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RESEARCH PAPER

Bisperoxovandium (pyridin-2-squaramide) targets both PTEN and ERK1/2 to confer neuroprotection

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BACKGROUND AND PURPOSE

We and others have shown that inhibiting phosphatase and tensin homolog deleted on chromosome 10 (PTEN) or activating ERK1/2 confer neuroprotection. As bisperoxovanadium compounds are well-established inhibitors of PTEN, we designed bisperoxovandium (pyridin-2-squaramide) [bpV(pis)] and determined whether and how bpV(pis) exerts a neuroprotective effect in cerebral ischaemia–reperfusion injury.

EXPERIMENTAL APPROACH

Malachite green-based phosphatase assay was used to measure PTEN activity. A western blot assay was used to measure the phosphorylation level of Akt and ERK1/2 (p-Akt and p-ERK1/2). Oxygen–glucose deprivation (OGD) was used to injure cultured cortical neurons. Cell death and viability were assessed by LDH and MTT assays. To verify the effects of bpV(pis) *in vivo*, Sprague–Dawley rats were subjected to middle cerebral artery occlusion, and brain infarct volume was measured and neurological function tests performed.

KEY RESULTS

bpV(pis) inhibited PTEN activity and increased p-Akt in SH-SY5Y cells but not in PTEN-deleted U251 cells. bpV(pis) also elevated p-ERK1/2 in both SH-SY5Y and U251 cells. These data indicate that bpV(pis) enhances Akt activation through PTEN inhibition but increases ERK1/2 activation independently of PTEN signalling. bpV(pis) prevented OGD-induced neuronal death *in vitro* and reduced brain infarct volume and promoted functional recovery in stroke animals. This neuroprotective effect of bpV(pis) was blocked by inhibiting Akt and/or ERK1/2.

CONCLUSIONS AND IMPLICATIONS

bpV(pis) confers neuroprotection in OGD-induced injury *in vitro* and in cerebral ischaemia *in vivo* by suppressing PTEN and activating ERK1/2. Thus, bpV(pis) is a bi-target neuroprotectant that may be developed as a drug candidate for stroke treatment.

Abbreviations

bpV(pis), bisperoxovandium (pyridin-2-squaramide); MCAO, middle cerebral artery occlusion; OGD, oxygen–glucose deprivation; PTEN, phosphatase and tensin homolog deleted on chromosome 10

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Tables of Links

TARGETS	
Akt (PKB)	PI3K
ERK1	PTEN
ERK2	

LIGANDS
Akt Inhibitor IV
LY294002
U0126

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual phosphatase that dephosphorylates both protein and lipid substrates (Schmid *et al.*, 2004). PTEN displays its lipid phosphatase activity by antagonizing Akt-dependent cell survival-promoting signalling via dephosphorylation of PIP_3 (phosphatidylinositol (3,4,5)-trisphosphate) (Maehama and Dixon, 1998; Yamada and Araki, 2001; Milella *et al.*, 2015). Akt is a multi-isoform serine/threonine kinase and a direct downstream target of PI3K (Schmid *et al.*, 2004; Chang *et al.*, 2007), and plays a pivotal role in promoting cell survival and growth (Chang *et al.*, 2007). There is substantial evidence indicating that inhibiting PTEN confers neuroprotection in ischaemia–reperfusion injury (Ning *et al.*, 2004; Chang *et al.*, 2007; Zhang *et al.*, 2007; Liu *et al.*, 2010a; Shi *et al.*, 2011; Zheng *et al.*, 2012). PTEN deletion or down-regulation has also been found to enhance axon regeneration after spinal cord injury (Liu *et al.*, 2010b; Zukor *et al.*, 2013; Lewandowski and Steward, 2014; Ohtake *et al.*, 2014; Singh *et al.*, 2014; Geoffroy *et al.*, 2015).

ERK1/2 is believed to mediate cell survival, neuronal plasticity and migration (Roux and Blenis, 2004; Kim *et al.*, 2005; Segarra *et al.*, 2006; Drosten *et al.*, 2010; Hao and Rockwell, 2013). In both *in vitro* and *in vivo* ischaemia stroke models, ERK1/2 signalling exerts neuroprotective effects in cerebral ischaemia–reperfusion injury (Chang *et al.*, 2004; Kilic *et al.*, 2005; Meller *et al.*, 2005; Zhang and Chen, 2008; Atif *et al.*, 2013; Nakano *et al.*, 2014; Buendia *et al.*, 2015; Koh, 2015).

In this study, we synthesized a new compound, bisperoxovandium (pyridin-2-squaramide) [bpV(pis)] and showed that it confers neuroprotection in ischaemia–reperfusion injury through inhibiting PTEN and activating ERK1/2.

Methods

The synthesis of bpV(pis)

V_2O_5 (1.43 g, 7.84 mmol) was dissolved in a solution of KOH (1.04 g, 18.53 mmol) in water (15 mL) and stirred for 5 min. Then 2 mL of a 30% ($w.v^{-1}$) aqueous solution of H_2O_2 was added to the resulting green solution, which resulted in effervescence. The mixture was next stirred for 25 min, which produced a progressive change in colour to orange and the complete dissolution of all solids. A further 12 mL of a 30% ($w.v^{-1}$) aqueous solution of H_2O_2 was added, and the reaction mixture was stirred for 15 min. At this point, a solution of pyridine-2-squaramide (2.98 g, 15.68 mmol) in a mixture of water (20 mL) and ethanol (5 mL) was added to the reaction mixture, which was stirred at room temperture (RT) for 30 min, after which consistent cloudiness was noted. The resulting pale yellow precipitate was filtered through a glass microfibre filter paper, washed with cold water and diethyl ether and dried overnight under reduced pressure (Rosivatz *et al.*, 2006).

PTEN activity assay

The ability of bpV(pis) to inhibit PTEN activity was assessed using a Malachite green-based phosphatase assay kit (Echelon Biosciences, Salt Lake City, UT, USA) following the manufacturer's instruction. PTEN enzyme and PIP_3 substrate were purchased from Echelon Biosciences (Salt Lake City, UT). This assay is based on the quantification of the phosphate liberated from $PI(3,4,5)P_3$, which forms a coloured complex with molybdate/malachite green after 20 min at room temperature; this is quantified by reading the absorbance at 620 nm. The % conversion of PIP_3 was determined by using the formula according to the manufacturer's instruction. Control wells containing PIP_3 only were set at 0% PTEN activity, and wells containing PTEN + PIP_3 were set at 100% PTEN activity. The IC_{50} value was calculated from the concentration–response curves generated using GraphPad Prism 5 software.

ERK1/2 activity assay

Recombinant ERK1/2 protein was purchased from Active Motif (Carlsbad, CA, USA). The ERK1/2 activity was tested using an ERK1/2 Assay Kit (Merck Millipore, Billerica, MA, USA) following the manufacturer's instructions. The activity of recombinant ERK1/2 was measured in assay dilution buffer I, pH 7.2, containing 75 mM magnesium chloride, 500 μM ATP, 2 $mg{\cdot}mL^{-1}$ dephosphorylated myelin basic protein (MBP) and bpV(pis) (0–100 μM). After a 20–30 min incubation at 30°C, 2.5 μL of the reaction mixture was removed and placed in a centrifuge tube. TBS and laemmli sample buffer were added. The sample was loaded for SDS-PAGE and immunoblot analysis, and probed with a monoclonal phospho-specific MBP antibody. The ERK activity is based

on the phosphorylation of MBP. The control group, which did not contain bpV(pis) was set at 100% of ERK1/2 activity. All experiments were repeated in triplicate. The quantification of immunoblot data was performed using ImageJ software.

Detection of bpV(pis) in SH-SY5Y cells

The SH-SY5Y cells were maintained at 8×10^5 cells per dish in 60 mm dishes. When the confluence of the SH-SY5Y cells reached 80–90%, the cells were washed three times in extracellular solution (ECS) (137 mM NaCl, 2.0 mM Ca^{2+}, 5.4 mM KCl, 1.0 mM $MgCl_2$, 25 mM HEPES and 33 mM glucose, titrated to pH 7.4 with an osmolarity of 300–320 mOsm) and then treated with bpV(pis) for 30 min. Excess bpV(pis) was removed by washing for five times with ECS, and then cells were collected for MS analysis.

