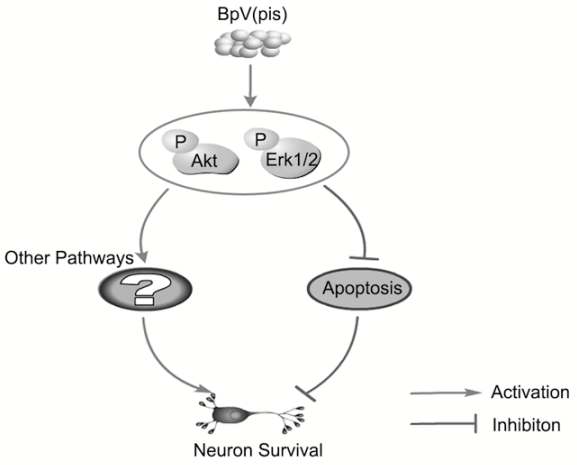

Figure 6. The mechanism of bpV(pis)-mediatized neuroprotection in 2VO-induced injury. The neuroprotective effect of bpV(pis) is partly due to apoptosis inhibition by activating Akt and Erk1/2, and partly due to activating other pro-survival signaling pathways mediated by PI3K/Akt and MAPK/ERK pathways.

Although this study shows that bpV(pis) confers neuroprotection against 2VO injury in VD male rats, there are still some shortcomings. Female rats, for instance, were not involved in the study. Research has shown that a combined rehabilitation paradigm, including physical (wheel running) and cognitive activity (modified Hebb--Williams maze), attenuates neuronal damage for male VD rats, but not for female VD rats. This indicates that it is necessity to consider bisexual animals in related preclinical experiments (Langdon et al., 2014). Additionally, older female rats may suffer various neurologic disorders due to cessation of ovarian estrogen production (Harrod et al., 2005; Henderson, 2005). However, it is worth mentioning that some studies have found that activation of PI3K/Akt and Erk1/2 pathways reduce the cognitive deficits of ovariectomized female rats (Kamel et al., 2018; Salas-Ramirez et al., 2015; Sun et al., 2019). Our results have shown that bpV(pis) also activates Akt and Erk1/2 pathways. Therefore, bpV(pis) may have further neuroprotective effects on female VD rats.

In conclusion, we have demonstrated bpV(pis) confers neuroprotection against 2VO injury through Akt and Erk1/2 activation. This finding provides experimental evidence that bpV(pis) may be as a potential drug candidate for VD.

Conflict of interest

The authors do not declare a conflict of interest.

Acknowledgments

This work was supported by grants from the Natural Science Foundation of Hubei Provincial Department of Education (B2018105), the Faculty Development Grants from Hubei University of Medicine (2017QDJZR11, 2018QDJZR09) and the Scientific and Technological Project of Shiyan City of Hubei Province (19Y10).

Submitted: October 07, 2019
Accepted: December 16, 2019
Published: December 30, 2019

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