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RESEARCH ARTICLE

Indirubin-3′-monoxime suppresses amyloid-beta-induced apoptosis by inhibiting tau hyperphosphorylation

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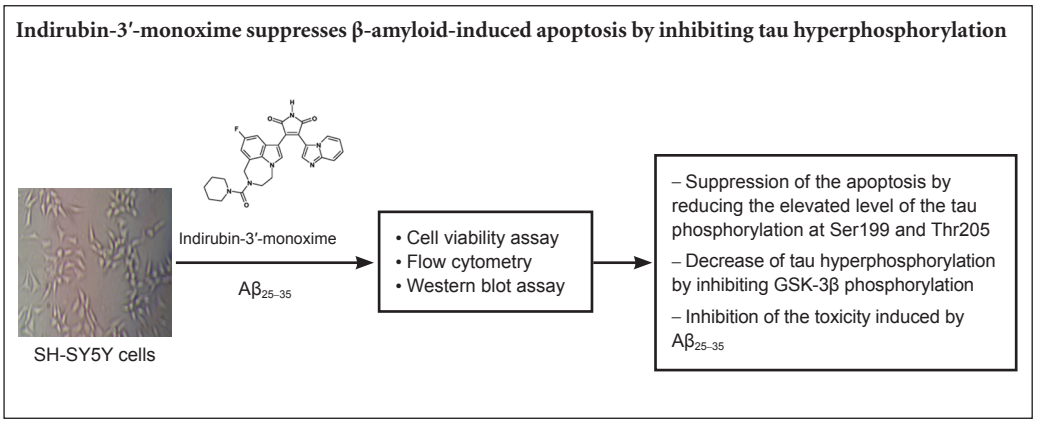
Graphical Abstract



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Abstract

Indirubin-3′-monoxime is an effective inhibitor of cyclin-dependent protein kinases, and may play an obligate role in neuronal apoptosis in Alzheimer's disease. Here, we found that indirubin-3′-monoxime improved the morphology and increased the survival rate of SH-SY5Y cells exposed to amyloid-beta 25–35 ($A\beta_{25–35}$), and also suppressed apoptosis by reducing tau phosphorylation at Ser199 and Thr205. Furthermore, indirubin-3′-monoxime inhibited phosphorylation of glycogen synthase kinase-3β (GSK-3β). Our results suggest that indirubin-3′-monoxime reduced $A\beta_{25–35}$-induced apoptosis by suppressing tau hyperphosphorylation *via* a GSK-3β-mediated mechanism. Indirubin-3′-monoxime is a promising drug candidate for Alzheimer's disease.

Key Words: *nerve regeneration; indirubin-3′-monoxime; amyloid-beta; Alzheimer's disease; neuronal apoptosis; tau hyperphosphorylation; phosphorylated glycogen synthase kinase-3β; phosphorylated c-Jun N-terminal kinase; neural regeneration*

Introduction

The recognition of pathologic features in patients with Alzheimer's disease (AD) has provided clues to the mechanisms of neuronal apoptosis, and drawn attention to new prospects for AD therapy. A number of experimental models have shown that neuronal death occurs alongside the elicitation of proteins involved in the cell cycle (Lim and Qi, 2003; Colacurcio et al., 2015). Damaged neurons, instead of continuing with the cell cycle after mitosis, initiate abortive processes that result in apoptotic cell death (Xu et al., 2008; Absalon et al., 2013). Cyclin-dependent kinase (CDK) is involved in such processes, and CDK inhibitors might reduce neuronal loss in AD (Zhang et al., 2004; Johnson et al., 2005). There is an urgent need to develop safe, effective, and selective CDK inhibitors that can pass the blood-brain barrier.

Indirubin is a selective CDK inhibitor, which suppresses the activities of CDK1, CDK2, and CDK5. However, it has poor water solubility and liposolubility (Absalon et al., 2013). Indirubin-3′-monoxime (IMX), also a CDK inhibitor, has a low molecular weight and better solubility than indirubin (Zahler et al., 2010; Liao and Leung, 2013). It is nontoxic and acts by competition with adenosine triphosphate at the catalytic site of CDKs (Shelton et al., 2004).

The compound was found to suppress tau phosphorylation in Sf9 cells expressing human tau 23 (Leclerc et al., 2001) and, in cerebellar granular neurons, it reduced apoptosis initiated by withdrawal of potassium (Xie et al., 2004). Systemic administration of IMX (20 mg/kg; 3 times per week for 2 months) in APP transgenic mice attenuated spatial memory deficits and decreased presenilin 1 (PS1) mutations in several AD-like phenotypes (Ding et al., 2010).

