Beta-Asarone Improves Cognitive Function by Suppressing Neuronal Apoptosis in the Beta-Amyloid Hippocampus Injection Rats

Yutao Geng, Chengchong Li, Jicheng Liu, Guihua Xing, Li Zhou, Miaoqian Dong, Xueyan Li, and Yingcai Niu*

The Institute of Medicine, Qiqihar Medical University; Qiqihar 161006, China.
Received December 20, 2009; accepted February 16, 2010; published online February 17, 2010

Elevated levels of β-amyloid (Aβ) in the brains being a hallmark of Alzheimer’s disease (AD) have been believed to play a critical role in the cognitive dysfunction that occurs in AD. Recent evidence suggests that Aβ induces neuronal apoptosis in the brain and in primary neuronal cultures. In this study, we investigated the effects of β-asarone, the major ingredient of Acorus Tatarinowii Schott, on cognitive function and neuronal apoptosis in Aβ hippocampus injection rats and its mechanism of action. The results show that the Aβ (1—42)-induced cognitive impairment caused impairments in spatial reference memory in a Morris water maze task and apoptosis in hippocampus. Oral administration of β-asarone with three different dose (12.5, 25, or 50 mg/kg) for 28 d ameliorated Aβ (1—42)-induced cognitive impairment and reversed the increase of apoptosis in the hippocampus. Aβ-induced c-Jun N-terminal kinase (JNK) results in phosphorylation, subsequent down-regulation of Bcl-2 and Bcl-w expression, and caspase-3 activation. Beta-asarone attenuate Aβ (1—42)-induced neuronal apoptosis in hippocampus by reversal down-regulation of Bcl-2, Bcl-w, caspase-3 activation and JNK phosphorylation. These results suggest that β-asarone may be a potential candidate for development as a therapeutic agent to manage cognitive impairment associated with conditions such as Alzheimer’s disease.

Key words Alzheimer’s disease; β-asarone; β-amyloid; c-Jun N-terminal kinase; apoptosis

Alzheimer’s disease (AD), a slowly progressive neuro-psychiatric illness, principally characterized by memory deficits. The deposition of amyloid β peptide (Aβ) within the senile plaques that are a hallmark of AD is thought to be a primary cause of the cognitive dysfunction that occurs in AD. The evidence that Aβ accumulation is a determining factor in AD makes it important to determine the mechanism by which Aβ induces neuronal cell death. Recent studies have shown that in AD brains and in cultures of neurons exposed to Aβ, the dying cells display the characteristics of apoptosis. The involvement of apoptosis has been corroborated by studies showing that Aβ alters expression of the Bcl-2 family of apoptosis-related genes. The c-Jun N-terminal kinase (JNK) signaling is linked to transcriptional regulation of members of the Bcl-2 family. In addition, Aβ-induced neuronal cell apoptosis is inhibited by treatment with a JNK inhibitor or by the targeted disruption of c-Jun or JNK3. Therefore, reagents that suppress neuronal apoptosis may be promising candidates for AD therapy.

The acetylcholinesterase inhibitors, such as donepezil, antioxidants such as vitamin E, and inhibitors to N-methyl-D-aspartate (NMDA) excitotoxicity are clinically used in AD therapy, because they marginally delay disease progression. However, currently there is no curative therapy for AD. Traditional use and clinical reports suggest that Acorus tatarinowii Schott may be effective for patients with mild to moderate AD. Substantial experimental evidence indicates that β-asarone (for its structure, see Fig. 1), the major ingredient of Acorus tatarinowii Schott, have neuroprotective effects in vitro and in vivo. So far, whether β-asarone might be beneficial to AD by suppressing the apoptosis factors expression still remains to be elucidated.

In this study, we investigated whether β-asarone exerted therapy against Aβ-induced neurotoxicity by using a developed rat model of hippocampal dysfunction induced by intrahippocampal injection of Aβ (1—42). We further investigated the effect of β-asarone on the Aβ-induced neuronal apoptosis and to identify signaling protein kinase cascades that may be responsible for the putative effect of β-asarone.

