Cell Mol Neurobiol (2017) 37:655–664
DOI 10.1007/s10571-016-0402-z

ORIGINAL RESEARCH

Indirubin-3-Oxime Prevents H_2O_2-Induced Neuronal Apoptosis via Concurrently Inhibiting GSK3β and the ERK Pathway

Jie Yu[1] · Jiacheng Zheng[1] · Jiajia Lin[1] · Linlu Jin[2] · Rui Yu[1] · Shinghung Mak[3] · Shengquan Hu[3] · Hongya Sun[4] · Xiang Wu[1] · Zaijun Zhang[5] · Mingyuen Lee[6] · Wahkeung Tsim[7] · Wei Su[2] · Wenhua Zhou[1] · Wei Cui[1] · Yifan Han[3] · Qinwen Wang[1]

Received: 23 April 2016 / Accepted: 30 May 2016 / Published online: 13 July 2016

Abstract Oxidative stress-induced neuronal apoptosis plays an important role in many neurodegenerative disorders. In this study, we have shown that indirubin-3-oxime, a derivative of indirubin originally designed for leukemia therapy, could prevent hydrogen peroxide (H_2O_2)-induced apoptosis in both SH-SY5Y cells and primary cerebellar granule neurons. H_2O_2 exposure led to the increased activities of glycogen synthase kinase 3β (GSK3β) and extracellular signal-regulated kinase (ERK) in SH-SY5Y cells. Indirubin-3-oxime treatment significantly reversed the altered activity of both the PI3-K/Akt/GSK3β cascade and the ERK pathway induced by H_2O_2. In addition, both GSK3β and mitogen-activated protein kinase inhibitors significantly prevented H_2O_2-induced neuronal apoptosis. Moreover, specific inhibitors of the phosphoinositide 3-kinase (PI3-K) abolished the neuroprotective effects of indirubin-3-oxime against H_2O_2-induced neuronal apoptosis. These results strongly suggest that indirubin-3-oxime prevents H_2O_2-induced apoptosis via concurrent inhibiting GSK3β and the ERK pathway in SH-SY5Y cells, providing support for the use of indirubin-3-oxime to treat neurodegenerative disorders caused or exacerbated by oxidative stress.

Keywords Indirubin-3-oxime · H_2O_2 · GSK3β · PI3-K · ERK

Abbreviations

ANOAVA	Analysis of variance
CGNs	Cerebellar granule neurons
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FDA	Fluorescein diacetate
GSK3β	Glycogen synthase kinase 3β
H_2O_2	Hydrogen peroxide
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase

✉ Wei Cui
cuiwei@nbu.edu.cn

✉ Yifan Han
bcyfhan@polyu.edu.hk

✉ Qinwen Wang
wangqinwen@nbu.edu.cn

[1] Ningbo Key Laboratory of Behavioral Neuroscience, Zhejiang Provincial Key Laboratory of Pathophysiology, School of Medicine, Ningbo University, Ningbo 315211, China

[2] Ningbo Xiaoshi High School, Ningbo 315010, China

[3] Department of Applied Biology and Chemistry Technology, Institute of Modern Chinese Medicine, The Hong Kong Polytechnic University, Hong Kong SAR, China

[4] The Affiliated Yinzhou Hospital, School of Medicine, Ningbo University, Ningbo 315211, China

[5] Institute of New Drug Research, Guangdong Province Key Laboratory of Pharmacodynamic, Constituents of Traditional Chinese Medicine and New Drug Research, College of Pharmacy, Jinan University, Guangdong, China

[6] State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau, China

[7] Division of Life Science and Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China

MTT	3(4,5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide
NO	Nitric oxide
PBS	Phosphate-buffered saline
PI	Propidium iodide
PI3-K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species

Introduction

Oxidative stress plays an important role in the loss of neurons in many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and cerebral ischemia (Kim et al. 2015). Many reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), nitric oxide (NO), and highly reactive hydroxyl and monoxide radicals can be released immediately after injury of neurons during oxidative stress (Bhat et al. 2015). This excessive ROS further induces neuronal apoptosis via their interactions with macromolecules as well as their ability to regulate both pro-survival and pro-apoptotic signaling pathways (Yoon et al. 2002). H_2O_2, an uncharged and freely diffusible molecule, is widely used as a neurotoxin to establish in vitro models of oxidative stress-induced neuronal apoptosis (Lee et al. 2007). Neuronal apoptosis can be regulated by the inhibition of pro-survival signaling pathways such as the phosphoinositide 3-kinase (PI3-K)/Akt cascade and the activation of pro-apoptotic signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway. Inhibition of PI3-K/Akt led to the activation of glycogen synthase kinase 3β (GSK3β), and was reported to be involved in H_2O_2-induced neuronal apoptosis (Gao et al. 2012; Lin et al. 2016). Moreover, inhibition of extracellular signal-regulated kinase (ERK), a key intermediate of the MAPK signaling pathway, has been proposed as a pro-apoptotic mechanism underlying H_2O_2-induced neuronal apoptosis (Yang et al. 2005; Lin et al. 2016).

Indirubin-3-oxime is a derivative of indirubin, an active constituent of the traditional Chinese medicine recipe Danggui Longhui Wan used to treat chronic myelocytic leukemia (Smith et al. 2006). Interestingly, a recent pharmacokinetics study has shown that indirubin-3-oxime could easily cross the blood–brain barrier, suggesting that this chemical might be used to treat brain disorders (Selenica et al. 2007). Previous studies have demonstrated that indirubin-3-oxime inhibits neuronal apoptosis induced by β-amyloid, 6-hydroxydopamine, and potassium deprivation in vitro (Zhang et al. 2009; Hu et al. 2015). Moreover, indirubin-3-oxime prevents behavioral abnormities induced by many neurotoxins in rodents, possibly by inhibiting oxidative stress-induced neuronal loss (Wang et al. 2007; Ding et al. 2010). However, the underlying molecular mechanisms by which indirubin-3-oxime protects against oxidative stress-induced neuronal apoptosis are largely unknown.

SH-SY5Y cells are rat human neuroblastoma which has been reported to be sensitive to oxidants (Li et al. 1996). Therefore, SH-SY5Y cells could be used for studying the molecular mechanisms of drugs against oxidative stress-induced neuronal apoptosis (Nirmaladevi et al. 2014; Tian et al. 2015). In addition, homogenous cerebellar granule neurons (CGNs) are easy to be acquired because more than 90 % of neurons in cerebellum are CGNs (Gonzalez-Polo et al. 2004). Therefore, CGNs could be used to investigate the effects of neuroprotective drugs on primary neurons.

In this study, we have shown that indirubin-3-oxime effectively prevents H_2O_2-induced neuronal apoptosis in both SH-SY5Y cells and primary CGNs. Moreover, our results have demonstrated that indirubin-3-oxime protects against H_2O_2-induced apoptosis via concurrent inhibiting GSK3β and the ERK pathway.