The cells were incubated for 1 min with 0.25% trypsin, resuspended in 2 mL of DMEM and centrifuged at 1000 x *g* for 3 or 5 min. The precipitate was resuspended in 200 μL of chilled saline solution (0.9%) and ultrasonicated. The mixture was then centrifuged at 14000× *g* for 20 min at 4°C. The supernatant was mixed with 2 × volume of methanol and centrifuged to remove protein. [^{13}C]-bpV(pis) (purchased from Beita Pharmatech co. , LTD, Jiangyin, China) was dissolved in a mixture of acetonitrile and water (1:1, v:v) to a concentration of 5 μM. The supernatant (20 μL) was mixed with 5 μM [^{13}C]-bpV(pis) solution (20 μL) in a 500 μL centrifuge tube. The mixture (5 μL) was injected into the MS system. The signal intensity of [^{13}C]-bpV(pis) and bpV(pis) in the mixture was measured, and the intensity ratios of the signals produced by bpV(pis) relative to that of [^{13}C]-bpV(pis) (internal standard) were calculated. These ratios were assumed to reflect the relative proportions of the various compounds contained in the mixture. The relative intensity of [^{13}C]-bpV(pis) was 100%. The higher the ratio, the more bpV(pis) had crossed through the cell membrane.

Cell culture, transfection and treatment

For cell culture, SH-SY5Y and U251 cells were seeded in a 6-well plate (8×10^5 cells per well) in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U·mL^{-1}), streptomycin (100 mg·mL^{-1}) and L-glutamine (2.0 mM) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air (Wang *et al.,* 2015). When the confluence of cells reached 80–90% on the day of treatment, cells were washed with standard ECS for 60 min and then treated with bpV(pis) for 30 min. The cells were then collected for western blot analysis.

When the confluence of SH-SY5Y cells reached 60–70% on the treatment day, cells were transfected with human PTEN siRNA (siRNApten) and non-targeting control siRNA (NsiRNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 8 h. The medium was then replaced with normal growth medium for 24 h. On the following day, the cells were treated with standard ECS for 60 min and then treated with bpV(pis) for 30 min. The cells were then collected for western blot analysis.

Cortical neuron culture and OGD insult

The cortical neuronal cultures were prepared from Sprague–Dawley (SD) rats at gestation day 17 as described previously (Brewer *et al.,* 1993; Shan *et al.,* 2009). The pregnant rats were anaesthetized with 4% isoflurane in 70% N_2O and 30% O_2 and killed by cervical dislocation. The rats were sprayed with 70% ethanol, and the embryos were removed. The embryos were quickly decapitated, and the cortices were placed in ice-cold plating medium (Neurobasal medium, 2% B-27 supplement, 0.5% FBS, 0.5 mM L-glutamax and 25 mM glutamic acid) following the removal of the meninges. The cortical neurons were suspended in plating medium and plated on Petri dishes coated with poly-D-lysine. Half of the plating medium was removed and replaced with maintenance medium (Neurobasal medium, 2% B-27 supplement and 0.5 mM L-glutamine) in the same manner every 3 days. After 12 days, the cultured neurons were used for the experiments (Brewer *et al.,* 1993; Shan *et al.,* 2009).

For the oxygen–glucose deprivation (OGD) challenge, cells were transferred to deoxygenated, glucose-free, extracellular solution (in mM: 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.0 NaH_2PO_4, 1.8 $CaCl_2$ and 26 $NaHCO_3$), placed in a specialized, humidified chamber (Plas-Labs, Lansing, USA), and maintained at 37°C in 85% N_2/10% H_2/5% CO_2 for 60 min (Liu *et al.,* 2006; 2010a). Then the medium was replaced with fresh maintenance medium containing an appropriate concentration of reagents for 24 h during the recovery period in a 95% O_2/5% CO_2 incubator. The control cultures were firstly transferred to another extracellular solution (in mM: 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.0 NaH_2PO_4, 1.8 $CaCl_2$, 26 $NaHCO_3$ and 33 glucose), and then placed in the humidified chamber, which was maintained at 37°C in 95% O_2/5% CO_2 for 60 min (Liu *et al.,* 2006; 2010a). Then the medium was replaced with fresh maintenance medium for the whole period at 37°C in a 95% O_2/5% CO_2 incubator.

Western blot

Western blotting was performed as described previously (Ning *et al.,* 2004). Briefly, the PVDF membrane (Millipore, USA) was incubated with a primary antibody against phospho-ERK1/2 (Thr^{202}/Tyr^{204}), ERK1/2 from Cell Signaling Technology (Boston, MA) and an antibody against PTEN, phospho-Akt (Ser ^{473}), Akt, GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies were labelled with horseradish peroxidase-conjugated secondary antibody, and protein bands were imaged using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, USA). The EC3 Imaging System (UVP, USA) was used to obtained blot images directly from the PVDF membrane. The western blot data were quantified using ImageJ software.

Neuronal viability assays

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme retained by viable cells with intact plasma membranes and released from cells with damaged membranes. LDH release was measured using a CytoTox 96 Cytotoxicity kit by following the manufacturer's instructions (Promega, USA). The levels of maximal LDH release were measured by treating the cultures with 10 × lysis solution (provided by the manufacturer) to yield complete lysis of the cells. Absorbance data were obtained using a 96-well plate reader (Molecular Devices, USA) at 490 nm. According to the manufacturer's instructions, the LDH release (%) was measured

by calculating the ratio of experimental LDH release to maximal LDH release (Shan *et al.,* 2009).

The viability of the cells in the neuronal cultures was assessed by their ability to take up thiazolyl blue tetrazolium bromide (MTT). The cells were incubated with MTT for 1 h, then lysed with DMSO and left at room temperature in the dark overnight. The lysates were then read on a plate reader (PowerWave X, Bio-Tek, Winooski, State, USA) at an absorbance wavelength of 540 nm (Qu *et al.,* 2009; Chien *et al.,* 2015).

Animals

Adult male SD rats, weighing 250 to 300 g, were group-housed with 2–3 rats per cage on a 12 h light/dark cycle in a temperature-controlled room (23–25°C) with free access to food and water. Animals were allowed at least 3 days to acclimatize before experimentation. The total number of male rats used in our *in vivo* experiments was 224. Adult pregnant female rats (n = 10) and 36 embryos were used for cortical neuronal cultures. All animal use and experimental protocols were approved and carried out in compliance with the IACUC guidelines and the Animal Care and Ethics Committee of Wuhan University School of Medicine. Randomization was used to assign samples to the experimental groups and to collect and process data. The experiments were performed by investigators blinded to the groups for which each animal was assigned. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Focal cerebral ischaemia and infarct measurement

Transient focal cerebral ischaemia was induced using the suture occlusion technique (Longa *et al.,* 1989). Male SD rats weighing 250–300 g were anaesthetized with 4% isoflurane in 70% N_2O and 30% O_2 by using a mask. A midline incision was made in the neck, the right external carotid artery (ECA) was carefully exposed and dissected and a 3–0 monofilament nylon suture was inserted from the ECA into the right internal carotid artery to occlude the origin of the right middle cerebral artery (approximately 22 mm). After 90 min of occlusion, the suture was removed to allow reperfusion, the ECA was ligated and the wound was closed. Sham-operated rats underwent identical surgery and/or i.c.v. injections except that the suture was inserted and withdrawn immediately. Rectal temperature was maintained at 37.0 ± 0.5°C using a heating pad and heating lamp. At 24 h after middle cerebral artery occlusion (MCAO), rats (n=24) were decapitated after being reperfused with ice-cold 0.9% saline after anaesthetized with 4% isoflurane in 70% N_2O and 30% O_2, and the brains were rapidly removed for western blot analysis and 2,3,5-triphenyltetrazolium chloride (TTC) staining.

The peri-infarct tissues of ipsilateral hemispheres were homogenized in RIPA buffer using a tissue grinder on ice for 30 min. Then, the tissue lysates were centrifuged at 12000× g for 15 min at 4 C, and the total protein concentrations were assessed with a bicinchoninic acid protein assay kit.

Other groups of rats (n = 136) were killed, and the brains were removed for TTC staining to evaluate cerebral infarct volumes (Wexler *et al.,* 2002). The brain was placed in a cooled matrix, and 2 mm coronal sections were cut. Individual sections were placed in 10 cm petri dishes and incubated for 30 min in a solution of 2% TTC in PBS at 37°C. The slices were fixed in 4% paraformaldehyde at 4°C. All image collection, processing and analysis were performed in a blind manner and under controlled environmental lighting. The scanned images were analysed using ImageJ software, and the infarct data for all groups were expressed as the ratios of the infarcted areas to the total brain section areas (Wexler *et al.,* 2002).

Technique for i.c.v. administration

First the rat was anaesthetized with a mixture of 4% isoflurane in 70% N_2O and 30% O_2 in a sealed perspective plastic box. Once the rat was deeply anaesthetized, its head was shaved and secured in position in a stereotaxic frame using the ear bars and upper incisor bar; the rat was continuously under anaesthesia with 4% isoflurane using a mask. Then, a small mid-sagittal incision was made, and bregma was located as the anatomical reference point. Drug infusion, at a rate of 1.0 $\mu L \cdot min^{-1}$, to the cerebral ventricle (from the bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) was performed using a 23 gauge needle attached via polyethylene tubing to a Hamilton microsyringe. Proper needle placement was verified via withdrawing a few microlitres of clear cerebrospinal fluid into the Hamilton microsyringe.