The aim of the present study was to investigate the neuroprotective effect of IMX against amyloid-beta (Aβ)-induced apoptosis in cultured SH-SY5Y neuroblastoma cells. In addition, we explored the effect of IMX on tau hyperphosphorylation and putative related mechanisms.

Materials and Methods

SH-SY5Y cell culture

SH-SY5Y cells (Beijing Union Medical College Cell Center, Beijing, China) were grown on RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 15% (v/v) fetal bovine serum (Gibco BRL), 2 mM L-glutamine, 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA), and 100 U/mL penicillin (Sigma) in a humidified atmosphere at 5% CO_2 and 37°C. The medium was replaced every 2 days; cells were passaged every 3–4 days.

Cell viability assay

Aβ peptide fragment 25–35 ($Aβ_{25–35}$; Sigma) was dissolved in sterile deionized water and stored at −20°C. IMX (Sigma) was dissolved in dimethyl sulfoxide to 10 mM, stored in aliquots at −20°C, and diluted in medium as necessary. The cells were divided into five groups in a 96-well plate: control (untreated); Aβ (20 μM $Aβ_{25–35}$); IMX0.2 (0.2 μM IMX + $Aβ_{25–35}$); IMX0.5 (0.5 μM IMX + $Aβ_{25–35}$); and IMX1 (1.0 μM IMX + $Aβ_{25–35}$). Cells in the Aβ groups were incubated with $Aβ_{25–35}$ at 37°C for 7 days. Cells in the IMX groups were incubated at 37°C for 2 days. We used 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium (WST-8, Cell Counting Kit-8; Dojindo, Kumamoto, Japan; Tsukatani et al., 2011) to examine the effect of IMX on $Aβ_{25–35}$-induced changes in SH-SY5Y cells. After treatment, the medium was replaced by Dulbecco's modified Eagle's medium to stop the effects of IMX and $Aβ_{25–35}$. WST-8 (10 μL) was added to each well, and the plate was incubated for 4 hours. Absorbance was measured at 450 nm using a Dynatech MR5000 reader (BMG Labtech, Offenburg, Germany) with a reference wavelength of 630 nm.

Flow cytometry

To determine the effect of IMX on early and late apoptosis and necrosis triggered by $Aβ_{25–35}$, we examined SH-SY5Y cells using flow cytometry. The cells were transferred into six-well plates and incubated with IMX (0, 0.5, and 1.0 μM) for 24 hours. $Aβ_{25–35}$ (20 μM) was then added and mixed. Forty-eight hours later, the cells that remained fixed to the plates were collected in phosphate buffered saline (PBS) and combined with the floating dead cells. Cells (approximately 1×10^6) were washed twice with cold PBS and resuspended in 200 μL cold 1× binding buffer. Annexin V–FITC (10 μL) and propidium iodide (PI; 5 μL) were admixed and incubated in the dark at room temperature for 15 minutes, then 300 μL binding buffer was added. Cells were quantified instantly in a flow cytometer (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), using emission filters at 525 and 575 nm. Cells negative for both annexin V and PI (V^-/PI^-) were considered normal, whereas a V^+/PI^- result was considered a criterion of early apoptosis, and V^+/PI^+ was assumed to indicate late apoptotic-like cell death and necrosis. A minimum of 10,000 cells per experiment were assayed in the three separate trials.

