European Journal of Pharmacology 635 (2010) 96–102

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and Analgesia

Beta-asarone protection against beta-amyloid-induced neurotoxicity in PC12 cells via JNK signaling and modulation of Bcl-2 family proteins

Chengchong Li [1], Guihua Xing [1], Miaoxian Dong, Li Zhou, Jiaming Li, Gang Wang, Dejia Zou, Rui Wang, Jicheng Liu, Yingcai Niu *

The Institute of Medicine, Qiqihar Medical University, Qiqihar 161042, China

ARTICLE INFO

Article history:
Received 11 December 2009
Received in revised form 15 February 2010
Accepted 3 March 2010
Available online 20 March 2010

Keywords:
Alzheimer's disease
Apoptosis
Beta-asarone
Beta-amyloid
C-Jun N-terminal kinase

ABSTRACT

Neurodegenerative brain disorders such as Alzheimer's disease have been well investigated. However, significant methods for the treatment of the promotion and progression of Alzheimer's disease are unavailable to date. Apoptosis is a crucial pathway in neuronal loss in Alzheimer's disease patients. Thus, the suppression of apoptosis may be an effective therapeutic strategy for Alzheimer's disease. In this study, we evaluated the effect of β-asarone on β-amyloid (Aβ)-induced toxicity in cultured PC12 cells. Our data show significant induction of apoptosis in PC12 cells incubated with Aβ peptide, and this effect was reduced by β-asarone. Beta-asarone reduced Aβ-induced JNK activation. In addition, β-asarone attenuates Aβ-induced down-regulation of Bcl-w and Bcl-xL in a JNK-dependent manner, and subsequent inhibition mitochondrial release of cytochrome c and activation of caspase-3. Together, these findings indicate that Aβ-induced apoptosis of PC12 cells proceeds through mitochondrial pathway. Further, the JNK signaling cascade plays a role in regulating the anti-apoptotic effects of β-asarone. Thus, our results indicate that β-asarone might be a potentially therapeutic compound for Alzheimer's disease.

1. Introduction

Alzheimer disease, the most common form of age-related dementia, affects approximately 15 million people worldwide (Cuenco et al., 2008). Recent studies have suggested that β-amyloid (Aβ) plays a critical role in the pathogenesis of Alzheimer's disease as a neurotoxic agent (Hellström-Lindahl et al., 2009). Aβ accumulation has been causally implicated in the neuronal dysfunction and neuronal loss that underlies the clinical manifestations of Alzheimer's disease (Basha et al., 2005). Apoptosis in neurons may contribute to the neuronal degeneration in Alzheimer's disease. Brain tissue from Alzheimer's disease patients contains deposits of oxidized Aβ and activated caspase-3, a cysteine protease that mediates mitochondrion-initiated apoptosis (Abrahamson et al., 2006). Other members of the caspase family have been found to mediate apoptosis resulting from Aβ cytotoxicity (Cecchi et al., 2007). Both necrotic and apoptotic processes are thought to occur in primary neurons and neuronal cell lines after exposure to Aβ as well as in Alzheimer's disease brains (Neumann et al., 2008). As a result, therapeutic strategies that address the toxicity of Aβ may foster novel developments for the treatment of Alzheimer's disease.

In fact, there is good evidence to support the mitochondrial pathway of apoptosis in Aβ-induced neurotoxicity. For example, intracellular Aβ aggregates or granules have been detected in the brains of patients with Alzheimer's disease (Xu et al., 2002). Microinjection of Aβ peptide rapidly induces cell death of primary human neurons through the p53-Bax apoptotic pathway (Zhang et al., 2002). Mitochondrial dysfunction and DNA damage are triggered in cells exposed to Aβ (Xu et al., 2009). The regulation of mitochondrial membrane integrity and the release of apoptogenic factors from mitochondria are key components of the apoptosis repertoire, and are tightly controlled by the Bcl-2 family of proteins (Yamaguchi et al., 2007). The antiapoptotic members, such as Bcl-w and Bcl-xL, localize predominantly at the mitochondrial membrane and promote cell survival. Recently, the involvement of apoptosis has been corroborated by studies showing that Aβ alters expression of the Bcl-2 family of apoptosis-related genes (Wang et al., 2007a,b). The c-Jun N-terminal kinase (JNK) signaling is linked to transcriptional regulation of members of the Bcl-2 family (Yao et al., 2005). Interestingly, JNK activation is observed in cultured neurons after Aβ exposure, and its inhibition significantly attenuates Aβ toxicity (Longpré et al., 2006).

The role of apoptosis in Aβ-induced toxicity suggests that its modulation may slow the neurodegenerative process. Traditional use and clinical reports suggest that *Acorus tatarinowii* Schott may be effective for patients with mild to moderate Alzheimer's disease (Liao

* Corresponding author. 333 BuKui Street, JianHua District, Qiqihar, The Institute of Medicine, Qiqihar Medical Univeristy, Qiqihar, 161006, China. Tel./fax: +86 452 6720289.
E-mail address: nyc1968@sohu.com (Y. Niu).
[1] All authors contributed equally for this work.

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.03.013

et al., 2005). Substantial experimental evidence indicates that β-asarone (for its structure, see Fig. 1), the major ingredient of *A. tatarinowii* Schott, have neuroprotective effects in vitro and in vivo (Fang et al., 2008; Chen et al., 2007). However, the cellular and molecular mechanisms remain unclear. There is much to be learned about the pathophysiology of the disease and, importantly, about possible novel therapeutic approaches from analyzing the pharmacological mechanisms of this traditional remedy.

In this study, we examined the regulatory effects of β-asarone on expression of Bcl-xL and Bcl-w in PC12 cells after insult with Aβ. Furthermore, we investigated both the upstream (e.g., JNK signaling) and downstream (e.g., cytochrome *c*) components of this antiapoptotic pathway.

2. Materials and methods

2.1. Cell culture

PC12 cells, a rat pheochromocytoma, obtained from the cell bank of Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) were maintained in Dulbecco's modified Eagle medium (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum, 50 U/ml penicillin (Invitrogen, Carlsbad, CA, USA), and 100 mg/ml streptomycin (Invitrogen). The cells were seeded in φ60 mm dishes (Nalge Nunc Int., Rochester, NY, USA) at 1×10^4 cells/cm^2 and maintained at 37 °C in a humidified atmosphere of 5% CO_2. PC12 cells were incubated and differentiated by the addition of nerve growth factor for 48 h before all the experiments performed in the present study.