Materials and Methods

Chemicals and Reagents

H_2O_2 was obtained from Calbiochem (San Diego, CA, USA). Indirubin-3-oxime and SB415286 were obtained from Sigma Chemicals (St Louis, MO, USA). PD98059, U0126, Wortmannin, and LY294002 were purchased from LC Laboratories (Woburn, MA, USA). Antibodies against pSer473-Akt, Akt, pSer9 glycogen synthase kinase 3β (GSK3β), GSK3β, phospho-Thr202/Tyr204-p44/42 (pERK), and ERK were obtained from Cell Signaling Technology (Beverly, MA, USA). Unless otherwise noted, all media and supplements used for cell cultures were purchased from Invitrogen (Carlsbad, CA, USA).

SH-SY5Y Cells Culture

SH-SY5Y cells were purchased from the Shanghai Institute of Cell Biology (Chinese Academy of Sciences), and cultured in high glucose modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and penicillin (100 U/ml)/streptomycin (100 μg/ml). The cells were cultured in an incubator at 37 °C and 5 % CO_2. The medium was replaced every other day. For the experiments with H_2O_2, SH-SY5Y cells (1×10^5 cells/ml) in DMEM with low serum content (1 % FBS) were seeded in 6-well or 96-well plates. All experiments were carried out 24 h after the cells were seeded.

Primary CGNs Culture

CGNs were prepared from 8-day-old Sprague–Dawley rats as described in our previous publication (Luo et al. 2010). Briefly, neurons were seeded at a density of 2.7×10^5 cells/cm^2 in basal modified Eagle's medium containing 10 % FBS, 25 mM KCl, 2 mM glutamine, and penicillin (100 U/ml)/streptomycin (100 µg/ml). Cytosine arabinoside (10 µM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. Granule cells were identified by a combination of several criteria including their size, shape and relative proportion of the total cell population as determined by phase contrast microscopy.

Measurement of Cell Viability

Cell viability was determined by the activity of mitochondrial dehydrogenases via the 3(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay as previously described (Cui et al. 2013, 2014). Briefly, after treatment, 10 µl of 5 mg/ml MTT solution was added to each well. Plates were incubated at 37 °C for 4 h in a humidified incubator. 100 µl of the solvating solution (0.01 N HCl in 10 % SDS solution) was then added to each well for 16–20 h. The absorbance of the samples was measured at a wavelength of 570 nm with 655 nm as a reference wavelength. Unless otherwise indicated, the extent of MTT conversion in cells without treatment is expressed as a percentage of the control.

Measurements of Lactate Dehydrogenase (LDH) Release

Cytotoxicity was evaluated by measuring the release of LDH after drug treatment for 24 h. Cells were incubated with 2 % (v/v) Triton X-100 in culture medium for 30 min at 37 °C to obtain a maximal LDH release as the positive control with 100 % toxicity. Extracellular LDH release was further determined in the conditioned media collected from the culture dishes using the LDH assay kit (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instructions. Briefly, 50 µl culture supernatants were collected from each well with the addition of 50 µl reaction buffer. Thirty minutes after mixing at room temperature, the release of LDH was measured at a wavelength of 490 nm with 655 nm as a reference wavelength.

Fluorescein Diacetate/Propidium Iodide Double Staining Assay

Viable cells were visualized by the fluorescein formed from fluorescein diacetate (FDA) by esterase activity in viable cells. Non-viable cells were stained by propidium iodide (PI) intercalation into DNA, which only penetrates the cell membranes of dead cells. Briefly, after incubation with 10 µg/ml of FDA and 5 µg/ml of PI for 15 min, the cells were examined and images were acquired using UV light microscopy for comparison with photos taken under phase contrast microscopy. To quantitative evaluate cell viability, photos of each well were taken from five random fields and the number of PI-positive cells and FDA-positive cells was counted. % of cell viability = [number of FDA-positive cells/(number of PI-positive cells + number of FDA-positive cells)] × 100 % was then averaged (Jones and Senft 1985).

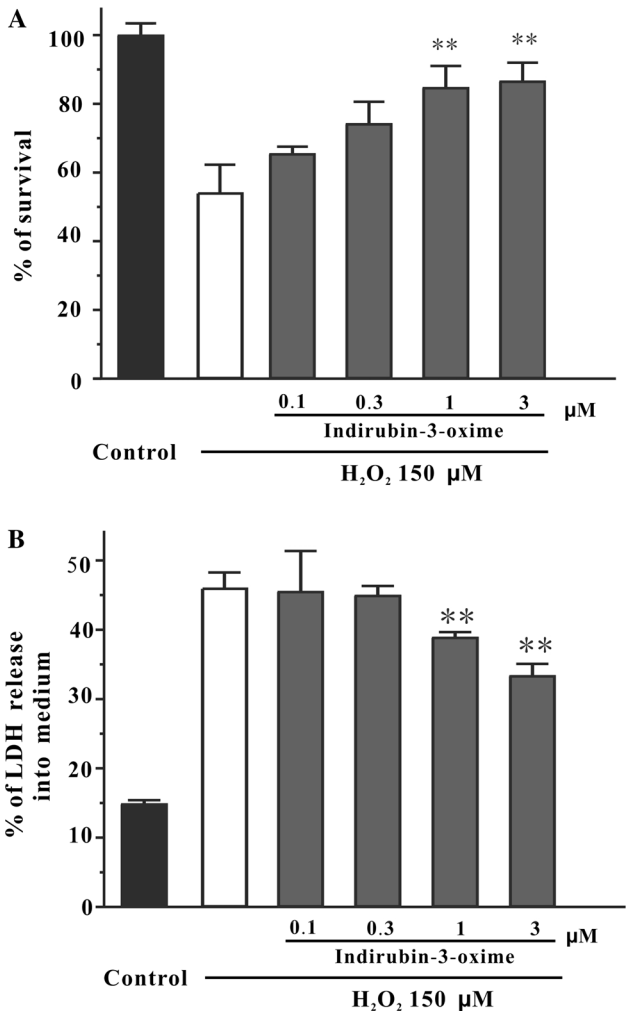

Fig. 1 Indirubin-3-oxime prevents H_2O_2-induced neuronal death in SH-SY5Y cells in a dose-dependent manner. SH-SY5Y cells were pre-treated with indirubin-3-oxime at the indicated concentrations for 2 h, and then exposed to 150 µM H_2O_2. Cell viability was measured by **a** the MTT assay or **b** the LDH assay at 24 h after H_2O_2 challenge. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ versus the H_2O_2-treated group (ANOVA and Dunnett's test)

Hoechst Staining

Chromatin condensation was detected by staining the cell nuclei with Hoechst-33342 as described previously (Cui et al. 2012; Hu et al. 2014). After treatment, cells grown in six-well plates were washed with ice-cold phosphate-buffered saline (PBS), fixed with 4 % formaldehyde in PBS for 15 min, membrane permeabilized in 0.1 % Triton X-100 for 15 min, and blocked in 1 % bovine serum albumin (BSA) for 15 min. Cells were then stained with Hoechst-33342 (5 μg/ml) at 4 °C for 5 min. Images were acquired using a fluorescence microscope (Nikon Instruments Inc. Melville, NY, USA) at 100× magnification. Ultraviolet excitation and emission wavelengths were used to obtain images of nuclei labeled with Hoechst-33342. To quantify the percentage of apoptotic nuclei in each group, photos of each well were taken from five random fields and the number of pyknotic nuclei and total nuclei counted. The percentage of pyknotic nuclei was then averaged.