Western blot assay

To evaluate the effects of $Aβ_{25–35}$ and IMX on the phosphorylation of tau, glycogen synthase kinase 3β (GSK-3β) and c-Jun N-terminal kinase (JNK) in SH-SY5Y cells, the cells were incubated with a mixture of 20 μM $Aβ_{25–35}$ and 0.5 μM IMX for 6 hours. They were then washed twice with cold PBS, and lysed in cell lysis buffer for 30 minutes on ice. The soluble portion was produced by centrifugation (15,000 × *g* for 15 minutes at 4°C). Protein concentrations were established using the bicinchoninic acid method (Yalamati et al., 2015) (Pierce Biotechnology, Rockford, IL, USA). Aliquots of total protein were boiled for 10 minutes in loading buffer and subsequently separated in 10% sodium dodecyl sulfate-polyacrylamide gel. Next, the proteins were transferred onto nitrocellulose membranes by electroporation (Immobilon TMP, Millipore Corp., Bedford, MA, USA) using a Trans-Blot system (Bio-Rad, New York, NY, USA). The membranes were then blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 (TTBS; 10 mM Tris-HCl, 150 mM NaCl, 0.2% Tween-20) for 1 hour at room temperature. Samples were incubated with monoclonal primary antibodies (β-actin, 1:10,000, Sigma; polyclonal anti-tau pS199, 1:1,000, BioSource Int., Camarillo, CA, USA; anti-tau pT205, 1:1,000, Bioworld Technology (St. Louis Park, MN, USA); Ser-9-phosphorylated GSK-3β (p-GSK-3β Ser9), phosphorylated JNK (p-JNK), and JNK, all 1:1,000; Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. The membranes were washed twice with TTBS, and incubated with the secondary antibody (anti-rabbit-horseradish peroxidase, 1:5,000; Cell Signaling Technology) at room temperature for 1 hour. Proteins were then visualized using the ECL Advanced Western Blotting Detection kit (Amersham Biosciences Ltd., Amersham, UK), and the mean optical density of each band was calculated using a Fluor-S MultiImager (Bio-Rad Laboratories (Shanghai) Co., Ltd., Shanghai, China) with Quantity One software (Bio-Rad Laboratories (Shanghai) Co., Ltd.).

Statistical analysis

The data, presented as the mean ± SEM, were analyzed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Means were compared by one-way analysis of variance and the least significant difference *post hoc* test. $P < 0.05$ was considered statistically significant.

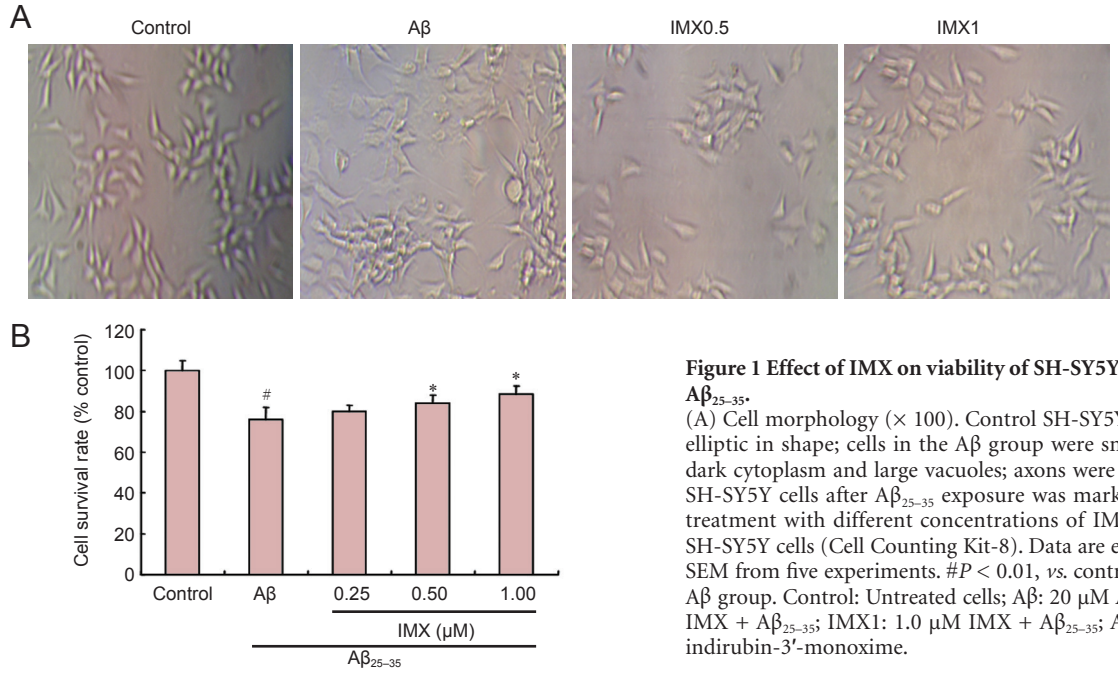

Figure 1 Effect of IMX on viability of SH-SY5Y cells exposed to $A\beta_{25–35}$.

(A) Cell morphology (× 100). Control SH-SY5Y cells were fusiform or elliptic in shape; cells in the Aβ group were small and speckled, with dark cytoplasm and large vacuoles; axons were absent. Morphology of SH-SY5Y cells after $A\beta_{25–35}$ exposure was markedly improved by pretreatment with different concentrations of IMX. (B) Survival rate of SH-SY5Y cells (Cell Counting Kit-8). Data are expressed as the mean ± SEM from five experiments. #$P < 0.01$, *vs.* control group; *$P < 0.01$, *vs.* Aβ group. Control: Untreated cells; Aβ: 20 μM $A\beta_{25–35}$; IMX0.5: 0.5 μM IMX + $A\beta_{25–35}$; IMX1: 1.0 μM IMX + $A\beta_{25–35}$; Aβ: amyloid-beta; IMX: indirubin-3′-monoxime.