Administration of bpV(pis) i.p.

bpV(pis) (20 mM) was dissolved in sterile saline containing DMSO and i.p. administered to rats at 1.0 h after ischaemia–reperfusion at concentrations of 20, 200 and 2000 $\mu g \cdot kg^{-1}$. Rats in the vehicle group received i.p. injections of the same volume of sterile saline containing a corresponding proportion of DMSO solution without bpV(pis).

Neurobehavioral tests

A total of 56 rats were used for neurobehavioral tests.

Neurological severity scores: The rats were subjected to a modified neurological severity score test as reported previously (Chen *et al.,* 2001). These tests are a battery of motor, sensory, reflex and balance tests, which are similar to the contralateral neglect tests in humans. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).

Beam walk test: The beam walk test measures the animals' complex neuromotor function (Aronowski *et al.,* 1996; Petullo *et al.,* 1999). The animal was timed as it walked a (100 × 2 cm) beam. A box for the animal to feel safe was placed at one end of the beam. A loud noise was created to stimulate the animal to walk towards and into the box (Aronowski *et al.,* 1996; Petullo *et al.,* 1999). Scoring was based upon the time it took the rat to go into the box (Petullo *et al.,* 1999). The higher the score, the more severe the neurological deficit.

Adhesive-removal test: A modified sticky-tape test was performed to evaluate forelimb function (Sughrue *et al.,* 2006). A sleeve was created using a 3.0 × 1.0 cm piece of yellow paper tape and was subsequently wrapped around

the forepaw so that the tape attached to itself and allowed the digits to protrude slightly from the sleeve. The typical response is for the rat to vigorously attempt to remove the sleeve by either pulling at the tape with its mouth or brushing the tape with its contralateral paw. The rat was placed in its cage and observed for 30 s. Two timers were started: the first ran without interruption and the second was turned on only while the animal attempted to remove the tape sleeve. The ratio of the left (affected)/right (unaffected) forelimb performance was recorded. The contralateral and ipsilateral limbs were tested separately. The test was repeated three times per test day, and the best two scores of the day were averaged. The lower the ratio, the more severe the neurological deficit.

Statistics

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis (Curtis *et al.,* 2015). The group size per experiment was based on a power analysis (Charan and Kantharia, 2013). The criterion for significance (α) was set at 0.05. The power of 80% was considered to yield a statistically significant result. Student's *t*-test or ANOVA was used where appropriate to examine the statistical significance of the differences between groups of data. Bonferroni tests were used for *post hoc* comparisons when appropriate. All results are presented as mean ± SEM. Significance was placed at $P < 0.05$.

Materials

bpV(pis) was synthesized in the Faculty of Pharmacy, Wuhan University School of Medicine. The PI3K inhibitor LY294002 was purchased from Cell Signaling Technology (Boston, MA). Akt Inhibitor IV was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ERK1/2 inhibitor U0126 was purchased from Selleckchem (Houston, TX).

Results

bpV(pis) enhances Akt activation through PTEN inhibition

To find a potential neuroprotectant that exerts its effect through PTEN inhibition, we designed and synthesized bpV(pis). The chemical equation and structure of bpV(pis) are shown in Figure 1A. Compound characterization of bpV(pis) was tested by infrared spectra analysis. The result obtained was shown as IR v (KBr) in cm^{-1}: v (NH_2) 3194,v (CN) 1617,v (VO) 960,v (OO) 816 cm^{-1} (Figure 1B). The molecular mass of bpV(pis) was tested by MS analysis. The result obtained was: MS (ESI) calcd for $C_9H_5KN_2O_8V$ $[M + H]^+$ 359.2, found 359.2; yield 76%.

We then measured the percentage inhibition of PTEN activity by using the Malachite green-based phosphatase assay. The IC_{50} value of bpV(pis) for PTEN activity was 39.44 ± 5.92 nM, indicating that bpV(pis) is markedly potent as inhibiting PTEN (Figure 1C).

Before testing the effects of bpV(pis) in cell and animal experiments, we examined whether bpV(pis) is able to traverse the cell membrane. As described in the section, we injected [^{13}C]-bpV(pis) and the SH-SY5Y cell extracts treated with bpV(pis) into a MS system. The signal intensity of [^{13}C]-bpV(pis) and bpV(pis) in the cell extracts was measured, and the intensity ratios of these signals relative to that of [^{13}C]-bpV(pis) (internal standard) were calculated. The higher the ratio, the more bpV(pis) passed through the cell membrane. The results showed that the signal intensity was detected in the cells treated with 10, 100 and 1000 nM bpV(pis), indicating that bpV(pis) can traverse the cell membrane (Figure 1D).

As PTEN negatively regulates Akt activation (Maehama and Dixon, 1998), we also tested the effect of bpV(pis) on PTEN inhibition by measuring Akt activation. Akt activation was quantified by measuring Akt phosphorylation (p-Akt) on Ser^{473} in a western blot assay (Shan *et al.,* 2009). The human neuroblastoma SH-SY5Y cells were washed with standard ECS for 60 min and then treated with bpV(pis) for 30 min. We showed that bpV(pis) markedly increased the level of p-Akt in SH-SY5Y cells (Figure 2A). Using a PTEN knockdown approach (Figure 2B), we showed that PTEN suppression by siRNApten increased the level of p-Akt (Figure 2C). However, bpV(pis) did not cause a further increase in p-Akt in SH-SY5Y cells transfected with siRNApten [siRNApten + bpV(pis) group in Figure 2C] compared with bpV(pis) treated SH-SY5Y cells transfected with the NsiRNA [NsiRNApten + bpV(pis) group in Figure 2C]. These results suggest that the bpV(pis)-induced increase in Akt activation in SH-SY5Y cells is mediated by the inhibition of PTEN.

To provide further evidence for the inhibition of PTEN by bpV(pis), SH-SY5Y cells were treated with LY294002 (10^{-2}–10^{2} μM) and incubated with or without 200 nM bpV(pis) for 30 min, followed by washing with standard ECS for 60 min. Western blotting assay was used to measure the percentage of p-Akt inhibition. As expected, bpV(pis) attenuated LY294002 (<10 μM)-induced Akt inhibition in SH-SY5Y cells (Figure 2D).

We also tested the effect of bpV(pis) on Akt phosphorylation in PTEN-deficient human glioblastoma U251 cells. We first confirmed that there was no PTEN expression in U251 cells (Figure 2E). We then showed that bpV(pis) (0.1, 1, 10, 100 and 1000 nM) did not affect Akt phosphorylation in U251 cells (Figure 2F). Together, these results indicate that bpV(pis) inhibits PTEN to enhance Akt activation.

bpV(pis) increases ERK1/2 activation independently of PTEN inhibition

bpV(pis) induced a marked elevation of ERK1/2 activation in SH-SY5Y cells (Figure 3A). The activation of ERK1/2 was measured by quantifying ERK1/2 phosphorylation (p-ERK1/2) on Thr^{202}/Tyr^{204} in a western blot assay (Figure 3A). We also measured the effect of PTEN knockdown on ERK1/2 phosphorylation in SH-SY5Y cells. PTEN suppression by siRNApten had no effect on p-ERK1/2 (Figure 3B). The bpV(pis)-induced increase in ERK1/2 phosphorylation was not affected by siRNApten transfection (Figure 3B). These data suggest that the elevation in ERK1/2 phosphorylation induced by bpV(pis) is independent of PTEN inhibition.