Western Blot Analysis

Western blotting was performed as previously described (Hu et al. 2013). Protein lysates were separated on SDS-polyacrylamide gels and transferred onto polyvinyldifluoride membranes. After membrane blocking, proteins were detected using primary antibodies. After incubation at 4 °C overnight, signals were obtained after binding to HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed using the enhanced chemiluminescence plus kit (Amersham Bioscience, Aylesbury, UK) and exposed to autoradiographic film.

Statistical Analysis

Results are expressed as mean ± SEM. Analysis of variance (ANOVA) followed by Dunnett's or Tukey's test was used for statistical comparisons. Levels of $p < 0.05$ were considered to be of statistical significance.

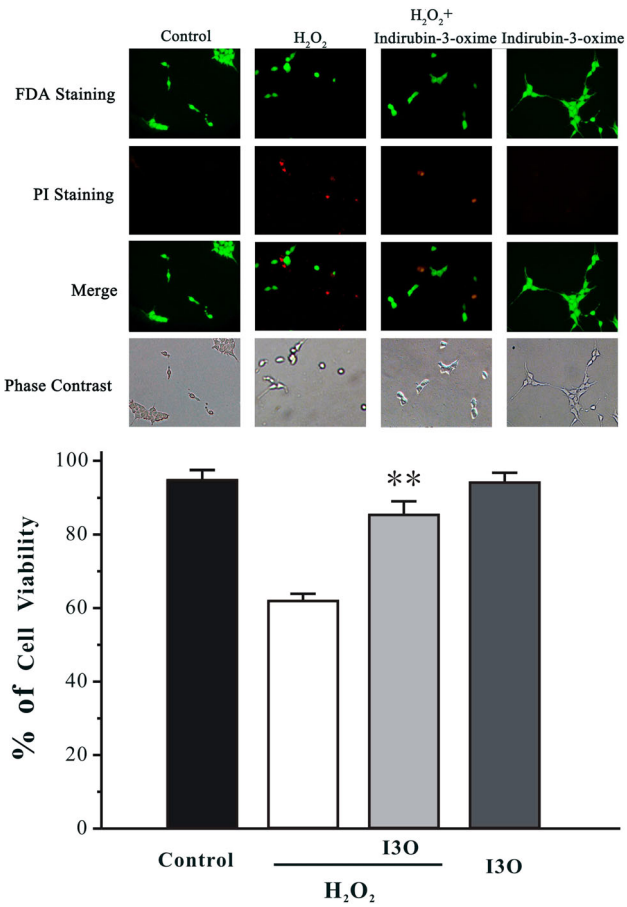

Fig. 2 Indirubin-3-oxime blocks H_2O_2-induced cell death in SH-SY5Y cells. SH-SY5Y cells were pre-treated with 3 μM indirubin-3-oxime (I3O) or vehicle control for 2 h, and then exposed to 150 μM H_2O_2. After 24 h of H_2O_2 challenge, SH-SY5Y cells were assayed with FDA/PI double staining. Cell viability was analyzed from representative photomicrographs. Data were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ versus the H_2O_2-treated group (ANOVA and Dunnett's test)

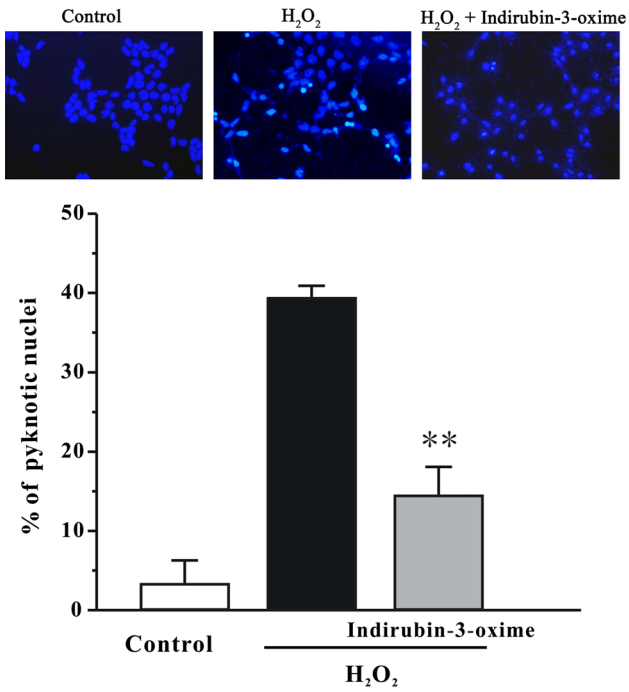

Fig. 3 Indirubin-3-oxime significantly prevents H_2O_2-induced apoptosis in SH-SY5Y cells. SH-SY5Y cells were pre-treated with 3 μM indirubin-3-oxime or vehicle control for 2 h, and then exposed to 150 μM H_2O_2. After 24 h of H_2O_2 challenge, SH-SY5Y cells were assayed with Hoechst staining. The number of pyknotic nuclei with condensed chromatin were counted from representative Hoechst staining photomicrographs and represented as a percentage of the total number of nuclei counted. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ versus the H_2O_2-treated group (ANOVA and Dunnett's test)

Results

Indirubin-3-Oxime Prevents H_2O_2-Induced Neuronal Apoptosis in SH-SY5Y Cells

It was previously reported that treatment with 150 μM H_2O_2 for about 24 h could induce typical apoptosis in SH-SY5Y cells (Tian et al. 2015). Therefore, we used this model to investigate the neuroprotective effects of indirubin-3-oxime. SH-SY5Y cells were pre-treated with a dose curve of indirubin-3-oxime (0.1–3 μM) for 2 h, followed by treatment with 150 μM H_2O_2 for 24 h. Cell viability was then measured using the MTT assay. It was found that indirubin-3-oxime significantly prevented H_2O_2-induced neuronal death in a dose-dependent manner (Fig. 1a). We also assayed LDH release to evaluate alterations in cell membrane permeability. As shown in Fig. 1b, indirubin-3-oxime (0.3–3 μM) significantly prevented the H_2O_2-induced increase of LDH release in a concentration-dependent manner, providing further support that indirubin-3-oxime can prevent H_2O_2-induced neuronal death. Treatment with 3 μM indirubin-3-oxime alone for 26 h was not cytotoxic and did not alter cell proliferation.