MATERIALS AND METHODS

Animals This study was approved by the Animal Care and Use Committee of Qiqihar Medical University. A total of 140 pathogen-free adult male Sprague-Dawley rats (weight range: 220 to 240 g) were employed in the study. The animals were obtained from the Beijing Vital River Experimental Animals Technology (Beijing, China), and were housed 6 per cage with free access to food and water, and were kept in a constant environment (22 ± 2 °C, 50% ± 5% humidity, 12 h light/dark cycle). The rats were randomly divided into seven groups, including the normal control group, sham control group, the Aβ-injection group, Aβ plus donepezil group, Aβ plus β-asarone (12.5 mg/kg) group, Aβ plus β-asarone (25 mg/kg) group, and Aβ plus β-asarone (50 mg/kg) group.

Aβ (1—42)-Induced AD Rat Model The Aβ (1—42) peptides (Sigma, St. Louis, MO, U.S.A.) were dissolved in 35% acetonitrile/0.1% trifluoroacetic acid at a concentration of 10 μg/μL, and incubated at 37 °C for 7 d to obtain the aggregated form. The rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal (i.p.), and placed on a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, U.S.A.). The scalp of each rat was incised, and the skull

* To whom correspondence should be addressed. e-mail: nyic1968@sohu.com

© 2010 Pharmaceutical Society of Japan
was adjusted to place bregma and lambda on the same horizontal plane. Small burr holes were drilled and peptides (1 µl = 10 µg) were injected into the bilateral hippocampuses (3.0 mm posterior to the bregma, 2.2 mm lateral to the midsagittal line, and 2.8 mm ventral to the surface of the skull, according to the brain atlas of Paxinos and Watson.16) The hole was blocked with dental acrylic cement and scalp was then closed with suture. Sham group rats received the same surgical procedures with injection of identical volume vehicle (acetonitrile/0.1% trifluoroacetic acid). After wound surgery, the animals were allowed to recover from surgery for 3 d. Donepezil hydrochloride (0.33 mg/kg) (Sigma, St. Louis, MO, U.S.A.) (referred to simply as donepezil) and β-asarone (Sigma, St. Louis, MO, U.S.A.) (12.5, 25, or 50 mg/kg) were administered intragastrically once daily for 28 d from 3 d after Aβ (1–42) hippocampus injection.

Morris Water Maze (MWM) Test The spatial learning abilities of rats were assessed in the MWM task. The water maze consisted of a metal circular pool (diameter, 120 cm; height, 35 cm), the upper part of which was surrounded by a circular white escape platform (10 cm in diameter) that was hidden 0.5 cm below the surface of the 30-cm-deep water which was made opaque with non-fat dry milk. The maze was located in an experimental room rich in environmental cues.

At 1 h after last intragastric administration of β-asarone, the rats were allowed to swim freely for 60 s and allowed the 20-s rest period on the platform. Memory-acquisition trials were performed in six daily sessions consisting of four trials that were started from four cardinal points of the compass. Rats were given 60 s to find the escape platform in the center of the south-west quadrant of the pool. If the rat did not find the platform within this limit, it was guided onto it. All rats were allowed to rest on the platform for 15 s. Trajectories were monitored with a computerized tracking system, and swim latencies to locate the platform were evaluated. Probe trials were run as fourth trials on day 6, during which the platform was removed from the maze and the rats were allowed to swim freely for 60 s before they were removed from the pool. The number of crossings over a point where the platform within this limit was calculated by computerized tracking system.

Tissue Processing After the behavioral experiment, rats were anesthetized and transcardially perfusion-fixed with 4% formaldehyde in phosphate-buffered saline. Whole brain was immediately removed and postfixed overnight at 4 °C in the same fixative. Then brain tissues were transferred to 70% alcohol and processed by standard procedures and paraffin-embedded. Five-micrometer sections were cut using a rotary microtome and placed on pretreated slides.