We further tested the effect of bpV(pis) (10, 20, 50, 100 and 200 nM) on ERK1/2 phosphorylation in PTEN-deficient U251 cells; bpV(pis) (20, 50, 100 and 200 nM) increased ERK1/2 phosphorylation in these cells (Figure 3C),

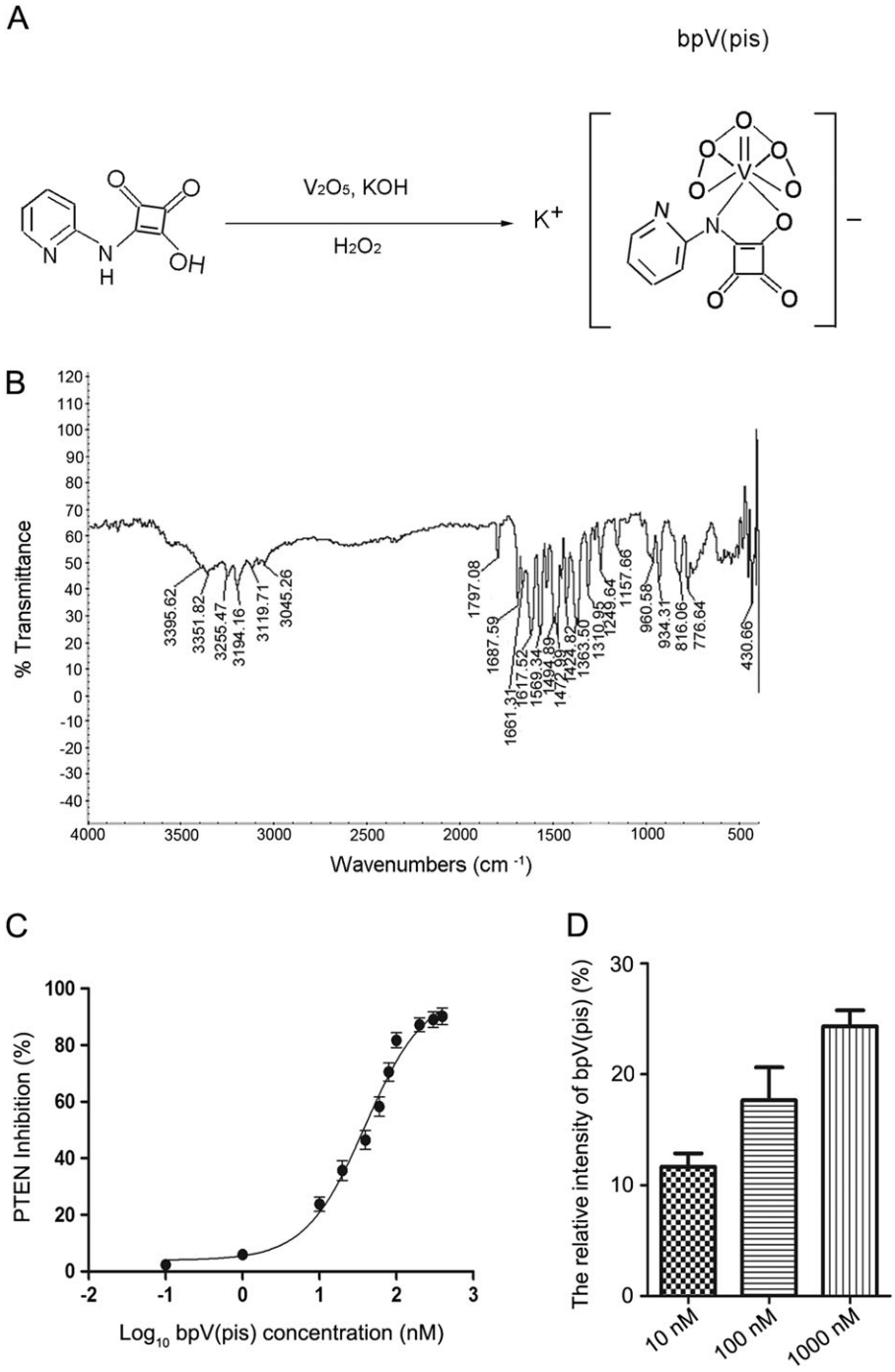

Figure 1

Characterization of bisperoxovandium (pyridin-2-squaramide) [bpV(pis)]. (A) The chemical equation and structure of bpV(pis). (B) Compound characterization of bpV(pis) was tested by infrared spectra analysis. The result was IR ν (KBr) in cm^{-1}: ν (NH_2) 3194,ν (CN) 1617,ν (VO) 960,ν (OO) 816 cm^{-1}. (C) Malachite green-based phosphatase assay showing the percentage of inhibition of PTEN activity by bpV(pis). The IC_{50} value of bpV(pis) for PTEN was 39.44 ± 5.92 nM. Three independent tests were performed. (D) bpV(pis) crosses through the cell membrane of SH-SY5Y cells. We used a MS system to detect the signal intensity of [^{13}C]-bpV(pis) and bpV(pis) from the cell extracts of SH-SY5Y cells treated with bpV(pis) (10, 100 and 1000 nM). The intensity ratios of these signals relative to that of [^{13}C]-bpV(pis) (internal standard) are shown; $n = 6$ independent cultures.

supporting the notion that bpV(pis) activates ERK1/2 independently of PTEN inhibition.

To verify whether bpV(pis) directly activates ERK1/2, we tested the effect of bpV(pis) on the activity of recombinant ERK1/2 *in vitro*. bpV(pis) did not alter the ERK1/2 activity (Figure 3D), indicating that another signalling pathway different from PTEN mediates the enhancement of ERK1/2 activation by bpV(pis) in SH-SY5Y and U251 cells.

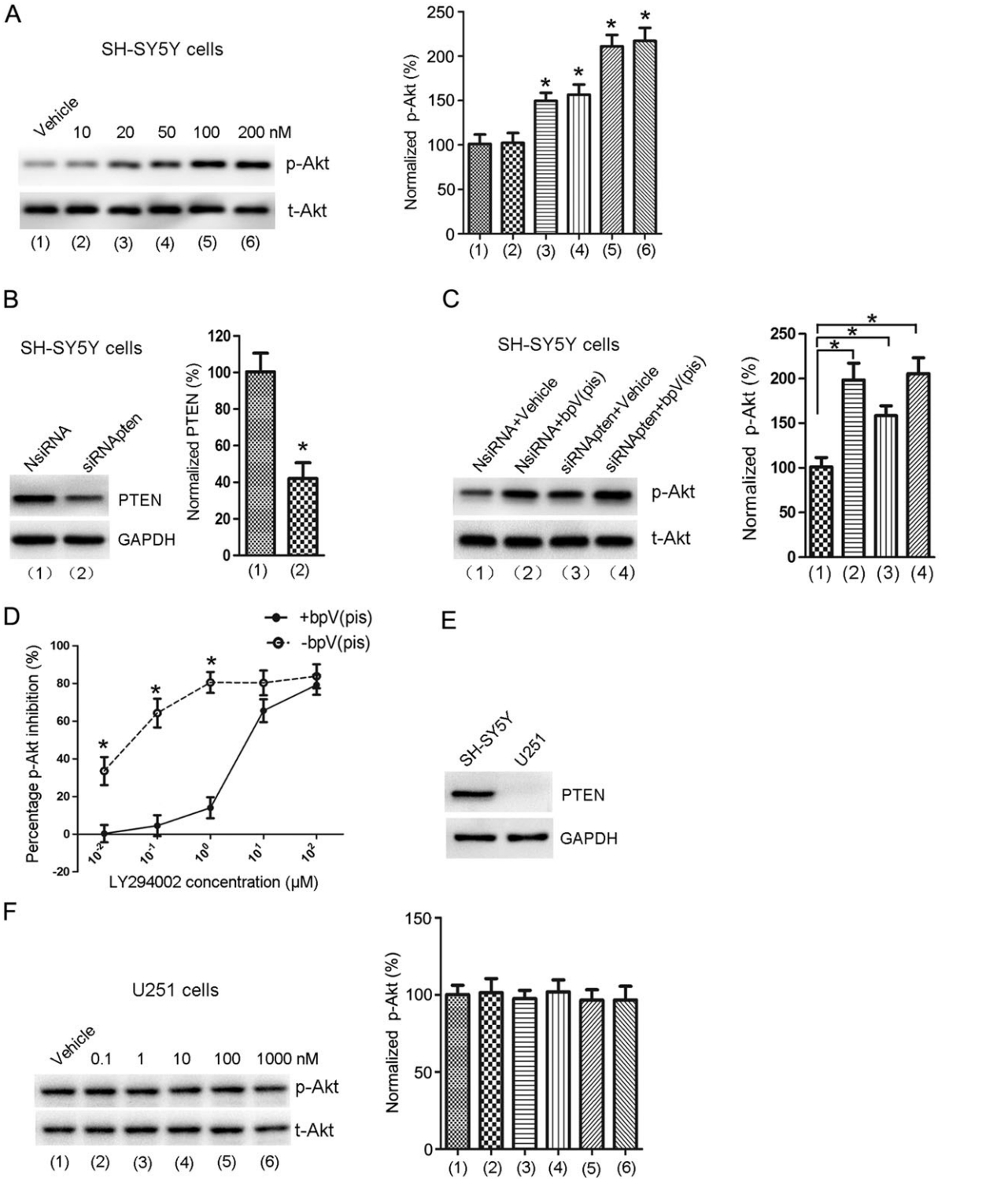

Figure 2

bpV(pis) enhances Akt phosphorylation through PTEN inhibition. (A) The level of p-Akt was increased in SH-SY5Y cells treated with bpV(pis) (20–200 nM). Data from each group are normalized to that of Vehicle group; $n = 7$ independent cultures, $^*P < 0.05$ versus Vehicle, one-way ANOVA test, followed by the Bonferroni *post hoc* test. (B) Treatment with siRNApten suppresses PTEN expression in SH-SY5Y cells. $n = 6$ independent cultures, $^*P < 0.05$ versus NsiRNA, Student's *t*-test. (C) The effect of bpV(pis) (200 nM) on p-Akt in SH-SY5Y cells transfected with siRNApten or NsiRNA; $n = 6$ independent cultures, $^*P < 0.05$ versus NsiRNA + Vehicle; one-way ANOVA test, followed by Bonferroni *post hoc* test. (D) bpV(pis) (200 nM) counteracts LY294002-induced inhibition of p-Akt in SH-SY5Y cells; $n = 7$ independent cultures, $^*P < 0.05$ versus bpV(pis), two-way ANOVAs, followed by Bonferroni *post hoc* test. (E) PTEN protein expression in SH-SY5Y and U251 cells. (F) p-Akt is not changed in U251 cells treated with bpV(pis) (0.1–1000 nM); $n = 6$ independent cultures.