To further characterize the protective effect of indirubin-3-oxime against neurotoxicity induced by H_2O_2, treated and control SH-SY5Y cells were examined by FDA/PI double staining. It was found that indirubin-3-oxime significantly blocked the loss of neurons induced by H_2O_2 (Fig. 2). Moreover, according to the number of pyknotic bodies stained by Hoechst-33342, indirubin-3-oxime significantly prevented apoptosis induced by H_2O_2 in SH-SY5Y cells (Fig. 3).

Indirubin-3-Oxime Prevents H_2O_2-Induced Neuronal Apoptosis in CGNs

We have reported that the treatment of 30 μM H_2O_2 for 6 h could lead to neuronal death in primary CGNs (Cui et al. 2011). In this study, we further investigated whether indirubin-3-oxime could produce neuroprotective effects in primary neurons. CGNs were pre-treated with indirubin-3-oxime for 2 h, and then treated with 30 μM H_2O_2 for another 6 h. CGNs were examined by MTT assay or FDA/PI double staining. It was found that indirubin-3-oxime significantly blocked neuronal death induced by H_2O_2 in CGNs (Fig. 4).

Activation of GSK3β and the ERK Signaling are Involved in the Neurotoxicity Induced by H_2O_2 in SH-SY5Y Cells

It has been reported that inhibition of the activation of GSK3β and the ERK signaling are involved in the

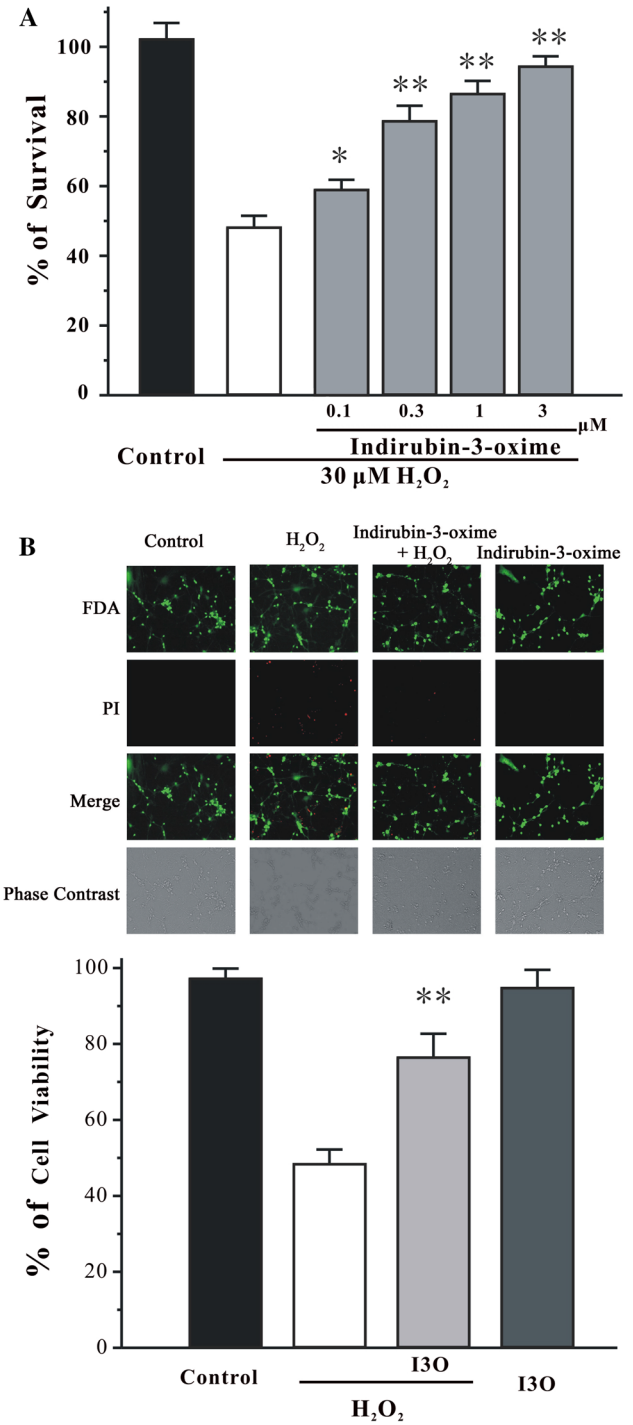

Fig. 4 Indirubin-3-oxime blocks H_2O_2-induced neuronal death in primary CGNs. **a** CGNs were pre-treated with indirubin-3-oxime (I3O) at the indicated concentrations for 2 h, and then exposed to 30 μM H_2O_2. Cell viability was measured by the MTT assay at 6 h after H_2O_2 challenge. **b** CGNs were pre-treated with 3 μM indirubin-3-oxime for 2 h, and then exposed to 30 μM H_2O_2. After 6 h of H_2O_2 challenge, CGNs were assayed with FDA/PI double staining. Cell viability was analyzed from representative photomicrographs. Data were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ versus the H_2O_2-treated group (ANOVA and Dunnett's test)

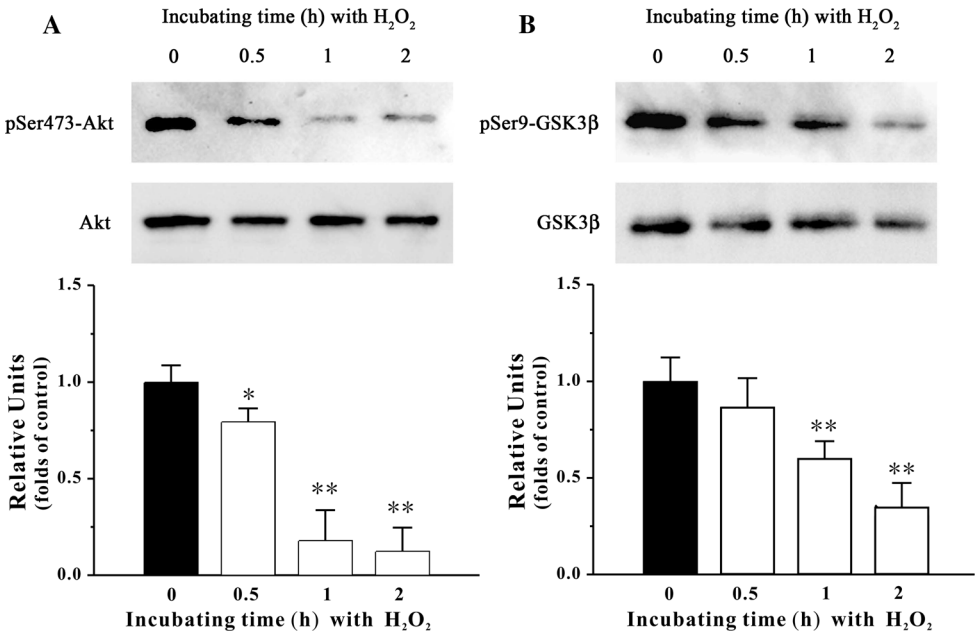

Fig. 5 H_2O_2 decreases the levels of pSer473-Akt and pSer9-GSK3β in a time-dependent manner in SH-SY5Y cells. SH-SY5Y cells were incubated with 150 μM H_2O_2 for various durations as indicated. Western blotting analysis was performed to detect protein expression of **a** pSer473-Akt and **b** pSer9-GSK3β. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $*p < 0.05$ and $**p < 0.01$ versus the control group (ANOVA and Dunnett's test)