Fluorescence Activated Cell Sorting (FACS) Analysis After the behavioral experiment, animals were euthanized by sodium pentobarbital overdose (125 mg/kg, i.p.). Brains were immediately removed, and the hippocampus was dissected out and kept on ice. They were cut into small pieces with scissors and subsequently processed to single-cell suspensions with a Medimachine (Becton Dickinson, San Jose, CA, U.S.A.). Annexin V assays were done using the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson, San Jose, CA, U.S.A.). Cells were washed twice with cold phosphate buffer saline (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, U.S.A.) and FlowJo software (Tree Star, San Carlos, CA, U.S.A.). FITC-conjugated annexin V-positive cells were considered as cells in the early stages of apoptosis. Cells distinguished by their ability to take up both FITC-annexin V and PI were considered as cells in the later stages of apoptosis. Live cells were those negatively stained for FITC-annexin V and PI.

Immunohistochemistry Analysis Brain sections (5 µm in thickness) were incubated at 4 °C overnight with primary antibody (caspase-3 antibody, Cell Signaling Technology Inc., Beverly, MA, U.S.A.; other antibody, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in concentrations of 1:100 (p-JNK), 1:200 (Bel-2), 1:150 (Bel-w) and 1:100 (caspase-3). As secondary antibody, horseradish peroxidase-conjugated immunoglobulin G (Medical Biological Laboratory, Nagoya, Japan), was used for 30 min at 37 °C. After further washing with Tris Buffered Saline, sections were incubated with Complex/horseradish peroxidase (1:200 dilution) for 30 min at 37 °C. Immunolocalization was performed by immersion in 0.05% 3,3'-diaminobenzidine tetrahydrochloride as chromagen. Slides were counterstained with hematoxylin before dehydration and mounting. Incubation without the primary antibody was performed as a control for the background staining.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR) Total RNA was extracted from 100 mg of frozen hippocampus tissues using RNAiso Reagent kit (Takara Biotechnology, Dalian, China), and cDNA was synthesized with SYBR ExScript™ reverse transcription (RT)-PCR kit (Takara Biotechnology, Dalian, China) according to the protocol provided by manufacturer. 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, deoxyribonucleotide triphosphates, ROX Reverence Dye and the corresponding primers. The cycles for PCR were as follows: 1 cycle of 95 °C for 10 s; 40 cycles of 5 s at 95 °C, 31 s at 60 °C. Melting curve analysis was always included to validate the specificity of the PCR products. Serial cDNA dilution curves were produced to calculate the amplification efficiency for all genes. A graph of threshold cycle (Ct) versus log 10 relative copy number of the sample from a dilution series was produced. The slope of the curve was used to determine the amplification efficiency. Reactions were performed in an ABI7300 Real-time PCR system (Applied Biosystems, CA, U.S.A.) and Ct data were collected with using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA, U.S.A.). GAPDH was used as an internal control. mRNAfold change relative to glyceraldehyde phosphate dehydrogenase (GAPDH) was calculated with the comparative Ct method of 2−DDCt.17) The following primers were used: 5'-GAC AAC TTT GTG ATC GTG GA-3' (sense) and 5'-ATG CAG GGA TGA TGT TCT GG-3' (antisense) for the GAPDH gene; 5'-GCA GCC TCA AAT TGT TGA CTA-3' (sense) and 5'-TGC TCC GGC TCA AAC CAT C-3' (antisense) for the caspase-3 gene; 5'-TGA ACC GGC
ATC TGC ACA C-3′ (sense) and 5′-CGT CCT CAG AGA CAG CCA GGA G-3′ (antisense) for the Bcl-2 gene; 5′-TGG AGA CAC GCT TGG CTG AC-3′ (sense) and 5′-TGT CCT CAC TGA TGC CCA GTT C-3′ (antisense) for the Bcl-w gene. mRNA levels were expressed as fold changes after normalization with GAPDH. All tests were done in triplicate to ensure reproducibility.