2.2. Determination of cell viability

Cell viability was measured by quantitative colorimetric assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as described previously (Hansen et al., 1989). Briefly, the cells were cultured at a density 5×10^4 cells per well in growth medium for 24 h in 96-well plates and then preincubated with or without β-asarone for 24 h following incubation with aggregated Aβ(25–35)(Sigma, St. Louis, MO. USA) prepared as described previously (Pike et al., 1993) for another 24 h. Twenty five μL/well of MTT solution (final concentration, 500 μg/ml) was added and cells were incubated at 37 °C for 4 h. Supernatants were then aspirated off and formazan crystals were dissolved with DMSO. The optical density of each well was determined at 570 nm using a microplate reader (Safire2, Tecan Group Ltd, Maennedorf, Switzerland).

2.3. Fluorescence activated cell sorting (FACS) analysis

PC12 cells were seeded in 6-well plates at a cell density of 1.5×10^5 cells/cm^2. Then the cells were treated with or without β-asarone at concentrations of 7.5, 15, and 30 μg/ml. Following 24 h incubation, aggregated Aβ(25–35) (20 μM) was added. The cells were collected 48 h later. Annexin V assays were done using the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson, San Jose, CA). Cells were washed twice with cold PBS and resuspended in binding buffer before addition of Annexin V-FITC and propidium iodide (PI). Cells were vortexed and incubated for 15 min in the dark at room temperature before analysis using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, San Carlos, CA).

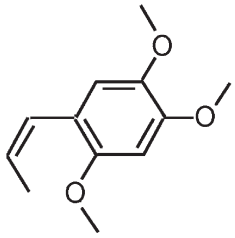

Fig. 1. Chemical structure of β-asarone.

2.4. RNA isolation and real-time PCR

PC12 cells were seeded in 6-well plates at a cell density of 1.5×10^5 cells/cm^2. Then the cells were treated with or without β-asarone at concentrations of 15 μg/ml. Following 24 h incubation, aggregated Aβ(25–35) (20 μM) was added. SP600125 (100 nM) (Calbiochem, La Jolla, CA), a selective JNK inhibitor, was added to cultures 1 h prior to Aβ(25–35). Cells were harvested by scraping into ice cold PBS 48 h later. Total RNA was extracted from cells using RNAiso Reagent kit (Takara Biotechnology, Dalian, China), and cDNA was synthesized with SYBR ExScriptTM RT-PCR kit (Takara Biotechnology, Dalian, China) according to the manufacturer' protocol. Reverse transcription was carried out as follows: 42 °C for 15 min, 95 °C for 2 min (one cycle). cDNA stored at −20 °C for PCR. Real-time PCR was performed on in a 50 μl of reaction solution containing 2×SYBR Premix Ex Taq polymerase, deoxynucleoside triphosphates, ROX Reverence Dye and the corresponding primers. The following sequences were used as primers: Bcl-xL sense primer, 5′-CAG CTT CAT ATA ACC CCA GGG AC-3′ and Bcl-xL antisense primer, 5′-GCT CTA GGT GGT CAT TCA GGT AGG; Bcl-w sense primer, 5′-GAG TTT GAG ACC CGC TTC C-3′ and Bcl-w antisense primer, 5′-GTC CTC ACT GAT GCC CAG TT; and GAPDH sense primer, 5′-GAC AAC TTT GGC ATC GTG GA-3′ and GAPDH antisense primer, 5′-ATG CAG GGA TGA TGT TCT GG. The thermal profile was as follows: 1 cycle of 95 °C for 10 s; 40 cycles of 5 s at 95 °C and 31 s at 60 °C. Reactions were performed in a ABI7300 real-time PCR system (Applied Biosystems, CA), and Ct (threshold cycle) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA). The Ct represents the cycle number at which a fluorescent signal rises statistically above background. Real-time PCR assay was performed in triplicate for each sample to ensure reproducibility. The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The fold change in target gene cDNA relative to the GAPDH internal control was determined by:

$$\text{Fold change} = 2^{-\Delta\Delta Ct},\ \text{where } \Delta\Delta Ct = \left(Ct_{\text{target gene}} - Ct_{GAPDH}\right) - \left(Ct_{\text{control}} - Ct_{GAPDH}\right)$$

2.5. Western blot

The cells were treated as described above for the real-time PCR analysis. Cytoplasm proteins were isolated from PC12 cells using Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Haimen, China), and protein concentrations were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer (Beyotime Biotechnology, Haimen, China), then they were aliquoted and stored. 100 μl of supernatant was added to an equal volume of 2×SDS sample buffer and boiled for 5 min at 100 °C. The samples were then stored at −80 °C until analyzed. Equal amounts of protein (100 μg/lane) were separated by 15% SDS-polyacrylamide gel electrophoresis and then electrotransferred onto a nitrocellulose filter membrane. After blocking for 4 h in a solution of 8% nonfat dry milk in Tris-buffered saline containing 0.1% tween (pH 7.6) at room temperature, membrane was then incubated overnight at 4 °C with primary antibody (caspase-3 antibody, Cell Signaling Technology Inc., Beverly, MA; other antibody, Santa Cruz Biotechnology, Santa Cruz, CA) in concentrations of 1:2000 (p-JNK), 1:2000 (JNK), 1:1500 (Bcl-xL), 1:1500 (Bcl-w), 1:1500 (caspase-3), and 1:3000 (GAPDH) in Tris-buffered saline with 0.1% Tween 20 containing 8 % nonfat dry milk. After washing four times, the membrane were incubated with Horseradish

Peroxidase Labeled Anti-Mouse IgG (10,000:1; Medical Biological Laboratory Co., Nagoya, Japan) at room temperature for 2 h and again washed four times. The blots were developed using an ECL western blotting kit (Amersham Biosciences, Piscataway, NJ, USA) as recommended by the manufacturer. GAPDH was probed as an internal control to confirm that an equal amount of protein was loaded in each lane. Band intensities were quantified by an AlphaImager™ 2200 using the SpotDenso function of AlphaEaseFC™ Software version 3.1.2 (Witec, Littau, Switzerland).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The cells were treated as described above for the real-time PCR analysis. Cells were collected, fractionated, and cytosolic cytochrome *c* was measured using the Quantikine M rat/mouse cytochrome *c* assay kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Cytosolic fractions were pipetted in triplicate onto a microplate precoated with rat/mouse cytochrome *c*. After a 2 h incubation and washing, substrate solution was added to each well. The reaction was stopped after 30 min and the optical density was measured at 540 nm using a microplate reader (Safire2, Tecan Group Ltd, Maennedorf, Switzerland). Cytochrome *c* concentrations expressed as nanograns/milliliter were extrapolated from the standard curves generated using reconstitute the r/m Cyt. *c* standard.