neurotoxicity induced by oxidative stress (Gao et al. 2012; Lin et al. 2016). To determine whether the alteration of these molecules is involved in our model, Western blotting analysis was used. As shown in Fig. 5, H_2O_2 decreased the levels of pSer473-Akt and pSer9-GSK3β in SH-SY5Y cells in a time-dependent manner. Moreover, the level of phospho-ERK increased significantly during the first hour post-treatment, although it returned to near basal level 2 h after H_2O_2 challenge (Fig. 6). Furthermore, SB415286, a specific inhibitor of GSK3β, and PD98059 and U0126, two specific inhibitors of MEK, significantly attenuated H_2O_2-induced neuronal death in SH-SY5Y cells (Fig. 7). These results suggest that the activation of GSK3β and MEK are involved in the neurotoxicity induced by H_2O_2 in SH-SY5Y cells. Interestingly, co-application of SB415286 and PD98059 significantly produced the neuroprotective effects, which are similar as those of indirubin-3-oxime.

Indirubin-3-Oxime Prevents the Activation of GSK3β by H_2O_2

The levels of pSer473-Akt and pSer9-GSK3β were determined in indirubin-3-oxime-treated cells by Western blotting. Pre-treatment with 3 μM indirubin-3-oxime prevented the decrease of both pSer473-Akt and pSer9-GSK3β that were caused by H_2O_2 (Fig. 8). Additionally, two PI3-K specific inhibitors, LY294002 and wortmannin, were used to investigate the mechanism of neuroprotection by indirubin-3-oxime. The inhibition of PI3-K by either

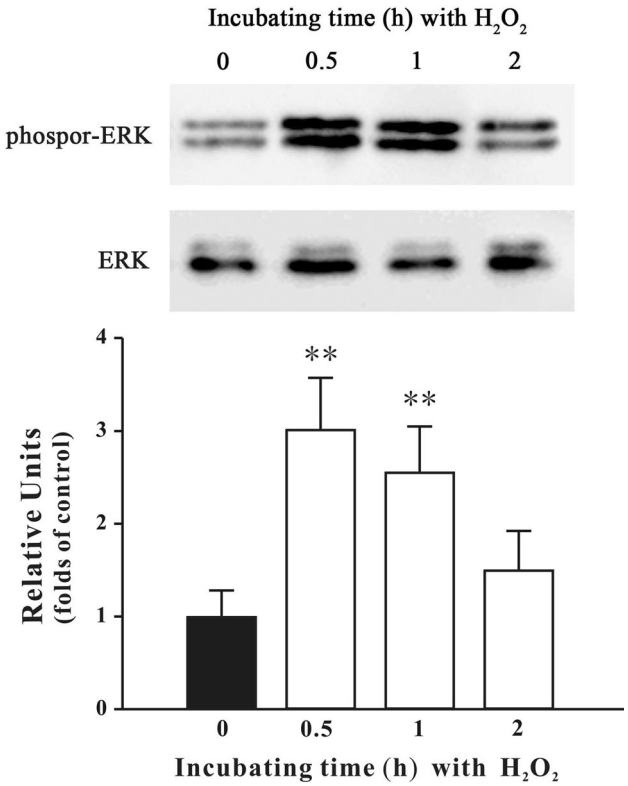

Fig. 6 H_2O_2 increases the levels of phospho-ERK in SH-SY5Y cells. SH-SY5Y cells were incubated with 150 μM H_2O_2 for various durations as indicated. Western blotting analysis was used to detect protein expression of phospho-ERK. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $**p < 0.01$ versus the control group (ANOVA and Dunnett's test)

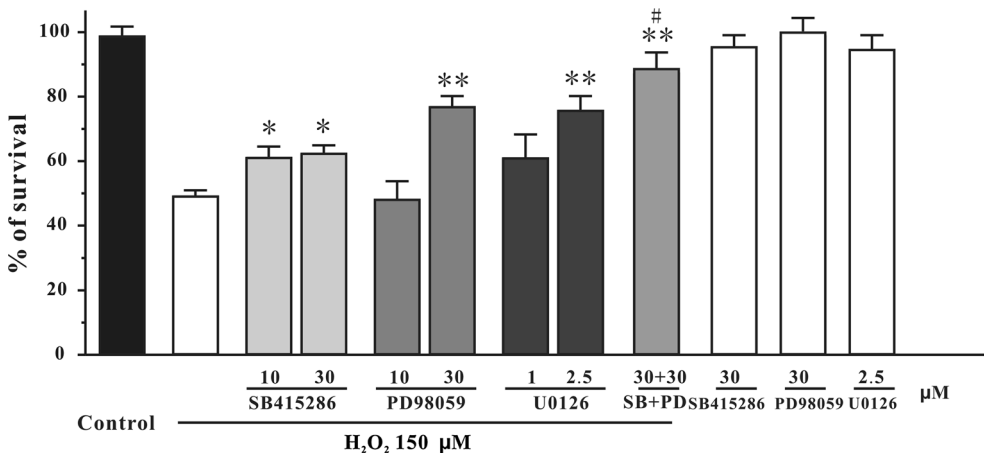

Fig. 7 Inhibition of GSK3β and MEK prevent H_2O_2-induced neuronal death. SH-SY5Y cells were pre-treated with SB415286, PD98059, U0126, or SB415286/PD98059 at the indicated concentrations for 2 h, and then exposed to 150 μM H_2O_2. Cell viability was measured by the MTT assay 24 h after H_2O_2 challenge. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus H_2O_2-treated group, $^{\#}p < 0.05$ versus H_2O_2 + 30 μM SB415286 group (ANOVA and Dunnett's test)