**Western Blot** Cytoplasmic proteins were isolated from 120 mg of frozen hippocampus tissues 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. One hundred microliters of supernatant was added to an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min at 100 °C. The samples were then stored at −80 °C until analyzed. The electrophoretic mobility of the proteins in this study was determined by SDS-polyacrylamide gel electrophoresis using 15% acrylamide concentrations. After electrophoresis, the proteins were transferred electrophoretically to a nitrocellulose filter membrane that was then blocked 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. The membrane was then incubated overnight at 4 °C with p-JNK antibody (2000:1; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), Bcl-w antibody (2000:1; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), Bcl-2 antibody (1500:1; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), caspase-3 antibody (1500:1; Cell Signaling Technology Inc., Beverly, MA, U.S.A.), and GAPDH antibody (2000:1; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), followed by being washed four times, after which they were incubated with horseradish peroxidase labeled Anti-Mouse immunoglobulin G (IgG) (10000:1; Medical Biological Laboratory Co., Nagoya, Japan) for 2 h and again washed four times. The blots were developed using an ECL Western blotting kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) as recommended by the manufacturer. GAPDH was probed as an internal control and was used to confirm that an equal amount of protein was loaded in each lane. Band intensities were determined using an AlphaImager™ 2200 using the SpotDenso function of AlphaEaseFC™ Software version 3.1.2 (Witec, Littau, Switzerland).

**Statistical Analysis** All values in the figures of present study indicate means±S.D. Group comparisons for escape latency were analyzed with two-way repeated-measures ANOVA, followed by a Student–Newman–Keuls post hoc test. 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). The data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, U.S.A.), and p<0.05 was assessed as statistically significant.

**RESULTS**

**Beta-Asarone Ameliorated Learning and Memory Impairment in Aβ Hippocampus Injection Model** The ability of rats to acquire, process, and recall spatial information was assessed in the MWM test by using escape latency and number of crossings over a point as indicator after discontinuance of drug treatment. Learning and memory deficits were revealed in Aβ (1—42)-injected rats. The ability of learning and memory of Aβ (1—42)-injected rats significantly improved when β-asarone (Figs. 2A, B) (12.5, 25, 50 mg/kg) was intragastrically administrated for 28 d and approached those of the donepezil treatment rats.

In the hidden platform test, the rats become more efficient at finding the platform on successive trial (Fig. 2A). Two-way ANOVA (7 groups×6 d) with repeated measures on days rendered that the main effect for groups was statistically significant (F(6, 126)=204.787, p<0.001). The main effect for day was also statistically significant (F(30, 620)=4961.745, p<0.001), and significant interaction (F(30, 630)=14.017, p<0.001). Post-hoc analysis (Student–Newman–Keuls) showed that the Aβ (1—42)-injected rats had significantly longer escape latency to find the platform than the sham-
operated rats on days 2—6 (all \( p<0.05 \)), and a significant decrease in escape latency when \( \beta \)-asarone (12.5, 25, or 50 mg/kg) was intragastrically administered as compared with the saline-administered group (\( p<0.05 \)).

In the probe trials (Fig. 2B), the number of crossings over a platform position was significantly decreased in the A\( \beta \) (1—42)-injected group as compared with that in the sham-operated group (4.79±2.07 versus 1.79±1.22, \( n=18 \) to 20, \( p<0.05 \)). The crossing number was recovered by treatment with \( \beta \)-asarone (12.5, 25, or 50 mg/kg) in a dose response manner and the recovery was significant (2.94±1.30, 3.67±1.18, and 4.16±1.70, \( n=18 \) to 20, all \( p<0.05 \)). Treatment with donepezil shows almost the same potencies with \( \beta \)-asarone on the probe trials. All rats showed normal swimming performance and constant increases in body weight. Locomotor activity did not show difference among groups (data not shown).