2.7. Statistical analysis

All values in the figures of present study indicate means ± standard deviation (S.D.), and all determinations were repeated three times. The one way analysis of variance (ANOVA) was used to evaluate the difference among multiple groups followed by a post hoc test (Student–Newman–Keuls), and the independent sample *t* test for differences between two treatment groups. The data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA), and $P<0.05$ was assessed as statistically significant.

3. Results

3.1. Preventive effect of β-asarone on Aβ-induced cell death

Cell viability detected by MTT revealed that Aβ(25–35) significantly increased PC12 cells death in a dose-dependent manner up to 20 μM, with no further decrease being observed at 100 μM concentrations. PC12 cells pretreated with β-asarone (7.5, 15, or 30 μg/ml) for 24 h prior to Aβ incubation produced dose-dependent attenuation of the Aβ toxicity (Fig. 2A). Thus 20 μM concentration of Aβ(25–35) and 15 μg/ml concentration of β-asarone was used in subsequent experiments.

We furthermore studied the time-dependent effect of Aβ (20 μM) on PC12 cells with or without β-asarone pretreatment (15 μg/ml). In the absence of β-asarone, the viability of PC12 cells was gradually decreased in a time-dependent manner up to 24 h, with no further decrease in 48 h (Fig. 2B). Thus 24 h treatment of Aβ was used in subsequent experiments. Beta-asarone pretreatment preserved the PC12 cells in the presence of Aβ(25–35).

3.2. Preventive effective of β-asarone on Aβ-induced cell apoptosis

Cell apoptosis was quantified by staining cell with annexin-V-FITC/PI (Fig. 3A). Quantitative analysis of Annexin V-positive cells revealed that treatment of PC12 cells with 20 μM Aβ for 24 h evoked marked cell apoptosis indicated by the percentage of Annexin V-positive cells. Pretreatment of PC12 cells with β-asarone at 7.5–30 μg/ml, prior to Aβ exposure, significantly decreased cell apoptosis (Fig. 3B). We further determined the activation of caspase-3 by western blot analysis (Fig. 4A). Cell treated with Aβ exhibited an increase in cleaved caspase-3, whose response was significantly depressed by the β-asarone pretreatment (Fig. 4B). In addition, pretreatment with the JNK inhibitor SP600125 also significantly decreased Aβ-induced cell apoptosis compared with Aβ alone (Fig. 4B).

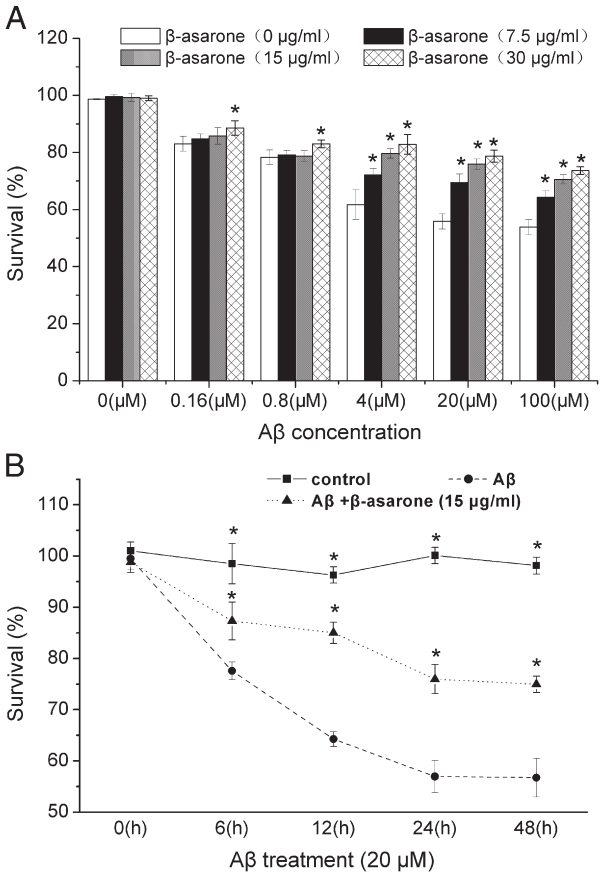

Fig. 2. Prevention of Aβ(25–35)-induced cell death by β-asarone. A) Dose-dependent change of cell viability of PC12 cells by Aβ treatment. PC12 cells were pretreated with β-asarone (0–30 μg/ml) for 24 h and exposed to aggregated Aβ(25–35) (0–100 μM) for 24 h, and cell viability was determined by MTT. B) Time-dependent change of cell viability of PC12 cells by Aβ treatment. PC12 cells were pretreated with β-asarone (15 μg/ml) for 24 h and exposed to aggregated Aβ(25–35)(20 μM) for 0–48 h. The data obtained from three separate experiments and are expressed as mean ± S.D.; *$P<0.05$ compared to Aβ(25–35) alone.

3.3. Preventive effect of β-asarone on Aβ-induced cytochrome c release

Cytochrome *c* concentration in cytosolic fractions was measured using a commercial ELISA kits. PC12 cells treated with Aβ for 24 h showed an increase in the cytosolic cytochrome *c* levels. Pretreatment with β-asarone attenuated the Aβ-induced increase in cytochrome *c* levels (Fig. 5). In addition, pretreatment with SP600125 also significantly decreased cytosolic cytochrome *c* levels as observed in effect of β-asarone.