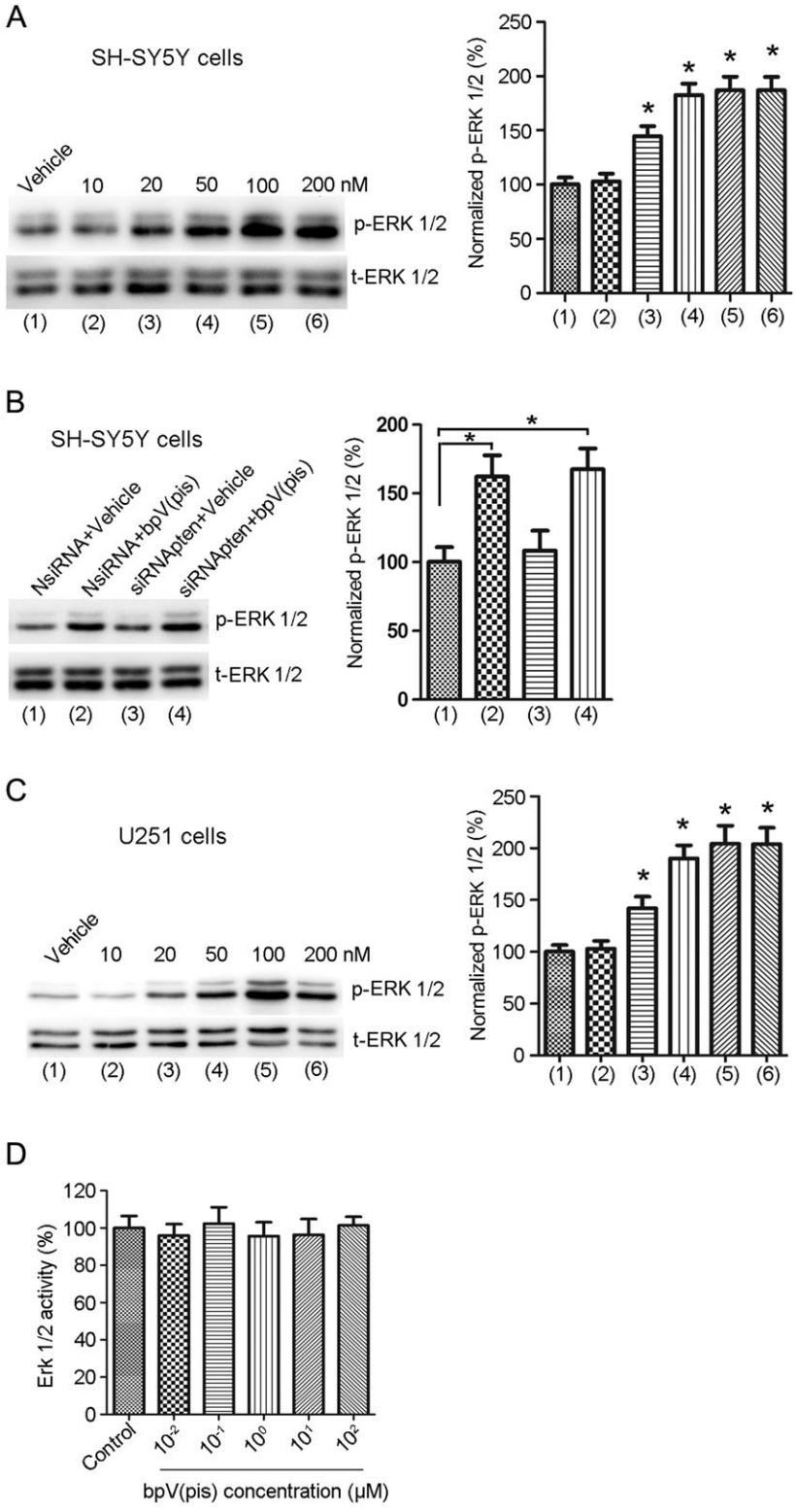

Figure 3

bpV(pis) increases p-ERK1/2 independently of PTEN inhibition. (A) The p-ERK1/2 is increased in SH-SY5Y cells treated with bpV(pis) (20–200 nM); $n = 7$ independent cultures, $^*P < 0.05$ versus Vehicle, one-way ANOVA, followed by the Bonferroni *post hoc* test. (B) The effect of bpV(pis) (200 nM) on p-ERK1/2 in SH-SY5Y cells transfected with siRNApten; $n = 6$ independent cultures, $^*P < 0.05$ versus NsiRNA + Vehicle; one-way ANOVA, followed by the Bonferroni *post hoc* test. (C) p-ERK1/2 is increased in U251 cells treated with bpV(pis) (20–200 nM); $n = 6$ independent cultures, $^*P < 0.05$ versus Vehicle, one-way ANOVA, followed by the Bonferroni *post hoc* test. (D) bpV(pis) does not activate ERK1/2 *in vitro*; $n = 6$ independent cultures.

bpV(pis) prevents the reduction in Akt and ERK1/2 phosphorylation in injured cortical neurons

The observed increase in p-Akt and p-ERK1/2 induced by bpV(pis) in normal conditions led us to determine whether bpV(pis) modulates Akt and ERK1/2 phosphorylation in injury conditions. To address this, we first tested the effect of bpV(pis) on p-Akt and p-ERK1/2 in OGD-induced neuronal injury, an *in vitro* model that mimics ischaemic neuronal injury. Both p-Akt and p-ERK1/2 were suppressed at 24 h after OGD injury in cortical neurons (Figure 4A, B). However, treatment with bpV(pis) at 30 min after re-oxygenation attenuated the reduction in p-Akt and p-ERK1/2 in OGD-treated neurons (Figure 4A, B).

Both PTEN/Akt and ERK1/2 signalling mediate bpV(pis)-induced neuroprotection in OGD insult

According the above findings (Figure 4A, B), we hypothesized that bpV(pis) might exert a neuroprotective effect in OGD-induced neuronal injury. We measured neuronal death/viability by LDH and MTT assay 24 h after OGD injury. Treatment with bpV(pis) (20, 50, 100 and 200 nM) 30 min after re-oxygenation decreased LDH release and increased the viability of the cells (Figure 5A, B). The beneficial effects of bpV(pis) (200 nM) were impaired after pretreatment with an Akt, Inhibitor IV (1.0 μM), and/or ERK1/2 inhibitor, U0126 (10 μM) (Figure 5C, D). Thus, both PTEN inhibition-mediated Akt activation and ERK1/2 activation contribute to bpV(pis)-induced neuroprotection.

bpV(pis) prevents the reduction in phosphorylation of Akt and ERK1/2 in rat cerebral ischaemia–reperfusion injury

To provide *in vivo* evidence for the role of bpV(pis) in neuroprotection, we tested the effect of bpV(pis) on p-Akt and p-ERK1/2 in the peri-infarct tissues of ischaemic brain in a rat MCAO model (Figure 6A) (Ashwal *et al.*, 1998; Sun *et al.*, 2003). At 1.0 h following ischaemia–reperfusion, bpV(pis) (100 μM, 5 μL) was administered into rat lateral ventricles. The phosphorylation of Akt and ERK1/2 was suppressed at 24 h after ischaemia onset (Figure 6B, C), whereas bpV(pis) treatment reduced the decrease in p-Akt and p-ERK1/2 in the ischaemic brain (Figure 6B, C).