MWM test indicating that injection of A\( \beta \) (1—42) efficiently impaired spatial working memory and \( \beta \)-asarone treatment suppressed this memory impairment.

**Beta-Asarone Attenuates Neuronal Apoptosis in Hippocampus of in A\( \beta \) Injection Model** To explore the effects of \( \beta \)-asarone on apoptosis in hippocampus induced by A\( \beta \) (1—42) in vivo, cell apoptosis was quantified by staining cell with annexin-V-FITC/PI (Fig. 3A). Quantitative analysis of Annexin V-positive cells (Fig. 3B) revealed that A\( \beta \) (1—42) injection resulted in 260% increase in the percentage of Annexin V-positive cells in hippocampus of the sham-operated rats (13.97±1.81% for A\( \beta \) (1—42)-injected rats versus 49.69±5.86 for sham-operated rats, \( n=5 \), \( p<0.05 \)). Normal rats were 15.15±1.48%. \( \beta \)-Asarone treatment (12.5, 25, or 50 mg/kg) attenuate the A\( \beta \) (1—42)-induced cell apoptosis effect in hippocampus, the percentage of Annexin V-positive cells to 43.25±3.30%, 42.33±4.42% and 37.10±2.32%, respectively. Donepezil had no effect on neuronal apoptosis (46.39±2.52%).

**Involvement of Caspase-3 Activation in the Suppressive Effect of \( \beta \)-Asarone against A\( \beta \) (1—42)-Induced Neu-**

![Fig. 3. Beta-Asarone Treatment Attenuation Neuronal Apoptosis Induced by A\( \beta \) (1—42) Injection](image-url)
Caspase-3, a key molecule in apoptotic signaling, is cleaved in response to activation by caspase-8 or caspase-9. We asked whether \( \beta \)-asarone suppresses neuronal apoptosis in rat hippocampus induced by A\( \beta \) (1—42) via caspase-3-mediated apoptosis pathways. The Western blot and immunohistochemistry result of caspase-3 in hippocampus (Fig. 4A, Fig. 5) showed A\( \beta \) (1—42)-injection insult in cleavage of caspase-3 (233.88±21.62% in the A\( \beta \) (1—42) injected rats).

**Fig. 4. Beta-Asarone Attenuation A\( \beta \) (1—42)-Induced Caspase-3 Activation, Downregulation of Bcl-2 and Bcl-w, and p-JNK Phosphorylation**

Treatment with \( \beta \)-asarone (12.5, 25, or 50 mg/kg), saline, and donepezil (0.33 mg/kg) given i.g. was initiated 3 d after the A\( \beta \) (1—42) hippocampus injection for 28 d. Pro-caspase-3, cleaved caspase-3, Bcl-2, Bcl-w, and p-JNK levels were determined by immunoblot analysis with antibody to caspase-3, Bcl-2 and Bcl-w, and p-JNK (A). The loading of the lanes was normalized to levels of GAPDH. Quantitated results of caspase-3 (B), Bcl-2 and Bcl-w (D), and p-JNK (F) are presented relative to sham. Densitometric analysis of western blot obtained from 5 rats per group. Total RNA was isolated from hippocampus using RNAiso reagent and used for cDNA synthesis. The mRNA levels of Bcl-2, Bcl-w, and caspase-3 were detected by real-time PCR. \( 2^{-\Delta\Delta C_t} \) analysis of PCR of Bcl-2, Bcl-w (E), and caspase-3 (C) obtained from 5 rats per group, and data are expressed as mean±S.D.; *\( p<0.05 \), §\( p<0.05 \), vs. A\( \beta \) (1—42)-injected rats.
injected group versus 84.54 ± 7.33% in sham group), treatment with β-asarone (12.5, 25 or 50 mg/kg, i.g.) once daily for 28 d dose-dependently attenuated Aβ (1—42)-induced cleavage of caspase-3 in hippocampus (146.26 ± 22.81%, 130.63 ± 24.50%, 122.37 ± 28.89%, approximately the inhibition being 37%, 44%, 47% respectively) (Fig. 4B). Consistent with the results of protein levels, real-time PCR revealed that β-asarone treatment (12.5, 25 or 50 mg/kg, i.g.) significantly decrease caspase-3 mRNA levels to 5.01 ± 0.56, 4.68 ± 0.27 and 2.60 ± 0.21 from 11.36 ± 3.13 of Aβ-injected rats (Fig. 4C).