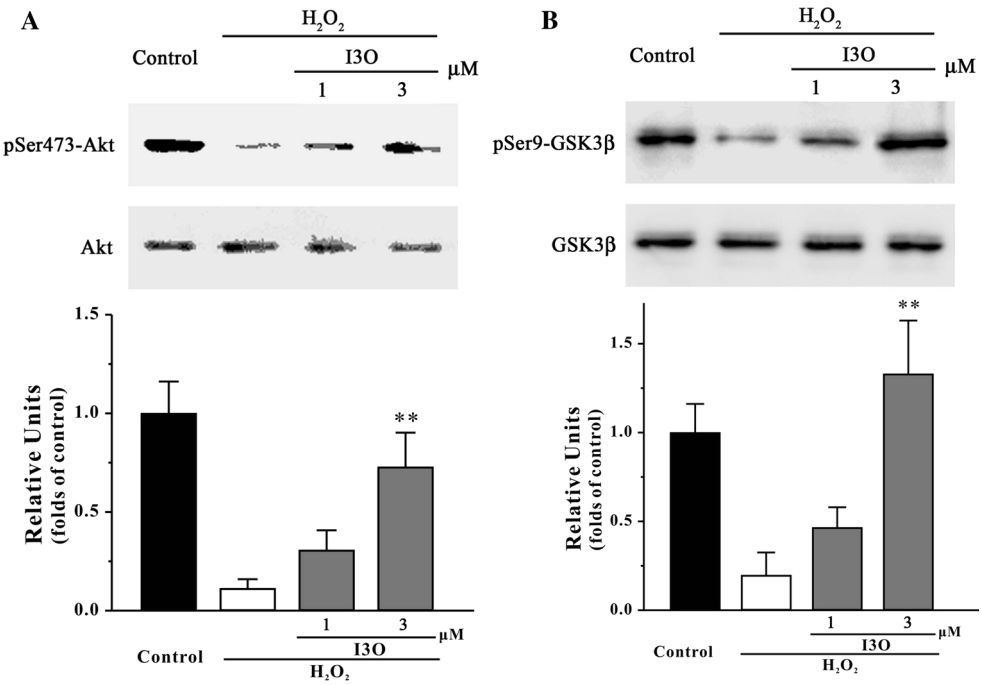

Fig. 8 Indirubin-3-oxime prevents the H_2O_2-induced decrease of pSer473-Akt and pSer9-GSK3β in SH-SY5Y cells. SH-SY5Y cells were pre-treated with indirubin-3-oxime (I3O) at the indicated concentrations for 2 h, and then exposed to 150 μM H_2O_2. Western blotting analysis was used at 2 h after H_2O_2 challenge to detect levels of **a** pSer473-Akt and **b** pSer9-GSK3β. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ versus H_2O_2-treated group (ANOVA and Dunnett's test)

10 μM LY294002 or 0.05 μM wortmannin significantly blocked the neuroprotective effects of indirubin-3-oxime against H_2O_2-induced neuronal death in our model (Fig. 9).

Indirubin-3-Oxime Inhibits the Activation of ERK Signaling Induced by H_2O_2

To further examine whether indirubin-3-oxime protected SH-SY5Y cells against neuronal death via inhibition of the ERK pathway, the level of phospho-ERK was also determined in indirubin-3-oxime-treated cells by Western blotting. As shown in Fig. 10, pre-treatment with indirubin-3-oxime at 3 μM for 2 h significantly prevented the increase of phospho-ERK that was induced by H_2O_2 at 0.5 h, suggesting that indirubin-3-oxime also prevents H_2O_2-induced neuronal death by preventing activation of the ERK pathway.

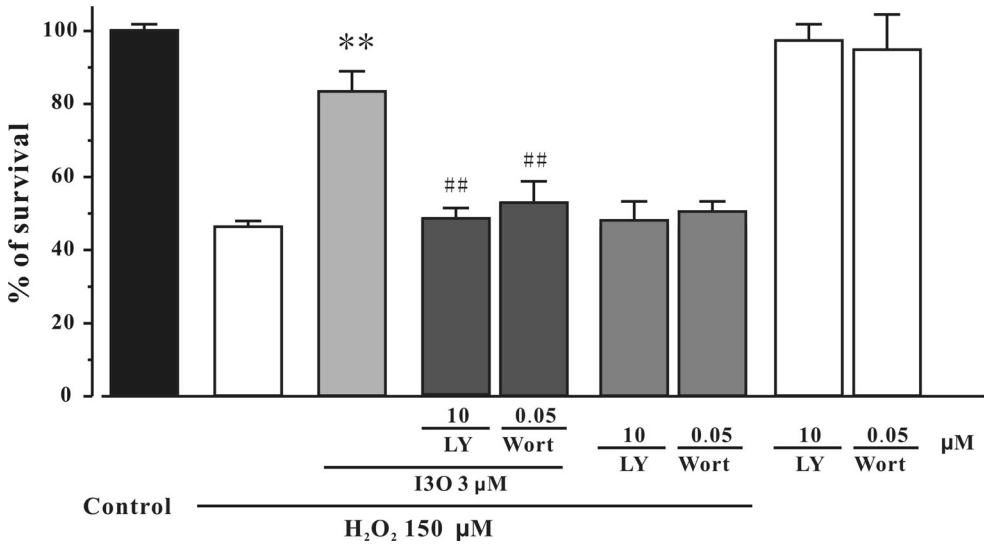

Fig. 9 PI3-K specific inhibitors abolish the neuroprotective effects of indirubin-3-oxime on H_2O_2-induced cell death in SH-SY5Y cells. SH-SY5Y cells were pre-treated with LY294002 (LY) or wortmannin (Wort) at the indicated concentrations for 0.5 h, and then supplemented with 3 μM indirubin-3-oxime (I3O) for 2 h before the exposure to 150 μM H_2O_2. Cell viability was then measured by the MTT assay 24 h after H_2O_2 challenge. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ versus H_2O_2-treated group, $^{\#\#}p < 0.01$ versus H_2O_2 plus I3O group (ANOVA and Tukey's test)

Discussion

In this study we have shown that indirubin-3-oxime effectively prevents H_2O_2-induced neuronal apoptosis. Moreover, our results suggest that the neuroprotective effects of indirubin-3-oxime were mediated through simultaneous inhibition of GSK3β and the ERK signaling.

H_2O_2-induced oxidative stress in neurons can lead to both apoptotic and necrotic death depending on the concentration of H_2O_2 used (Valencia and Moran 2004; Fatokun et al. 2007). Compounds with anti-apoptotic properties may have therapeutic utility in neurodegenerative diseases characterized by neuronal apoptosis (Bachis et al. 2001). In our study, we have shown that H_2O_2 could significantly increase the number of pyknotic bodies, suggesting that H_2O_2 mainly induces neuronal apoptosis rather than necrosis in SH-SY5Y cells, which is consistent with other reports that exposure of 150 μM H_2O_2 for 24 h produces neuronal apoptosis in SH-SY5Y cells (Tian et al. 2015).

Indirubin-3-oxime has previously been reported to have neuroprotective effects (Sharma and Taliyan 2014; 2015). It was reported that systemic administration of indirubin-3-oxime (30 mg/kg i.v. or 20 mg/kg i.p.) could easily cross the blood–brain barrier, and retain in the brain at 4 h after administration, suggesting that indirubin-3-oxime might be used in the treatment of neurological diseases (Selenica et al. 2007). Interestingly, previous studies have also shown that indirubin-3-oxime can suppress excessive ROS production in both lipopolysaccharide-induced primary microglia cultures and 6OHDA-induced PC12 cells, suggesting that its neuroprotective activity may be mediated by anti-oxidative properties (Jung et al. 2010; Hu et al. 2015). In our study, we demonstrated that indirubin-3-oxime prevented H_2O_2-induced neuronal death not only in SH-SY5Y cells, but also in primary CGNs, providing a strong support that indirubin-3-oxime could effectively protected against ROS-induced neuronal loss and may be used in the treatment of neurological diseases.