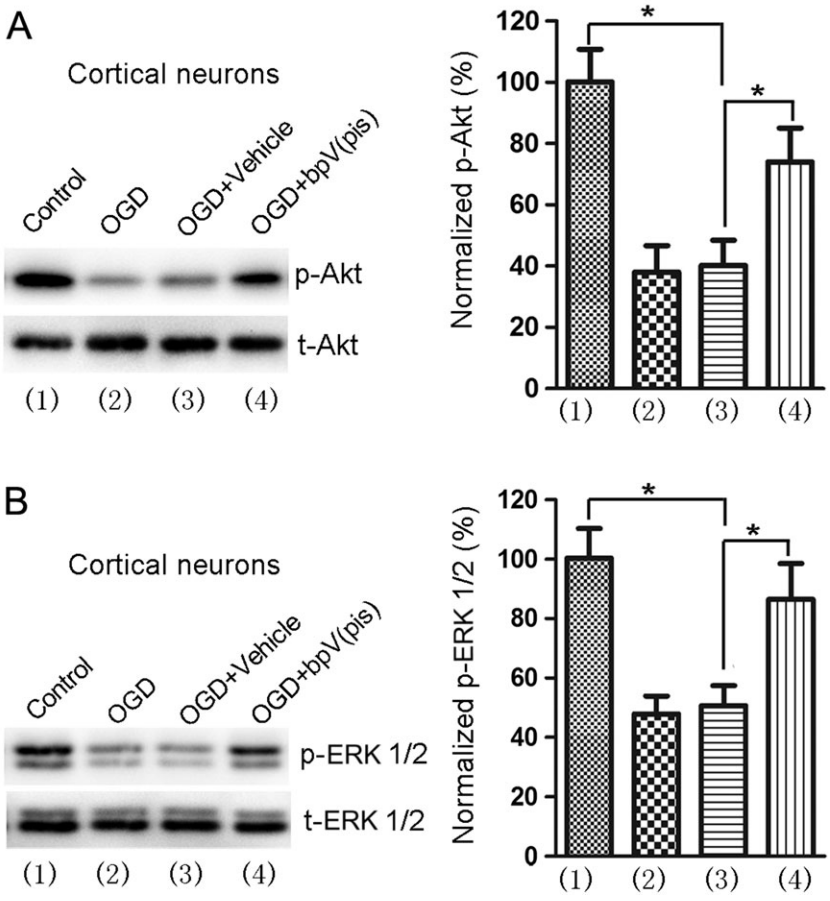

Figure 4

OGD-induced decrease in p-Akt and p-ERK1/2 is reduced by bpV(pis). (A), (B) bpV(pis) (200 nM) attenuates OGD-induced reduction of p-Akt and p-ERK1/2 in cortical neurons; $n = 6$ independent cultures, $^*P < 0.05$, one-way ANOVA, followed by the Bonferroni *post hoc* test.

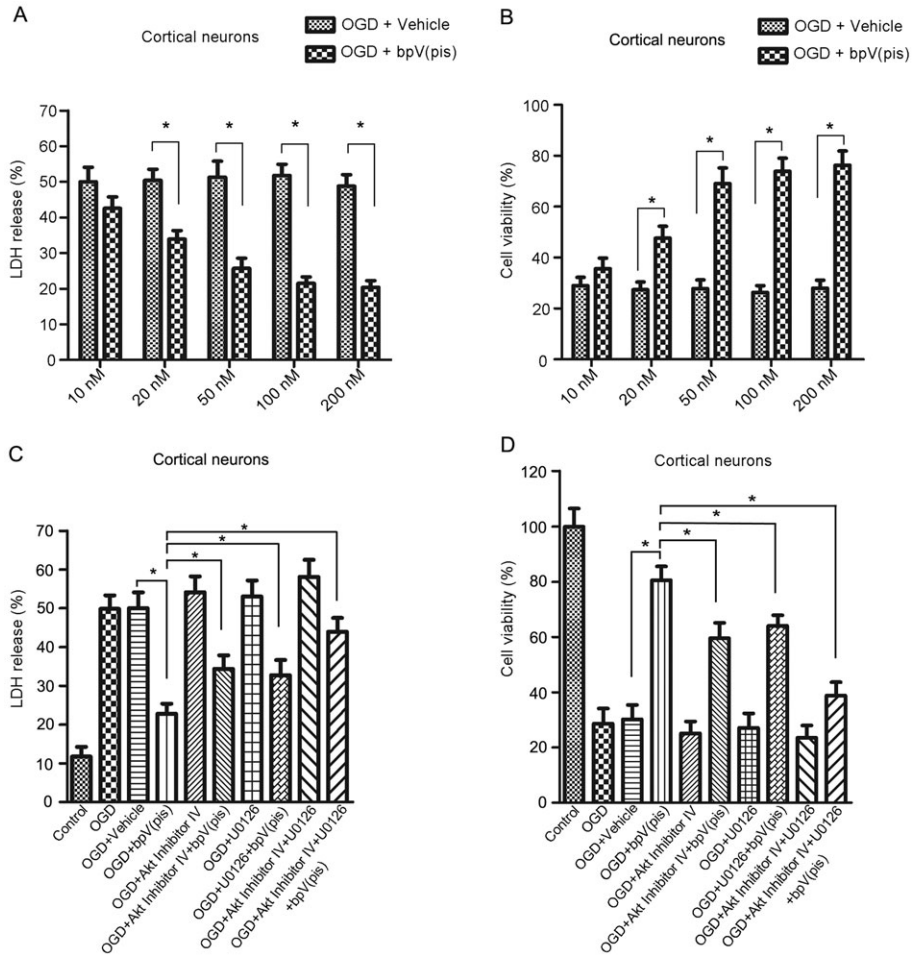

Figure 5

bpV(pis) protects against OGD-induced neuronal death through PTEN inhibition and ERK1/2 activation. (A, B) bpV(pis) (20–200 nM) decreased LDH release (A) and increased cell viability (B) during OGD-induced neuronal death; $n = 7$ independent cultures. The tests were performed in quintuplicate. *$P < 0.05$ versus OGD + Vehicle, two-way ANOVA, followed by the Bonferroni *post hoc* test. (C, D) Treatment with bpV(pis) (200 nM) at 30 min after re-oxygenation in cultured cortical neurons decreased LDH release (C) and increased cell viability (D), and the neuroprotective effect of bpV(pis) was blocked by Akt Inhibitor IV (1.0 μM) and ERK1/2 inhibitor U0126 (10 μM); $n = 7$ independent cultures. The tests were performed in quintuplicate. *$P < 0.05$, one-way ANOVA, followed by the Bonferroni *post hoc* test. The cortical neurons were treated with Akt Inhibitor IV and U0126 at 20 min before bpV(pis) treatment.

Both PTEN/Akt and ERK1/2 signalling mediate bpV(pis)-induced neuroprotection in cerebral ischaemia–reperfusion injury

To test whether bpV(pis) conferred neuroprotection in ischaemic stroke. We measured the effect of bpV(pis) on ischaemic neuronal death in rats subjected to MCAO. The experimental procedures are shown in Figure 7A. bpV(pis), (100 μM, 5 μL, i.c.v.) injected 1.0 h after ischaemia–reperfusion, decreased the infarct volume at 24 h after ischaemia onset (Figure 7B). Animals treated with bpV(pis) (20, 200 and 2000 $\mu g \cdot kg^{-1}$) by i.p. injections 1.0 h after ischaemia–reperfusion also had a significantly reduced infarct volume (Figure S1).

To investigate whether the decrease in infarct volume induced by bpV(pis) was mediated through both the PTEN/Akt and/or ERK1/2 signalling pathway, we suppressed the activity of Akt and ERK1/2 by injecting Akt Inhibitor IV (100 μM, 2 μL) and/or U0126 (500 μM, 2 μL) into the lateral ventricles 30 min before the bpV(pis) injection (Pignataro *et al.*, 2008; Liu *et al.*, 2013). This pre-injection of Akt Inhibitor IV and/or U0126 prevented the bpV(pis)-induced decrease of infarct volume (Figure 7B).

We also performed a battery of neurobehavioral tests to evaluate the functional effect of bpV(pis). As illustrated in Figure 8A–C, bpV(pis) improved the functional outcome of stroke animals by effects mediated through both the PTEN/Akt and ERK1/2 signalling pathways.