3.4. Effects of β-asarone on expression of Bcl-xL and Bcl-w

Given that Bcl-2 family proteins are important modulators of PC12 cells apoptosis induced by Aβ, we determined the effect of β-asarone on Bcl-w and Bcl-xL protein levels and the role of JNK signal pathway. Western blot results (Fig. 6A) shows that Aβ treatment significantly decreased Bcl-w and Bcl-xL protein levels in PC12 cells, and this decrease was significantly counteracted by β-asarone pretreatment (15 μg/ml for 24 h). The SP600125-mediated increase of Bcl-w and Bcl-xL levels was not affected by β-asarone (Fig. 6B). In addition, pretreatment with SP600125 also significantly increased Bcl-w and

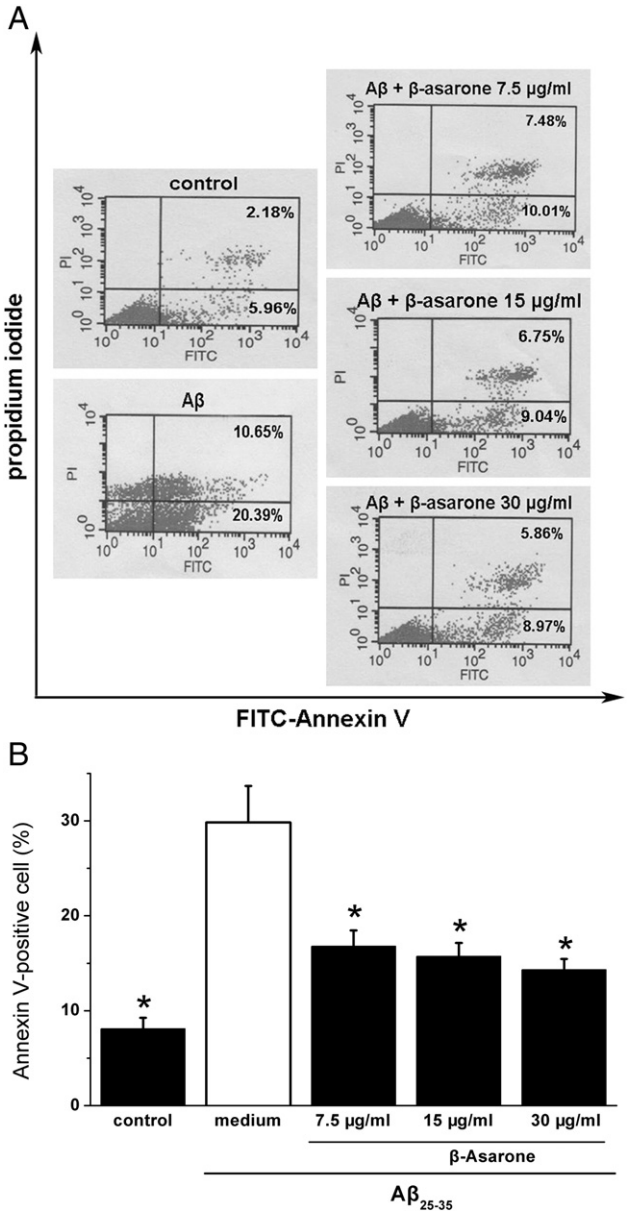

Fig. 3. Beta-asarone pretreatment attenuation Aβ(25–35)-induced cell apoptosis. PC12 cells were pretreated with 7.5, 15, or 30 μg/ml for 24 h and exposed to aggregated Aβ(25–35) (20 μM) for 24 h, and cells apoptosis was measured by labeling cells with annexin-V-FITC and counterstaining with propidium iodide (PI). A) Annexin-V-FITC/PI double staining of PC12 cells. The numbers indicate the percentage of cells in each quadrant (lower left: $FITC^-/PI^-$, intact cells; lower right: $FITC^+/PI^-$, apoptotic cells; upper left: $FITC^-/PI^+$, necrotic cells; upper right: $FITC^+/PI^+$, late apoptotic cells). B) The bar chart describes the percentage distribution of apoptotic cells. Percentage of annexin V-positive cells analysis of *FACS* obtained from three separate experiments and are expressed as mean ± S.D.; $^*P<0.05$ compared to Aβ(25–35) alone.

Bcl-xL protein levels in PC12 cells. Consistent with the results of protein levels, real-time PCR revealed that β-asarone pretreatment significantly increase Bcl-w and Bcl-xL mRNA levels (Fig. 6C).

3.5. Effect of β-asarone on Aβ-induced phosphorylation of JNK

We examined the effect of β-asarone regulating Aβ-induced JNK activation. Western blot results (Fig. 7A) show that Aβ (25–35) triggers JNK phosphorylation. Beta-asarone pretreatment inhibited Aβ-induced phosphorylation of JNK. The JNK inhibitor SP600125 blocked the inhibitory effect of β-asarone on Aβ-induced JNK phosphorylation of JNK (Fig. 7B).