How does indirubin-3-oxime produce neuroprotective effects in our model? We have evaluated the effects of indirubin-3-oxime on ROS scavenge using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and found that indirubin-3-oxime (0.1–10 μM) could not decrease DPPH radical, suggesting that the neuroprotective effects of indirubin-3-oxime might not due to ROS scavenge (Data not shown). We speculated that indirubin-3-oxime might regulate pro-survival and pro-apoptotic signaling pathways that are involved in H_2O_2-induced apoptosis. Indirubin-3-oxime is a potent and direct inhibitor of GSK3β with an IC_{50} of 22 nM (Eisenbrand et al. 2004). In our study, we found that indirubin-3-oxime prevents the H_2O_2-induced decrease of pSer9-GSK3β. We also showed that SB415286, another small molecule of GSK3β inhibitor, prevented H_2O_2-induced neuronal apoptosis, supporting the role of GSK3β inhibition in the neuroprotective effects of indirubin-3-oxime. However, indirubin-3-oxime (3 μM) could almost fully prevent H_2O_2-induced cell death. SB415286 could not fully reversed H_2O_2-induced cell death at high

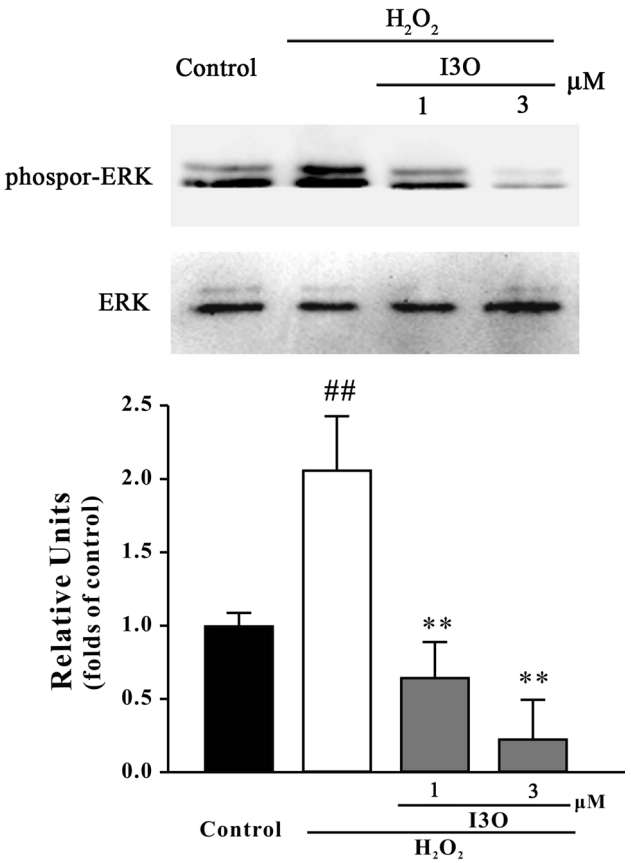

Fig. 10 Indirubin-3-oxime prevents the H_2O_2-induced increase of phospho-ERK in SH-SY5Y cells. SH-SY5Y cells were pre-treated with indirubin-3-oxime (I3O) at the indicated concentrations for 2 h, and then exposed to 150 μM H_2O_2. Western blotting analysis was used at 30 min after H_2O_2 challenge to detect levels of phospho-ERK. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; $^{**}p < 0.01$ versus H_2O_2-treated group (ANOVA and Dunnett's test)

concentrations, suggesting that indirubin-3-oxime might act on other neuroprotective target(s) besides GSK3β.

Activation of the ERK signaling has been regarded as one of the key pathways mediating neuronal apoptosis (Jiang et al. 2005). We found that indirubin-3-oxime prevents the H_2O_2-induced increase of pERK. Furthermore, we show that treatment with MEK inhibitors can also prevent H_2O_2-induced neuronal apoptosis, suggesting that inhibition of the ERK signaling may also be involved in the neuroprotective effects of indirubin-3-oxime. How could indirubin-3-oxime act on the ERK pathway? Indirubin-3-oxime could compete with ATP for binding to the catalytic subunit of kinase (Zhang et al. 2009). Due to the similarities of ATP binding site among different kinases, indirubin-3-oxime inhibits many kinases, including GSK3β, cyclin dependent kinase (CDK) 1, CDK2, and CDK5 (Rivest et al. 2011). We speculated that indirubin-3-oxime might directly act on the ATP binding site of the upstream kinases of ERK, e.g., MEK, and inhibit the phosphorylation of ERK. To further determine which kinase(s) are directly inhibited by indirubin-3-oxime, additional experiments are being undertaken in our lab.

How could PI3-K inhibitors abolish the neuroprotective effects of indirubin-3-oxime when GSK3β was inhibited by indirubin-3-oxime? Previous studies have shown that there is a crosstalk between the PI3-K and the ERK pathways (Frebel and Wiese 2006; Yang et al. 2005; Heras-Sandoval et al. 2014). Inactivation of PI3-K triggered the activation of the ERK pathway. We speculated that PI3-K inhibitors might abolish indirubin-3-oxime-induced neuroprotective effects independent of GSK3β activation, possibly via the activation of the ERK pathway.

In summary, we have found that indirubin-3-oxime prevents H_2O_2-induced neuronal apoptosis via concurrent inhibition of GSK3β and the ERK signaling. Our results also provide support for the use of indirubin-3-oxime or similar compounds in the treatment neurodegenerative disorders caused or exacerbated by oxidative stress.

Acknowledgments This work was supported by the Natural Science Foundation of Zhejiang Province (LY15H310007), Research Grants Council of Hong Kong (561011 & 15101014), the Applied Research Project on Nonprofit Technology of Zhejiang Province (2016C37110), the National Natural Science Foundation of China (U1503223, 81202150, 81471398), the Ningbo International Science and Technology Cooperation Project (2014D10019), Ningbo municipal innovation team of life science and health (2015C110026), Ningbo Natural Science Foundation (2015A610219), the Scientific Research Foundation for the Returned Overseas Chinese Scholars, the State Education Ministry, and the K. C. Wong Magna Fund in Ningbo University.