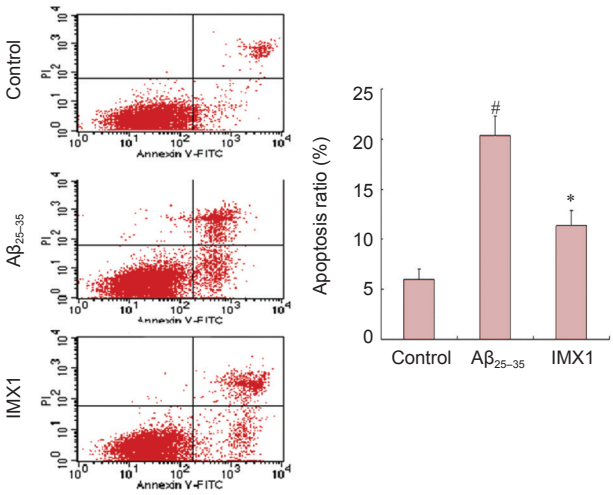

Figure 2 Apoptosis triggered by $A\beta_{25–35}$ was suppressed by IMX (flow cytometry).

The results are displayed as the mean ± SEM of five experiments. #$P < 0.01$, *vs.* control group; *$P < 0.01$, *vs.* Aβ group. Control: Untreated cells; Aβ: 20 μM $A\beta_{25–35}$; IMX1: 1.0 μM IMX + $A\beta_{25–35}$; Aβ: amyloid-beta; IMX: indirubin-3′-monoxime.

Results

IMX enhanced the viability of SH-SY5Y cells exposed to $A\beta_{25–35}$

Under an inverted phase contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan), SH-SY5Y cells in the control group appeared well-formed than those in the Aβ group. Pretreatment with different concentrations of IMX markedly improved morphology after $A\beta_{25–35}$ exposure (**Figure 1A**).

Cell viability after $A\beta_{25–35}$ exposure was significantly lower than in control cells ($P < 0.001$). However, pretreatment with IMX (0.5 μM and 1.0 μM) increased cell viability in a concentration-dependent manner ($P < 0.01$; **Figure 1B**).

IMX reduced neuronal apoptosis triggered by $A\beta_{25–35}$ in SH-SY5Y cells

Flow cytometry showed that there were more apoptotic cells after exposure to 20 μM $A\beta_{25–35}$ than in the control group ($P < 0.01$), but significantly fewer in the IMX group (12.4 ± 1.82%) than in the Aβ group (20.33 ± 2.02%; $P < 0.01$). This indicates that IMX protected cells against Aβ-induced apoptosis (**Figure 2**).

IMX decreased tau phosphorylation caused by $A\beta_{25–35}$

Western blot assay revealed significantly more tau phosphorylation at Ser199 and Thr205 in the Aβ group than in the control group ($P < 0.05$). This effect was markedly reduced by co-treatment with IMX at 0.5 μM and 1.0 μM ($P < 0.05$; **Figure 3**).

Effects of IMX on p-GSK-3β expression

p-GSK-3β (Ser9) expression was lower after $A\beta_{25–35}$ exposure than in control cells, indicating that GSK-3β activity was increased. However, p-GSK-3β (Ser9) was markedly overexpressed after pretreatment with IMX ($P < 0.05$; **Figure 4A**). These results suggest that GSK-3β is involved in the effect of IMX on Aβ-induced tau phosphorylation.

IMX treatment did not affect p-JNK expression

The expression of p-JNK in the Aβ group was markedly higher than that in the control group ($P < 0.05$). However, there were no significant changes after IMX treatment (**Figure 4B**), indicating that p-JNK is not involved in the effect of IMX on Aβ-induced tau phosphorylation.