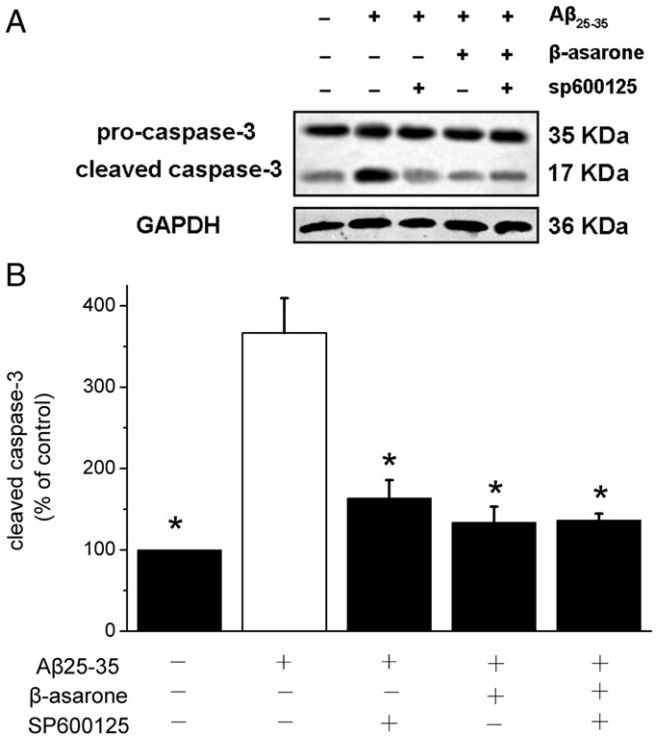

Fig. 4. Beta-asarone pretreatment attenuation Aβ(25–35)-induced caspase-3 activation in PC12 cells. PC12 cells were pretreated with or without β-asarone at concentrations of 15 μg/ml. Following 24 h incubation, aggregated Aβ(25–35) (20 μM) was added. SP600125 (100 nM) was added to cultures 1 h prior to Aβ(25–35). Pro-caspase-3 and cleaved-caspase-3 levels were determined by immunoblot analysis with antibody to caspase-3. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of cleaved-caspase-3 are presented relative to control. Densitometric analysis of western blot obtained from three separate experiments and are expressed as mean ± S.D.; $^*P<0.05$ compared to Aβ(25–35) alone.

4. Discussion

This study demonstrates for the first time neuroprotective effect of β-asarone against Aβ insult in PC12 cells, a typical model of Alzheimer's disease in a culture system, as evidenced by increased cell viability and

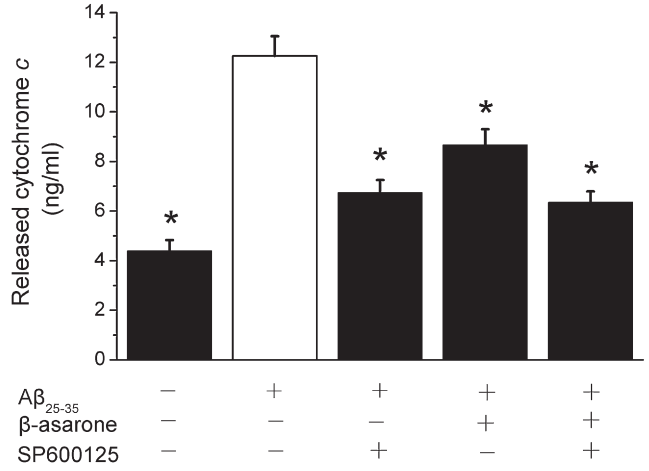

Fig. 5. Beta-asarone attenuation the cytosolic cytochrome *c* levels in PC12 cells. PC12 cells were pretreated with or without β-asarone at concentrations of 15 μg/ml. Following 24 h incubation, aggregated Aβ(25–35) (20 μM) was added. SP600125 (100 nM) was added to cultures 1 h prior to Aβ(25–35). The level of cytosolic cytochrome *c* was measured by ELISA. Values obtained from three separate experiments and expressed as mean ± S.D.; $^*P<0.05$ compared to Aβ(25–35) alone.

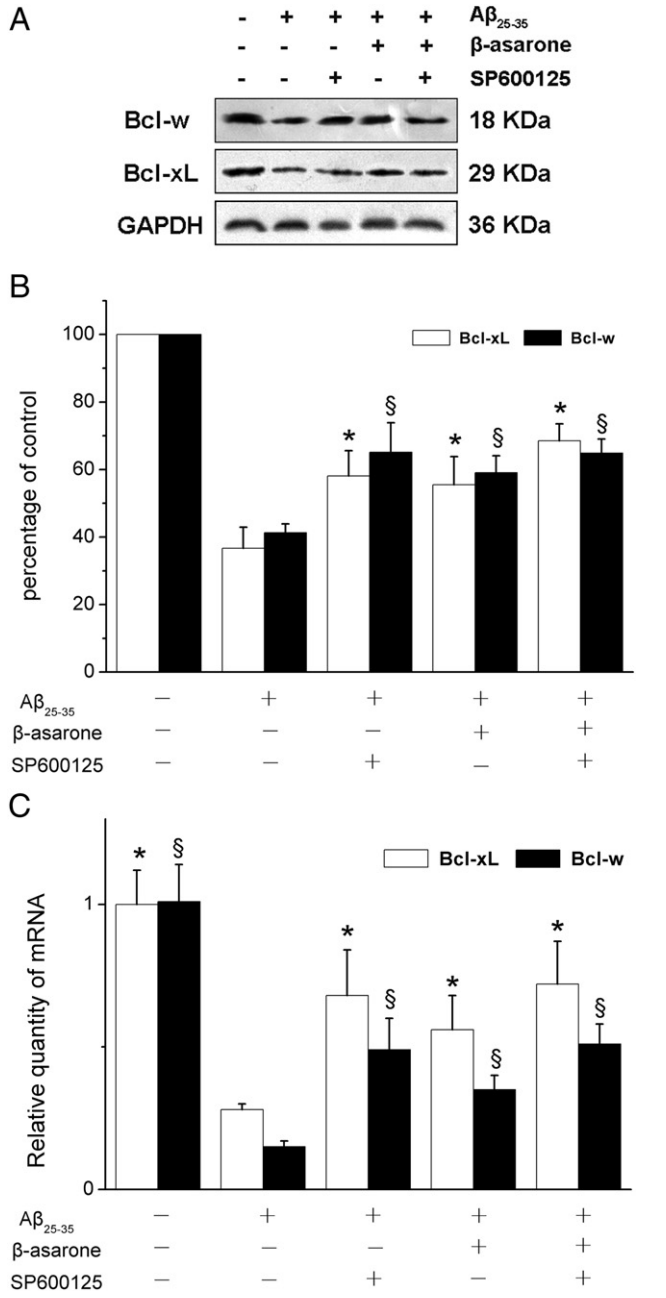

Fig. 6. Beta-asarone attenuation Aβ(25–35)-induced downregulation Bcl-xL and Bcl-w. PC12 cells were pretreated with or without β-asarone at concentrations of 15 μg/ml. Following 24 h incubation, aggregated Aβ(25–35) (20 μM) was added. SP600125 (100 nM) was added to cultures 1 h prior to Aβ(25–35). A) Bcl-xL and Bcl-w levels were determined by immunoblot analysis with antibody to Bcl-xL and Bcl-w. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of Bcl-xL and Bcl-w are presented relative to control. Densitometric analysis of western blot obtained from three separate experiments. C) Total RNA was isolated from PC12 cells using RNAiso reagent and used for cDNA synthesis. The mRNA levels of Bcl-xL and Bcl-w were detected by real-time PCR. $2^{-\Delta\Delta Ct}$ analysis of PCR obtained from three separate experiments, and data are expressed as mean ± S.D. *$P<0.05$ compared to Aβ(25–35) alone. #$P<0.05$ compared to Aβ + β-asarone treatment cells.