References

Bachis A, Colangelo AM, Vicini S et al (2001) Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity. J Neurosci 21:3104–3112

Bhat AH, Dar KB, Anees S et al (2015) Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases: a mechanistic insight. Biomed Pharmacother 74:101–110

Cui W, Li W, Zhao Y et al (2011) Preventing H_2O_2-induced apoptosis in cerebellar granule neurons by regulating the VEGFR-2/Akt signaling pathway using a novel dimeric antiacetylcholinesterase bis(12)-hupyridone. Brain Res 1394:14–23

Cui W, Zhang Z, Li W et al (2012) Unexpected neuronal protection of SU5416 against 1-methyl-4-phenylpyridinium ion-induced toxicity via inhibiting neuronal nitric oxide synthase. PLoS One 7:e46253

Cui W, Zhang Z, Li W et al (2013) The anti-cancer agent SU4312 unexpectedly protects against MPP+-induced neurotoxicity via selective and direct inhibition of neuronal NOS. Br J Pharmacol 168:1201–1214

Cui W, Zhang ZJ, Hu SQ et al (2014) Sunitinib produces neuroprotective effect via inhibiting nitric oxide overproduction. CNS Neurosci Ther 20:244–252

Ding Y, Qiao A, Fan GH (2010) Indirubin-3′-monoxime rescues spatial memory deficits and attenuates β-amyloid-associated

neuropathology in a mouse model of Alzheimer's disease. Neurobiol Dis 39:156–168

Eisenbrand G, Hippe F, Jakobs S, Muehlbeyer S (2004) Molecular mechanisms of indirubin and its derivatives: novel anticancer molecules with their origin in traditional Chinese phytomedicine. J Cancer Res Clin Oncol 130:627–635

Fatokun AA, Stone TW, Smith RA (2007) Cell death in rat cerebellar granule neurons induced by hydrogen peroxide in vitro: mechanisms and protection by adenosine receptor ligands. Brain Res 1132:193–202

Frebel K, Wiese S (2006) Signalling molecules essential for neuronal survival and differentiation. Biochem Soc Trans 34:1287–1290

Gao Y, Dong C, Yin J, Shen J, Tian J, Li C (2012) Neuroprotective effect of fucoidan on H_2O_2-induced apoptosis in PC12 cells via activation of PI3K/Akt pathway. Cell Mol Neurobiol 32:523–529

Gonzalez-Polo RA, Soler G, Fuentes JM (2004) MPP+: mechanism for its toxicity in cerebellar granule cells. Mol Neurobiol 30:253–264

Heras-Sandoval D, Perez-Rojas JM, Hernandez-Damian J, Pedraza-Chaverri J (2014) The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cell Signal 26:2694–2701

Hu SQ, Cui W, Xu DP et al (2013) Substantial neuroprotection against K+ deprivation-induced apoptosis in primary cerebellar granule neurons by novel dimer bis(propyl)-cognitin via the activation of VEGFR-2 signaling pathway. CNS Neurosci Ther 19:764–772

Hu S, Wang R, Cui W et al (2014) Inhibiting β-amyloid-associated Alzheimer's pathogenesis in vitro and in vivo by a multifunctional dimeric bis(12)-hupyridone derived from its natural analogue. J Mol Neurosci 55:1014–1021

Hu S, Cui W, Zhang Z et al (2015) Indirubin-3-oxime effectively prevents 6OHDA-induced neurotoxicity in PC12 cells via activating MEF2D through the inhibition of GSK3β. J Mol Neurosci 57:561–570

Jiang H, Zhang L, Koubi D et al (2005) Roles of Ras-Erk in apoptosis of PC12 cells induced by trophic factor withdrawal or oxidative stress. J Mol Neurosci 25:133–140

Jones KH, Senft JA (1985) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate–propidium iodide. J Histochem Cytochem 33:77–79

Jung HJ, Nam KN, Son MS et al (2010) Indirubin-3′-oxime inhibits inflammatory activation of rat brain microglia. Neurosci Lett 487:139–143

Kim GH, Kim JE, Rhie SJ, Yoon S (2015) The role of oxidative stress in neurodegenerative diseases. Exp Neurobiol 24:325–340

Lee KY, Koh SH, Noh MY, Park KW, Lee YJ, Kim SH (2007) Glycogen synthase kinase-3β activity plays very important roles in determining the fate of oxidative stress-inflicted neuronal cells. Brain Res 1129:89–99

Li X, Song L, Jope RS (1996) Cholinergic stimulation of AP-1 and NFκB transcription factors is differentially sensitive to oxidative stress in SH-SY5Y neuroblastoma: relationship to phosphoinositide hydrolysis. J Neurosci 16:5914–5922

Lin X, Wu S, Wang Q et al (2016) Saikosaponin-D Reduces HO-induced PC12 cell apoptosis by removing ROS and blocking MAPK-dependent oxidative damage. Cell Mol Neurobiol. doi:10.1007/s10571-016-0336-5

Luo J, Li W, Zhao Y et al (2010) Pathologically activated neuroprotection via uncompetitive blockade of *N*-methyl-D-aspartate receptors with fast off-rate by novel multifunctional dimer bis(propyl)-cognitin. J Biol Chem 285:19947–19958

Nirmaladevi D, Venkataramana M, Chandranayaka S, Ramesha A, Jameel NM, Srinivas C (2014) Neuroprotective effects of bikaverin on H_2O_2-induced oxidative stress mediated neuronal damage in SH-SY5Y cell line. Cell Mol Neurobiol 34:973–985

Rivest P, Renaud M, Sanderson JT (2011) Proliferative and androgenic effects of indirubin derivatives in LNCaP human prostate cancer cells at sub-apoptotic concentrations. Chem Biol Interact 189:177–185

Selenica ML, Jensen HS, Larsen AK et al (2007) Efficacy of small-molecule glycogen synthase kinase-3 inhibitors in the postnatal rat model of tau hyperphosphorylation. Br J Pharmacol 152:959–979

Sharma S, Taliyan R (2014) Neuroprotective role of indirubin-3′-monoxime, a GSKβ inhibitor in high fat diet induced cognitive impairment in mice. Biochem Biophys Res Commun 452:1009–1015

Smith PD, Mount MP, Shree R et al (2006) Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci 26:440–447

Tian X, Guo LP, Hu XL et al (2015) Protective effects of *Arctium lappa* L. roots against hydrogen peroxide-induced cell injury and potential mechanisms in SH-SY5Y cells. Cell Mol Neurobiol 35:335–344

Valencia A, Moran J (2004) Reactive oxygen species induce different cell death mechanisms in cultured neurons. Free Radic Biol Med 36:1112–1125

Wang W, Yang Y, Ying C et al (2007) Inhibition of glycogen synthase kinase-3β protects dopaminergic neurons from MPTP toxicity. Neuropharmacology 52:1678–1684

Yang LY, Ko WC, Lin CM et al (2005) Antioxidant *N*-acetylcysteine blocks nerve growth factor-induced H_2O_2/ERK signaling in PC12 cells. Ann N Y Acad Sci 1042:325–337

Yoon SO, Yun CH, Chung AS (2002) Dose effect of oxidative stress on signal transduction in aging. Mech Ageing Dev 123:1597–1604

Zhang S, Zhang Y, Xu L et al (2009) Indirubin-3′-monoxime inhibits β-amyloid-induced neurotoxicity in neuroblastoma SH-SY5Y cells. Neurosci Lett 450:142–146