Discussion

SH-SY5Y neuroblastoma cells are a well-characterized human cell model for investigating the pharmacological effects of IMX. In the present study, we exposed the cells to 20 μM

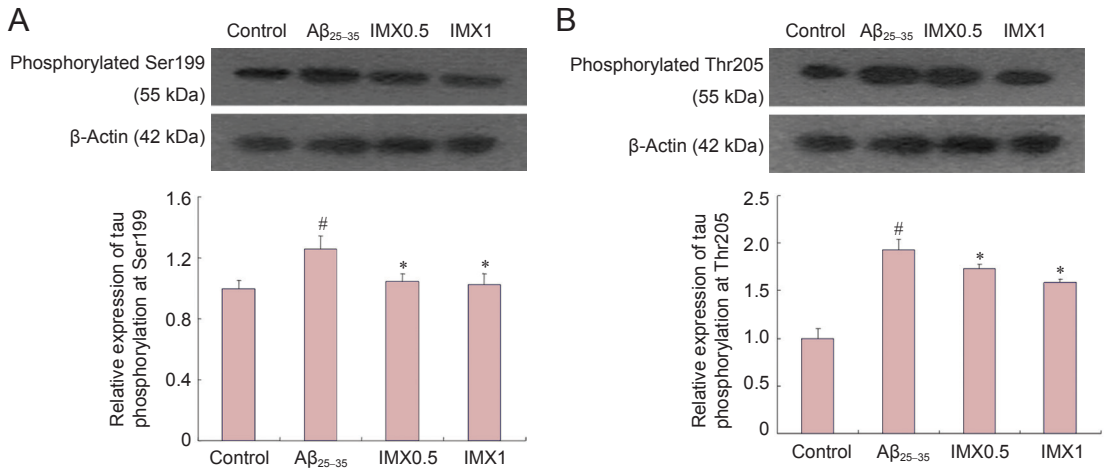

Figure 3 IMX decreased $A\beta_{25–35}$-induced tau phosphorylation at Ser (A) and Thr205 (B).
Phosphorylated tau expression was measured as the optical density of the bands normalized to β-actin. Data are presented as the mean ± SEM of three experiments. #$P < 0.01$, *vs.* control group; *$P < 0.01$, *vs.* Aβ group. Control: Untreated cells; Aβ: 20 μM $A\beta_{25–35}$; IMX0.5: 0.5 μM IMX + $A\beta_{25–35}$; IMX1: 1.0 μM IMX + $A\beta_{25–35}$; IMX: indirubin-3′-monoxime; Aβ: amyloid-beta.

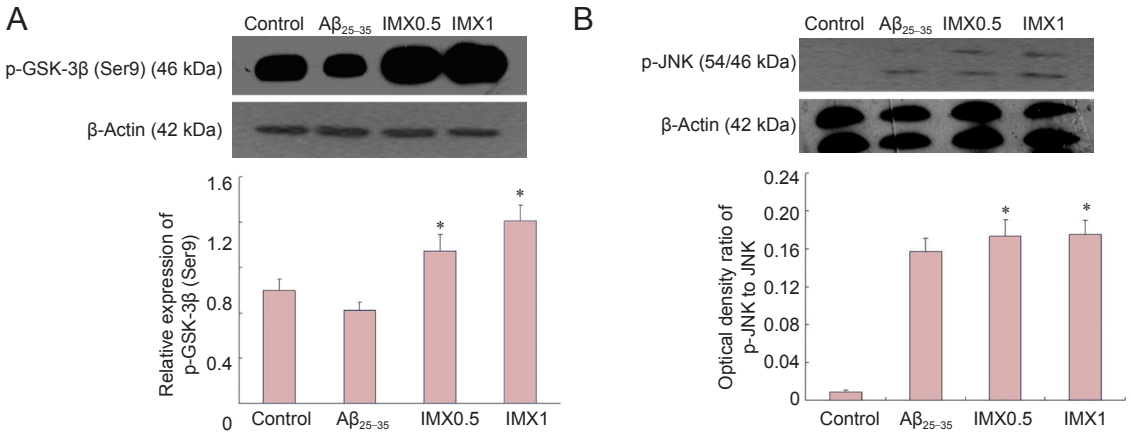

Figure 4 Effect of IMX on p-GSK-3β (A) and p-JNK (B) expression in the presence of $A\beta_{25–35}$.
p-GSK-3β (Ser9) and p-JNK expression levels were measured as the optical density of the bands normalized to β-actin and JNK, respectively. Data are expressed as the mean ± SEM of three experiments. *$P < 0.05$, *vs.* Aβ group. Control: Untreated cells; Aβ: 20 μM $A\beta_{25–35}$; IMX0.5: 0.5 μM IMX + $A\beta_{25–35}$; IMX1: 1.0 μM IMX + $A\beta_{25–35}$; (p-)GSK-3β: (phosphorylated) glycogen synthase kinase-3β; (p-)JNK: (phosphorylated) c-Jun N-terminal kinase; IMX: indirubin-3′-monoxime; Aβ: amyloid-beta.