Discussion

PTEN is a tumour suppressor but plays an important role in neurological diseases (Endersby and Baker, 2008; Kreis *et al.*,

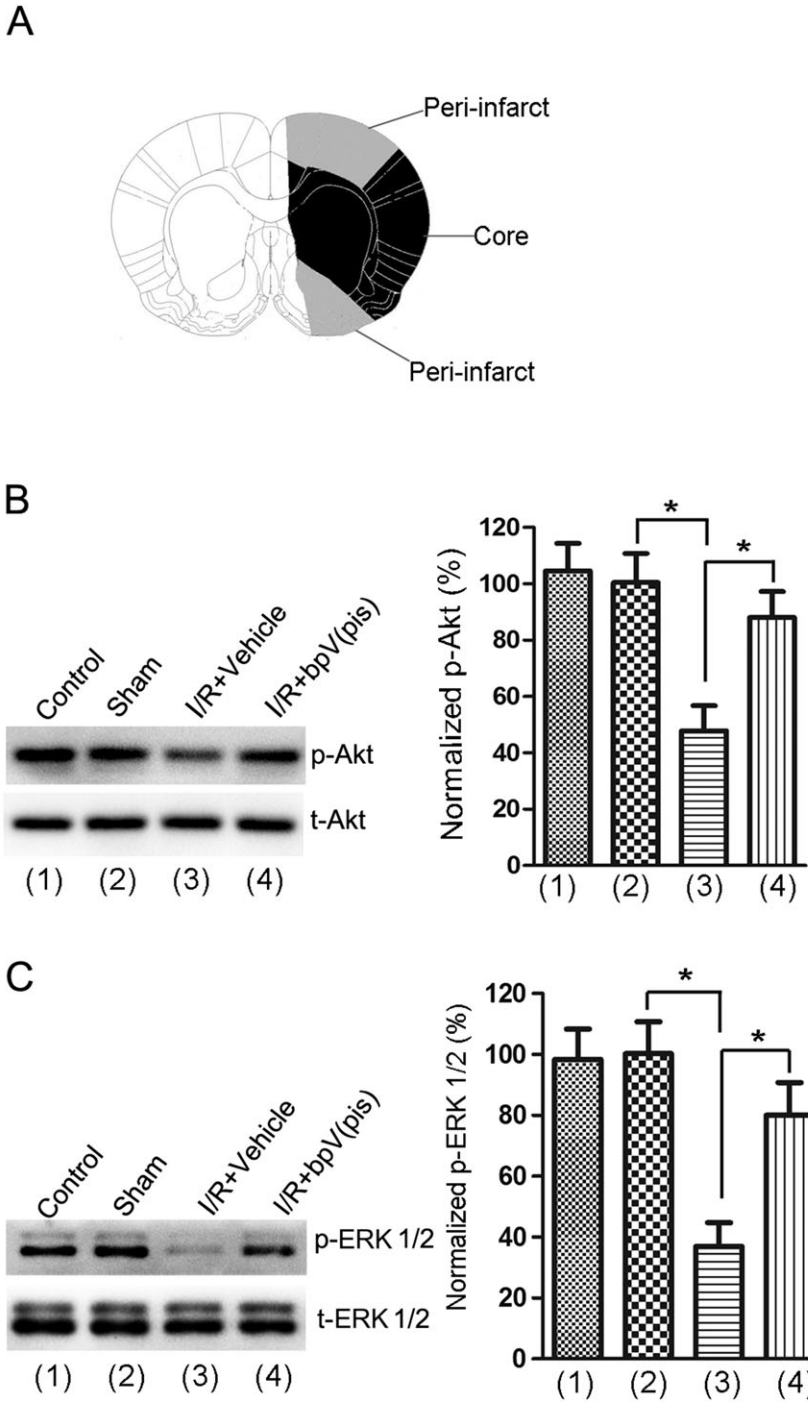

Figure 6

bpV(pis) blocked the reduction in p-Akt and p-ERK1/2 in rat cerebral ischaemia–reperfusion injury. (A) Diagram shows the dissected ischaemic peri-infarct. (B, C) At 1.0 h following ischaemia–reperfusion, treatment with bpV(pis) (100 μM, 5 μL) prevents the decrease in p-Akt (B) and p-ERK1/2 (C) at 24 h after ischaemia onset in the ischaemic peri-infarct (n = 8 for each group; *$P < 0.05$, one-way ANOVA, followed by the Bonferroni *post hoc* test). I/R, ischaemia–reperfusion.

2014; Weston *et al.,* 2014; Stavarache *et al.,* 2015). In our previous study, we demonstrated that suppressing the lipid phosphatase activity of PTEN activates Akt and suppressing the protein phosphatase activity of PTEN inhibits extrasynaptic GluN2B-containing NMDA receptors, which lead to the protection against ischaemic neuronal death (Ning *et al.,* 2004). We further showed that activation of GluN2A-containing NMDA receptors exerts

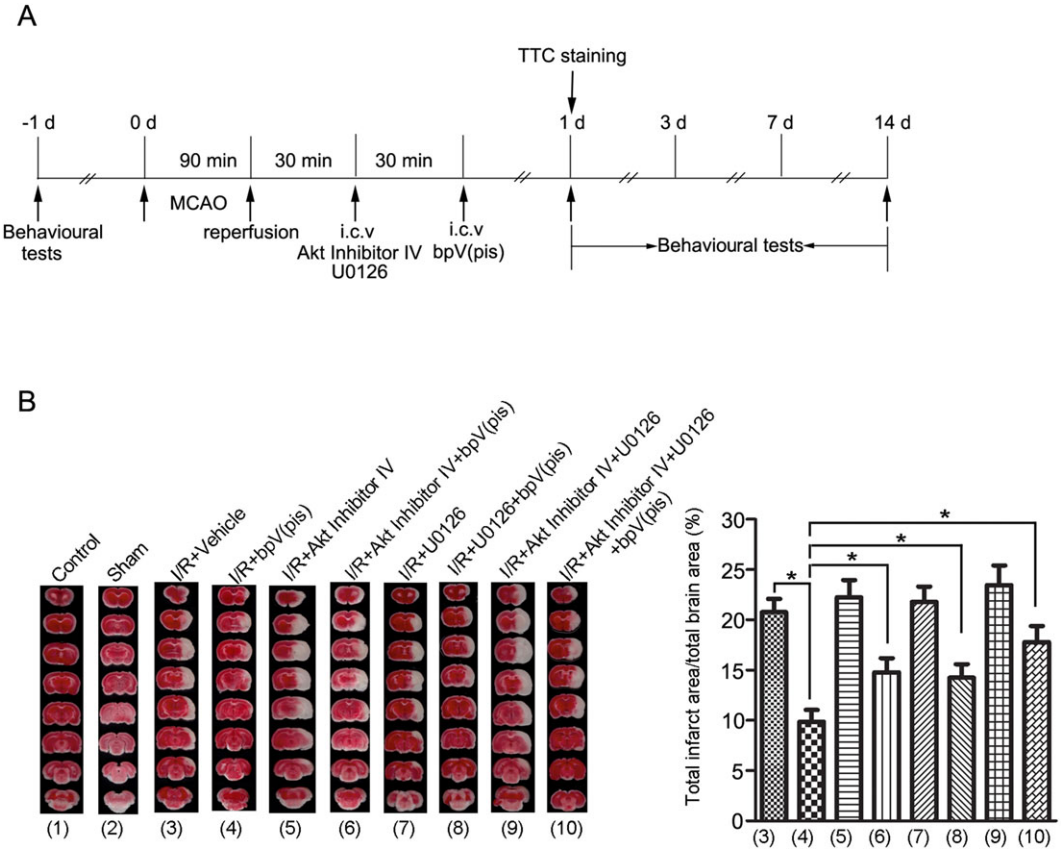

Figure 7

bpV(pis) reduces the infarct volume in MCAO rats through PTEN inhibition and ERK1/2 activation. (A) Diagram shows the experimental procedures in MCAO rats. All groups of animals were subjected to two i.c.v. injections. Akt Inhibitor IV (100 μM, 2 μL) or U0126 (500 μM, 2 μL)] was given at 30 min after reperfusion as the first injection, and bpV(pis) (100 μM, 5 μL) was given at 60 min after reperfusion as the second injection.(B) Sample images and the summarized data of TTC staining indicate that bpV(pis) reduces the infarct volume in the rat brain 24 h after ischaemia onset and that pretreatment with Akt Inhibitor IV and/or U0126 block the bpV(pis)-induced reduction in infarct volume ($n = 10$ for each group; $^*P < 0.05$, one-way ANOVA, followed by the Bonferroni *post hoc* test). I/R, ischaemia–reperfusion.

neuroprotective effects through suppression of PTEN and subsequent increase in nuclear TDP-43 (TAR DNA-binding protein-43) (Zheng *et al.,* 2012). We also showed that inhibiting PTEN, by enhancing the expression and function of the $GABA_A$ receptor, protects against neuronal death in the *in vitro* OGD and *in vivo* ischaemic stroke models (Liu *et al.,* 2010a). Other studies have provided substantial evidence to support the notion that inhibiting PTEN confers neuroprotection in ischaemia–reperfusion injury (Howitt *et al.,* 2012; Zhang *et al.,* 2013). The results of the present study suggest that bpV(pis) inhibits PTEN to confer neuroprotection.