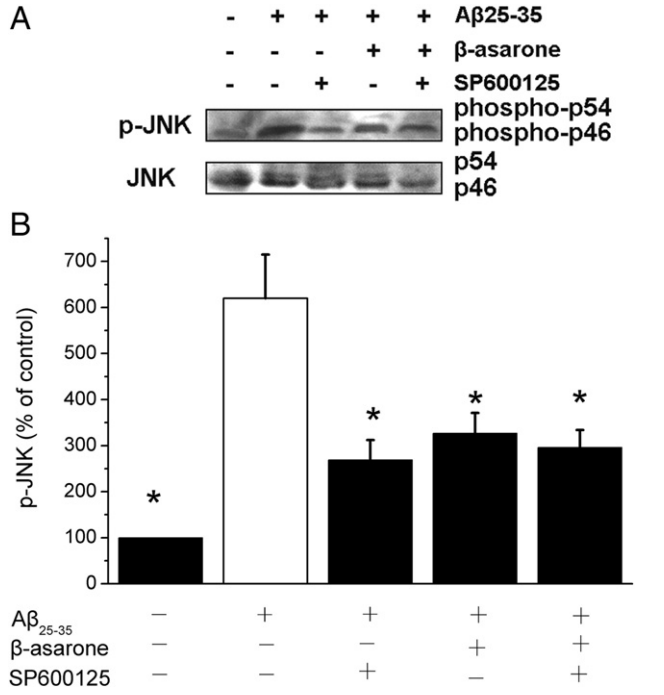

Fig. 7. Beta-asarone attenuation Aβ(25–35)-induced JNK phosphorylation. PC12 cells were pretreated with or without β-asarone at concentrations of 15 μg/ml. Following 24 h incubation, aggregated Aβ(25–35) (20 μM) was added. SP600125 (100 nM) was added to cultures 1 h prior to Aβ(25–35). A) JNK and p-JNK levels were determined by immunoblot analysis with antibody to JNK and p-JNK. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of p-JNK are presented relative to control. Densitometric analysis of western blot obtained from three separate experiments, and data are expressed as mean ± S.D. *$P<0.05$ compared to Aβ(25–35) alone. #$P<0.05$ compared to Aβ + β-asarone treatment cells.

decreased cells apoptosis. Beta-asarone significantly inhibited Aβ-induced downregulation of Bcl-xL and Bcl-w, thus inhibiting cytochrome *c* release, caspase activation. In addition, we found that JNK-dependent signaling pathway could be involved in neuroprotection of β-asarone against Aβ toxicity.

A. tatarinowii Schott has long been employed in the clinical treatment of Alzheimer's disease in chinese herbal books. The more recent results of Wu et al. confirmed that β-asarone, a component isolated from essential oil of *A. tatarinowii* Schott, is easy to pass through blood brain barrier, and brain is an major organ of distributing of it (Wu and Fang, 2004). Beta-asarone, however, has not yet been evaluated for actions on the Alzheimer's disease and its mechanism of action.

Apoptosis is a fundamental process of cell death that occurs via activation of distinct signaling pathways involving mitochondria, mitochondrial regulatory proteins, and activation of caspases (Cui et al., 2005). Apoptosis is actively executed by several members of the caspase family, including caspase-3, which is involved in the final execution phase of apoptosis (May and Madge, 2007). Increased neuronal caspase-3 expression is observed in the brain with Alzheimer's disease (Wai et al., 2009). Several studies presently indicate that apoptosis selectively increased in primary neuronal cultures exposed to Aβ and also augmented in brain tissue derived from Alzheimer's disease patients (Nie et al., 2008; Satoi et al., 2005). In this study, Aβ treatment of differentiated PC12 cells increased apoptosis and the level of cleaved caspase-3, a marker of apoptosis. Beta-asarone pretreatment attenuated PC12 cells apoptosis and level of cleaved caspase-3.

Therefore, we addressed whether or not β-asarone can regulate genes associated with apoptosis, such as Bcl-xL and Bcl-w. Bcl-xL and Bcl-w widely express in mammalian tissues, including CNS, and functions as a negative regulator of neuronal apoptosis (Akcali et al., 2005). Neural expression of Bcl-xL and Bcl-w are highest in the mature brain, suggesting that Bcl-xL and Bcl-w function may be particularly important in adulthood (Hamnér et al., 1999). The two best-studied pathways of caspase activation are the cell-surface–death-receptor pathway, i.e., Fas-mediated apoptosis, and the mitochondrion-initiated pathway. Many components of the mitochondrial apoptotic cascade appear to be involved in the neuronal

toxicity of Aβ peptides (Yin et al., 2002). Aβ can downregulate anti-apoptotic Bcl-xL and Bcl-w expression or require that to mediate neurotoxicity (Wei et al., 2003). The antiapoptotic protein Bcl-xL and Bcl-w resides in the outer mitochondrial wall and inhibits cytochrome c release (Zhang et al., 2007). Our results indicate that Aβ treatment caused a decrease expression of the antiapoptotic protein Bcl-xL and Bcl-w in PC12 cells. Beta-asarone preconditioning inhibited the decrease in the expression of Bcl-xL and Bcl-w. Pretreatment with the JNK pharmacological inhibitor SP600125 before β-asarone preconditioning blocked the inhibitory effects of β-asarone on Aβ-induced Bcl-xL and Bcl-w downregulation, suggesting that the effect of β-asarone is mediated through JNK signaling. In the mean time, the mRNA level of Bcl-xL and Bcl-w were also down-regulated by Aβ and were followed by the increased expression in β-asarone treatment cells prior to Aβ abuse.