$A\beta_{25–35}$, which showed a low level of neurotoxicity. Aggregated $A\beta_{25–35}$ reduced cell viability and initiated apoptosis. Notably, our results provide evidence for the dose-dependent protective effect of IMX against Aβ-induced cell death, consistent with the findings of our previous investigation (Zhang et al., 2009).

Aβ induces and maintains the pathogenic changes in AD, but tau protein also has an important role in the progression of the disease (Iqbal et al., 2014). Aβ amyloidosis triggers the starting phase of tau accumulation and phosphorylation at Ser199, Thr231, and Ser396 in APP Sw mice (Tomidokoro et al., 2001; Stein et al., 2004). In cultured Neuro-2a cells (Hu et al., 2004; Jung et al., 2012; Huang et al., 2014; Nicole et al., 2014; Deng et al., 2015; Zhang et al., 2015), SH-SY5Y cells, and hippocampal neurons (Lafay-Chebassier et al., 2005; Jin et al., 2011; Reifert et al., 2011; Doherty et al., 2013; Martins et al., 2013; Sui et al., 2015), Aβ markedly diminished cell viability, increased the number of apoptotic-like cells, and promoted tau phosphorylation. Inhibiting tau phosphorylation has become a viable approach to treating or even preventing AD. Here, $A\beta_{25–35}$ exposure elevated the rate of tau phosphorylation at pS199 and pT205 in serum-free cultured SH-SY5Y cells, supporting the findings of a previous study (Sun et al., 2008). We also found that IMX suppressed tau phosphorylation induced by $A\beta_{25–35}$. This suggests that the effect of IMX on $A\beta_{25–35}$-induced neurotoxicity may be *via* the inhibition of tau phosphorylation.

GSK-3β, also called Tau Protein Kinase I, is a proline-directed serine/threonine kinase, which phosphorylates tau at a number of AD-relevant epitopes *in vitro* and in transfected cells (Hanger et al., 1992; Ishiguro et al., 1992, 1993; Mandelkow et al., 1992; Mulot et al., 1994; Sperbera et al., 1995;

Bass et al., 2015). GSK-3β may have a pivotal function in the relationship between Aβ peptides and phosphorylated tau, and triggers a pathogenic cycle in AD (Wang et al., 2006; Dobarro et al., 2013; Hoppe et al., 2013; Xian et al., 2014; Amin et al., 2015; Kim et al., 2015; Vossel et al., 2015), resulting in a more important effect than those caused by other kinases such as cdk/MAPK (Elyaman et al., 2002; Liu et al., 2002). The activation of GSK-3β is reportedly related to its low rate of phosphorylation at residue Ser9 (Stambolic and Woodgett, 1994; Wang et al., 1994; Murai et al., 1996). In the present study, we used an antibody against p-GSK-3β Ser9 to investigate the activation of GSK-3β in SH-SY5Y cells. After exposure to $A\beta_{25–35}$, p-GSK-3β (Ser9) expression was decreased, indicating that GSK-3β activity was elevated. However, pretreatment with IMX led to a considerable rise in the expression of p-GSK-3β (Ser9), which suggests that GSK-3β contributes critically to the action of IMX on Aβ-initiated tau phosphorylation.

The constituents of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase, p38 MAPK, and JNK, are enzymes of major importance in the hyperphosphorylation of tau. Because the activation of JNK is critically involved in Aβ-induced cell death (Wei et al., 2002), we investigated the effects of IMX on JNK activation after $A\beta_{25–35}$ exposure. Expression of p-JNK in the $A\beta_{25–35}$-exposed cells was significantly higher than in control cells. However, there were no significant changes after IMX treatment, indicating that IMX did not influence JNK in our cell system.

In summary, IMX exerted neuroprotective effects by preventing Aβ-induced damage, *via* a mechanism that likely involves inhibition of tau phosphorylation. The suppression of GSK-3β signaling was the most important route by which IMX suppressed phosphorylation. Taking into consideration the important role of Aβ throughout the pathogenesis of AD, our results suggest that IMX is a promising drug candidate for the treatment of AD.

Author contributions: *YDZ, SGZ and XSW conceived and designed the study. XJL, SGZ, LGX and JL performed the experiments. SGZ, JPS, MQ and QD analyzed the data and wrote the paper. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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