Involvement of Bcl-2 Family Proteins in the Suppressive Effect of β-Asarone against Aβ (1—42)-Induced Neuronal Apoptosis  

The Bcl-2 family of proteins plays a key role in the apoptotic process. To investigate the effects of β-asarone on expression of antiapoptotic Bcl-2 and Bcl-w, we determined these proteins by Western blotting and immunohistochemistry. Bcl-2 and Bcl-w protein levels in Aβ-injected rats was significantly reduced as compared with that in sham-operated rats (35.38 ± 2.58% vs. 90.24 ± 3.49%; 37.72 ± 2.47% vs. 102.98 ± 7.48%; n = 5, p < 0.05, respectively). Treatment with β-asarone (12.5, 25, or 50 mg/kg) markedly increased the expression of Bcl-2 (56.74 ± 3.35%, 51.92 ± 3.22%, 83.24 ± 4.45%) and Bcl-w (51.28 ± 4.66%, 45.32 ± 3.64%, 60.72 ± 5.13%) compared with that in Aβ (1—42)-injected rats (n = 5, p < 0.05, respectively) (Figs. 4A, D, Fig. 5). Consistent with the results of protein levels, real-time PCR revealed that β-asarone treatment (12.5, 25 or 50 mg/kg, i.g.) significantly increased Bcl-2 mRNA levels to 0.29 ± 0.03, 0.49 ± 0.11 and 0.57 ± 0.04 from 0.15 ± 0.02 of Aβ-injected rats, and increased Bcl-w mRNA levels to 0.24 ± 0.02, 0.39 ± 0.05 and 0.44 ± 0.06 from 0.12 ± 0.01 (Fig. 4E). In addition, down-regulation of Bcl-2 and Bcl-w were not attenuated by donepezil.

Effect of β-Asarone Attenuation Aβ (1—42)-Induced Neuronal Apoptosis through a JNK Signal Pathway  

to determine whether the JNK pathway functions involvement in β-asarone attenuate Aβ (1—42)-induced neuronal apoptosis, we assessed p-JNK using Western blotting analysis. Western blotting and immunohistochemistry result revealed (Fig. 4A, Fig. 5) that p-JNK protein was constitutively expressed in both the normal hippocampus and the Aβ-induced hippocampus, although the expression was clearly up-regulated in the latter. However, the expression of p-JNK in Aβ-induced hippocampus was significantly down-regulated when the rats were treated with β-asarone (12.5, 25 or 50 mg/kg) once daily for 28 d (Fig. 4F). Donepezil (0.33 mg/kg) had no effect on the p-JNK expression.

DISCUSSION

We have clearly demonstrated for the first time in the present study that β-asarone ameliorated impairment of learning and memory in rats induced by hippocampus injection of Aβ (1—42). This study further suggests that β-asarone antagonized neurotoxicity of Aβ (1—42) in this model as evi-

![Fig. 5. Immunohistochemical Labeling of p-JNK, Bcl-2, Bcl-w, and Caspase-3 by Diaminobenzidine Reaction of Rat Hippocampus Tissue Paraffin Sections](image-url)
enced by attenuated neuronal apoptosis in rat hippocampus. JNK-mediated signal pathway is likely involved in mechanism of anti-apoptosis action of β-asarone.