Mitochondrial cytochrome *c* is a water-soluble protein loosely attached in the mitochondrial intermembrane space. In response to a variety of apoptosis-inducing agents, cytochrome *c* is released from mitochondria to the cytosol (Li et al., 2007). Cytochrome *c* participates in the formation of a cytosolic complex. Within this complex, caspase-9 is activated, leading to the downstream activation of caspase-3 (Twiddy et al., 2004). Activated caspase-3 ultimately leads to cell death**.** We found that cytochrome *c* levels in cytosolic of PC12 cells treatment with Aβ were significantly higher than in the blank control cells. Interestingly, the cytochrome *c* levels in cytosolic significantly decrease after β-asarone treatment as a manner similar to cells apoptosis. These findings imply that β-asarone might inhibit apoptosis involving release of cytochrome *c* in PC12 cells.

Concerted actions of molecular signaling networks determine cell fates (Jin and El-Deiry, 2005). Within the many stress-responsive signaling pathways, the JNK signaling cascade is crucial for the maintenance of cell homeostasis and controls many cellular processes, including cell growth, transformation, differentiation and apoptosis (Junttila et al., 2008). There is very strong evidence linking the activation of JNK to neuronal loss in response to Aβ neurotoxicity (Yao et al., 2005). JNK signaling promotes apoptosis which is linked to transcriptional regulation of many genes, including Bcl-xL and Bcl-w (Wang et al., 2007a,b). SP600125, JNK pharmacological inhibitor, effectively prevents alterations of Bcl-2 family protein expression induced by Aβ, indicating that this critical step in the Aβ induced-apoptosis pathway is dependent on JNK activation (Yao et al., 2007). Thus, the suppression of JNK-dependent apoptosis gene expression may be an extremely effective therapeutic strategy for preventing neuronal cell apoptosis. In the present study, we found that Aβ-induced PC12 cells apoptosis involves JNK-dependent downregulation of Bcl-w and Bcl-xL. Beta-asarone significantly attenuated Aβ-induced changes in Bcl-w and Bcl-xL expression. Notably, β-asarone also significantly reduced Aβ-induced JNK phosphorylation, suggesting that inhibitory effect of β-asarone on Aβ-induced changes in Bcl-w and Bcl-xL expression involves inhibition of JNK activation.

In conclusion, our results are consistent with the hypothesis that β-asarone potentially attenuates Aβ-induced PC12 cells death, at least in part, by inhibition of Aβ-induced JNK activation, subsequent JNK-dependent up-regulation of Bcl-w and Bcl-xL, and inhibiting mitochondrial release of cytochrome *c* and activation of caspase-3. Our findings suggest that β-asarone, an important active principal of *A. tatarinowii* Schott, might be a potential drug for the Alzheimer's disease to suppress neuronal cell apoptosis.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (No. 30873396), of the Heilongjiang Province (No. D200955), and the National Science Foundation for Post-doctoral Scientists of China (No. 20080430140).

References

Abrahamson, E.E., Ikonomovic, M.D., Ciallella, J.R., Hope, C.E., Paljug, W.R., Isanski, B.A., Flood, D.G., Clark, R.S., DeKosky, S.T., 2006. Caspase inhibition therapy abolishes brain trauma-induced increases in Abeta peptide: implications for clinical outcome. Exp. Neurol. 197, 437–450.

Akcali, K.C., Sahiner, M., Sahiner, T., 2005. The role of bcl-2 family of genes during kindling. Epilepsia 46, 217–223.

Basha, M.R., Murali, M., Siddiqi, H.K., Ghosal, K., Siddiqi, O.K., Lashuel, H.A., Ge, Y.W., Lahiri, D.K., Zawia, N.H., 2005. Lead (Pb) exposure and its effect on APP proteolysis and Abeta aggregation. FASEB J. 19, 2083–2084.

Cecchi, C., Fiorillo, C., Baglioni, S., Pensalfini, A., Bagnoli, S., Nacmias, B., Sorbi, S., Nosi, D., Relini, A., Liguri, G., 2007. Increased susceptibility to amyloid toxicity in familial Alzheimer's fibroblasts. Neurobiol. Aging 28, 863–876.

Chen, Y.Z., Fang, Y.Q., Wang, Q.W., Xie, Y.H., Kuang, Z.S., 2007. Effects of beta-asarone on morphology and cell viability in PC12 cells and cultured rat cortical neurons. Zhong Yao Cai 30, 317–321.

Cuenco, K.T., Green, R.C., Zhang, J., Lunetta, K., Erlich, P.M., Cupples, L.A., Farrer, L.A., DeCarli, C., MIRAGE Study Group, 2008. Magnetic resonance imaging traits in siblings discordant for Alzheimer disease. J. Neuroimaging 18, 268–275.

Cui, H., Li, T., Ding, H.F., 2005. Linking of N-Myc to death receptor machinery in neuroblastoma cells. J. Biol. Chem. 280, 9474–9481.

Fang, Y.Q., Fang, R.M., Fang, G.L., Jiang, Y., Fu, S.Y., 2008. Effects of beta-asarone on expression of c-fos in kindling epilepsy rat brain. Zhong guo Zhong Yao Za Zhi 33, 534–536.

Hamnér, S., Skoglösa, Y., Lindholm, D., 1999. Differential expression of bcl-w and bcl-x messenger RNA in the developing and adult rat nervous system. Neuroscience 91, 673–684.

Hansen, M.B., Nielsen, S.E., Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203–210.

Hellström-Lindahl, E., Viitanen, M., Marutle, A., 2009. Comparison of Abeta levels in the brain of familial and sporadic Alzheimer's disease. Neurochem. Int. 55, 243–252.

Jin, Z., El-Deiry, W.S., 2005. Overview of cell death signaling pathways. Cancer Biol. Ther. 4, 139–163.

Junttila, M.R., Li, S.P., Westermarck, J., 2008. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J. 22, 954–965.