ERK1/2 activation has been reported to have a neuroprotective effect. A number of growth factors are shown to reduce ischaemic damage by up-regulating ERK1/2 activity (Zhu *et al.,* 2002; Buisson *et al.,* 2003; Sansone *et al.,* 2013). Oestrogen, osteopontin, leptin, melatonin derivative Neu-P11 and apelin-13 are also known to confer protection through ERK1/2 activation (Guerra *et al.,* 2004; Meller *et al.,* 2005; Zhang and Chen, 2008; Yang *et al.,* 2014; Buendia *et al.,* 2015). Our findings indicate that bpV(pis) protects against ischaemic neuronal death by activating ERK1/2, providing evidence to support the neuroprotective role of ERK1/2 in ischaemic stroke.

However, the activation of ERK1/2 by bpV(pis) is neither through PTEN inhibition nor via direct activation. MAP kinase is a family of serine/threonine protein kinases. The Raf protein, an up-stream activator of MAP kinase, activates MEK1/2 through phosphorylation and then phosphorylated MEK1/2 phosphorylates and activates ERK1/2 (Lake *et al.,* 2016). It is possible that the activation of ERK1/2 by bpV(pis) might depend upon the Raf/MEK/ERK1/2 and other signalling pathways. The detailed mechanisms remain to be further explored.

Bisperoxovanadium compounds belong to the class of vanadate derivatives that are well-established inhibitors of protein tyrosine phosphatases and PTEN (Schmid *et al.,* 2004). However, the mechanisms underlying the effect of bisperoxovanadium compounds have not been clarified. Squaramide is a functional group existing in many bioactive compounds. Some squaramide-based compounds have anti-leishmanial activity (Marin *et al.,* 2016). Both *in vitro* and *in vivo* studies have confirmed that N,N′-squaramides are effective against Chagas disease (Olmo *et al.,* 2014). The squaric

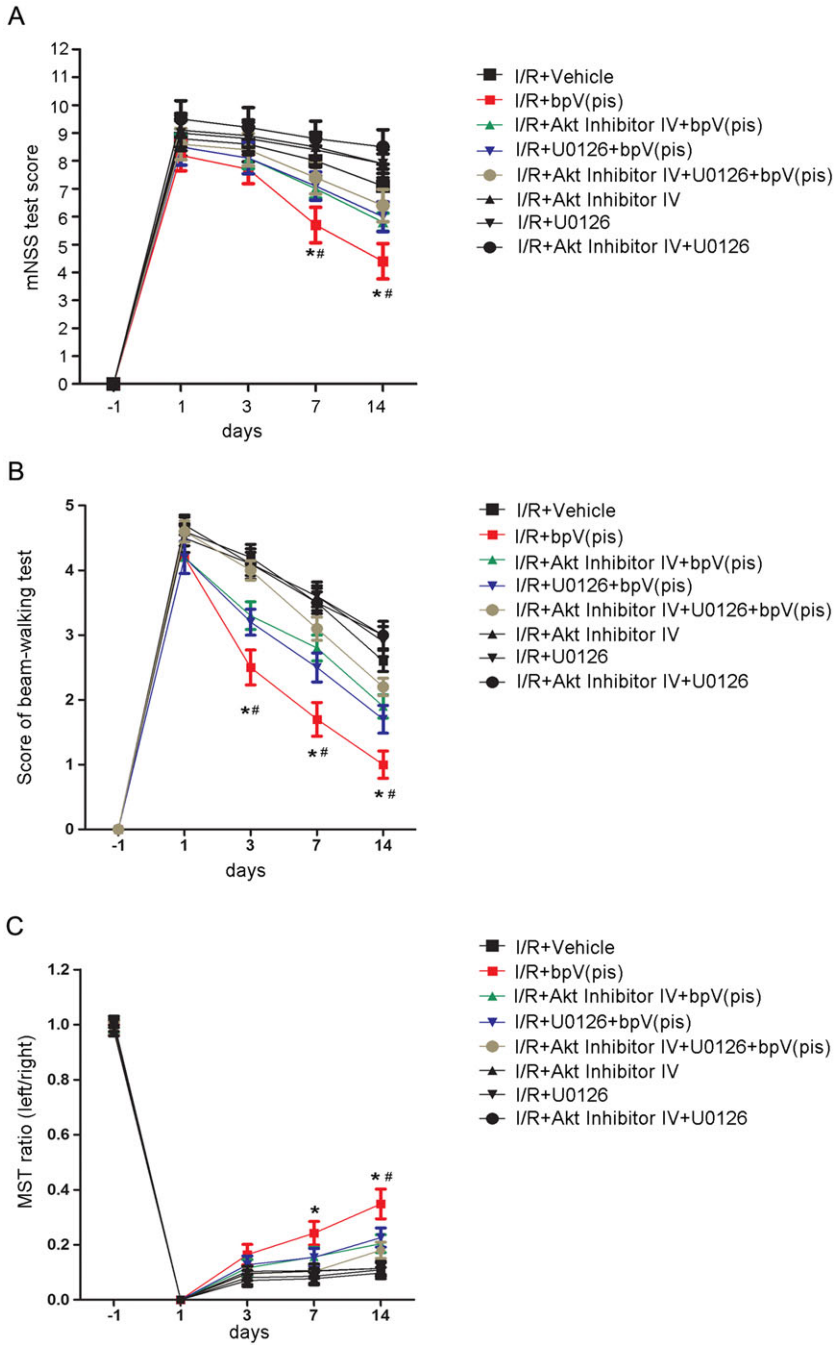

Figure 8

bpV(pis) improves neurological function in ischaemia–reperfusion injury by inhibiting PTEN and activating ERK1/2. (A) Animals treated with bpV(pis) have lower scores of modified neurological severity score (mNSS) test at days 7 and 14 after MCAO. Animals pre-injected with Akt inhibitor IV and/or U0126 have higher scores of mNSS test at days 7 and 14 after MCAO [$n = 7$ for each group; $^{*}P < 0.05$, vs. I/R + Vehicle, $^{\#}P < 0.05$, vs. I/R + bpV(pis), two-way ANOVA, followed by the Bonferroni *post hoc* test]. (B) Animals treated with bpV(pis) have lower scores in the beam-walking test at days 3, 7 and 14 after MCAO. Animals pre-injected with Akt inhibitor IV and/or U0126 have higher scores in the beam-walking test at days 3, 7 and 14 after MCAO [$n = 7$ for each group; $^{*}P < 0.05$, vs. I/R + Vehicle, $^{\#}P < 0.05$, vs. I/R + bpV(pis), two-way ANOVA, followed by the Bonferroni *post hoc* test]. (C) Animals treated with bpV(pis) have a higher ratio in the modified sticky-tape (MST) test at days 7 and 14 after MCAO. Animals pre-injected with Akt inhibitor IV and/or U0126 have lower ratio in the MST test at day 14 after MCAO [$n = 7$ for each group; $^{*}P < 0.05$, vs. I/R + Vehicle, $^{\#}P < 0.05$, vs. I/R + bpV(pis), two-way ANOVA, followed by the Bonferroni *post hoc* test]. I/R, ischaemia–reperfusion.

acid-containing glutamate analogue exhibits a high affinity for NMDA receptors (Chan *et al.*, 1995) and squaric acid dibutylester is a topical sensitizing agent utilized for the treatment of alopecia areata (Hill *et al.*, 2015). Hence, because of its functional activity, stability, low cost of starting materials and straightforward synthesis, squaramide is an

appropriate molecule for the development of affordable agents. We therefore designed and synthesized the compound, bpV(pis), in the presence of pyridine-2-squaramide and V_2O_5.

In conclusion, the results of the present study demonstrate for the first time that a new compound bpV(pis) confers neuroprotection both through suppressing PTEN and increasing the activation of ERK1/2 in cerebral ischaemia–reperfusion injury. These findings provide experimental evidence to support the development of bpV(pis) as a potential drug candidate for ischaemic stroke.

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Author contributions

Z.F.Z., J.C., X.H., Y.Z., H.B.L., R.X.L. and Z.F.W. conducted the experiments, acquired and analysed the data. Z.Q.L., W. J.L., F.L., H.B.Z. and Q.W. conceived the project and designed the experiments. Z.F.Z., J.C. and Q.W. wrote the manuscript. Z.Q.L., W.J.L., F.L. and H.B.Z. reviewed and edited the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Figure S1 Sample images (above) and the summarized data (below) of TTC staining show that intraperitoneal injections of bpV(pis) (20–2000 μg/kg) at 1.0 h after I/R reduce the infarct volume at 24 h after ischaemia onset (n = 8 for each group; *P < 0.05 vs. I/R + Vehicle, two-way ANOVA test, followed by Bonferroni *post hoc* test). Vehicle rats received intraperitoneal injections of corresponding proportion of DMSO solution dissolved in 0.9% saline. I/R: ischaemia–reperfusion.