Li, C.R., Zhou, Z., Lin, R.X., Zhu, D., Sun, Y.N., Tian, L.L., Li, L., Gao, Y., Wang, S.Q., 2007. beta-sitosterol decreases irradiation-induced thymocyte early damage by regulation of the intracellular redox balance and maintenance of mitochondrial membrane stability. J. Cell. Biochem. 102, 748–758.

Liao, W.P., Chen, L., Yi, Y.H., Sun, W.W., Gao, M.M., Su, T., Yang, S.Q., 2005. Study of antiepileptic effect of extracts from *Acorus tatarinowii* Schott. Epilepsia 46, 21–24.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Longpré, F., Garneau, P., Christen, Y., Ramassamy, C., 2006. Protection by EGb 761 against beta-amyloid-induced neurotoxicity: involvement of NF-kappaB, SIRT1, and MAPKs pathways and inhibition of amyloid fibril formation. Free Radic. Biol. Med. 41, 1781–1794.

May, M.J., Madge, L.A., 2007. Caspase inhibition sensitizes inhibitor of NF-kappaB kinase beta-deficient fibroblasts to caspase-independent cell death via the generation of reactive oxygen species. J. Biol. Chem. 282, 16105–16116.

Neumann, K.F., Rojo, L., Navarrete, L.P., Farías, G., Reyes, P., Maccioni, R.B., 2008. Insulin resistance and Alzheimer's disease: molecular links and clinical implications. Curr. Alzheimer Res. 5, 438–447.

Nie, B.M., Jiang, X.Y., Cai, J.X., Fu, S.L., Yang, L.M., Lin, L., Hang, Q., Lu, P.L., Lu, Y., 2008. Panaxydol and panaxynol protect cultured cortical neurons against Abeta25-35-induced toxicity. Neuropharmacology 54, 845–853.

Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G., Cotman, C.W., 1993. Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. J. Neurosci. 13, 1676–1687.

Satoi, H., Tomimoto, H., Ohtani, R., Kitano, T., Kondo, T., Watanabe, M., Oka, N., Akiguchi, I., Furuya, S., Hirabayashi, Y., Okazaki, T., 2005. Astroglial expression of ceramide in Alzheimer's disease brains: a role during neuronal apoptosis. Neuroscience 130, 657–666.

Twiddy, D., Brown, D.G., Adrain, C., Jukes, R., Martin, S.J., Cohen, G.M., MacFarlane, M., Cain, K., 2004. Pro-apoptotic proteins released from the mitochondria regulate the protein composition and caspase-processing activity of the native Apaf-1/caspase-9 apoptosome complex. J. Biol. Chem. 279, 19665–19682.

Wai, M.S., Liang, Y., Shi, C., Cho, E.Y., Kung, H.F., Yew, D.T., 2009. Co-localization of hyperphosphorylated tau and caspases in the brainstem of Alzheimer's disease patients. Biogerontology 10, 457–469.

Wang, X.T., Pei, D.S., Xu, J., Guan, Q.H., Sun, Y.F., Liu, X.M., Zhang, G.Y., 2007a. Opposing effects of bad phosphorylation at two distinct sites by Akt1 and JNK1/2 on ischemic brain injury. Cell. Signal. 19, 1844–1856.

Wang, Z., Zhang, X., Wang, H., Qi, L., Lou, Y., 2007b. Neuroprotective effects of icaritin against beta amyloid-induced neurotoxicity in primary cultured rat neuronal cells via estrogen-dependent pathway. Neuroscience 145, 911–922.

Wei, Z., Mousseau, D.D., Richardson, J.S., Dyck, L.E., Li, X.M., 2003. Atypical antipsychotics attenuate neurotoxicity of beta-amyloid(25–35) by modulating Bax and Bcl-X(l/s) expression and localization. J. Neurosci. Res. 74, 942–947.

Wu, H.B., Fang, Y.Q., 2004. Pharmacokinetics of beta-asarone in rats. Yao Xue Xue Bao 39, 836–838.

Xu, G., Gonzales, V., Borchelt, D.R., 2002. Rapid detection of protein aggregates in the brains of Alzheimer patients and transgenic mouse models of amyloidosis. Alzheimer Dis. Assoc. Disord. 16, 191–195.

Xu, S., Zhong, M., Zhang, L., Wang, Y., Zhou, Z., Hao, Y., Zhang, W., Yang, X., Wei, A., Pei, L., Yu, Z., 2009. Overexpression of Tfam protects mitochondria against beta-amyloid-induced oxidative damage in SH-SY5Y cells. FEBS J. 276, 2809–3800.

Yamaguchi, R., Andreyev, A., Murphy, A.N., Perkins, G.A., Ellisman, M.H., Newmeyer, D.D., 2007. Mitochondria frozen with trehalose retain a number of biological functions and preserve outer membrane integrity. Cell Death Differ. 14, 616–624.

Yao, M., Nguyen, T.V., Pike, C.J., 2005. Beta-amyloid-induced neuronal apoptosis involves c-Jun N-terminal kinase-dependent downregulation of Bcl-w. J. Neurosci. 25, 1149–1158.

Yao, M., Nguyen, T.V., Pike, C.J., 2007. Estrogen regulates Bcl-w and Bim expression: role in protection against beta-amyloid peptide-induced neuronal death. J. Neurosci. 27, 1422–1433.

Yin, K.J., Lee, J.M., Chen, S.D., Xu, J., Hsu, C.Y., 2002. Amyloid-beta induces Smac release via AP-1/Bim activation in cerebral endothelial cells. J. Neurosci. 22, 9764–9770.

Zhang, Y., McLaughlin, R., Goodyer, C., LeBlanc, A., 2002. Selective cytotoxicity of intracellular amyloid beta peptide1–42 through p53 and Bax in cultured primary human neurons. J. Cell Biol. 156, 519–529.

Zhang, H., Nimmer, P.M., Tahir, S.K., Chen, J., Fryer, R.M., Hahn, K.R., Iciek, L.A., Morgan, S.J., Nasarre, M.C., Nelson, R., Preusser, L.C., Reinhart, G.A., Smith, M.L., Rosenberg, S.H., Elmore, S.W., Tse, C., 2007. Bcl-2 family proteins are essential for platelet survival. Cell Death Differ. 14, 943–951.