*Acorus tatarinowii* SCHOTT has long been employed in the clinical treatment of AD in Chinese herbal books. It has been reported to be responsible for various pharmacological actions on the central nervous system (CNS).13) The rhizomes and leaves of *Acorus tatarinowii* SCHOTT are known to contain 0.11—0.42% of essential oil consisting of 30 kinds of compounds which may affect CNS.13) The more recent results of Wu et al. confirmed that β-asarone, a component isolated from essential oil of *Acorus tatarinowii* SCHOTT, is easy to pass through blood–brain barrier, and brain is an important organ of distributing of it.19) Beta-asarone, however, has not yet been evaluated for actions on the AD and its mechanism of action. In the present study, bilateral hippocampus injection of Aβ (1—42) produced behavioral results similar to those previously described.20) Rats receiving Aβ (1—42) injection alone without any treatment showed impairment of spatial memory. We treated the Aβ-injected rats with β-asarone at dose of 12.5, 25 or 50 mg/kg for 28 d significantly shortened escape latency on days 2—6 of place navigation test and increased the number of crossings over the platform position when compared to the untreated rats of spatial probe test. These results indicate that β-asarone has the ameliorating effect on cognitive impairment caused by Aβ (1—42).

Apoptosis, a sequence of regulated cellular events culminating in cell death, is a fundamental biological process that fulfills many important functions in the developing and adult organism.21) However, the aberrant induction of apoptosis can have dire consequences, particularly in largely irreparable cells, such as neurons. Several studies presently indicate that apoptosis might occur in, and contribute to, AD onset and progression.22) The role of apoptosis in in vitro models and transgenic animal models of neuro-degeneration has been largely documented, and direct neurotoxic effects of Aβ involve activation of apoptosis pathways.23,24) In the present study, Aβ hippocampus injection increased neuronal cell apoptosis, one of the most important pathological hallmarks of AD. Beta-asarone administration attenuated neuronal apoptosis in the hippocampus in a dose-dependent manner. Concomitantly β-asarone at the same dose ameliorated impairment of spatial memory induced by Aβ (1—42). Therefore, β-asarone improvement cognitive function might be due to its inhibition of neuronal apoptosis. The repair effect of donepezil, the drug most commonly used to treat AD patients in clinics, on memory impairment was almost the same with β-asarone. However, neuronal apoptosis were not attenuated by it. These results are in agreement with the findings of Li et al.25)

Apoptosis is actively executed by several members of the caspase family, including caspase-3, which is involved in the final execution phase of apoptosis.26) Caspase-3 is a type of apoptotic protein and caspase-3 precursor exits in the cytoplasm under normal condition. Once caspase-3 is activated, cell apoptosis is inevitable.27) Immunohistochemical and biochemical studies report the presence of active caspase(s) and caspase-cleaved substrates in neurons, around senile plaques and neurofibrillary tangles, and also in postsynaptic densities.28) In the present study, Aβ (1—42) hippocampus injection increased the level of cleaved a caspase-3, marker for caspase-3 activity, which is the feature of apoptosis in AD. Beta-asarone administration attenuated Aβ-induced expression of activated caspase-3 in the hippocampus, suggesting that it exerts protective effects against Aβ (1—42)-induced neuronal apoptosis by affecting the execution phase of apoptosis.

Members of the Bcl-2 family are pivotal regulators of the neuronal apoptosis process and include both proteins that promote cell survival (e.g., Bcl-2 and Bcl-w) and others that antagonize it (e.g., Bax).29) Bcl-2 and Bcl-w is widely expressed in mammalian tissues, including the central nervous system, and functions as a negative regulator of neuronal apoptosis.30) Neural expression of Bcl-2 is highest in the mature brain, suggesting that Bcl-2 function may be particularly important in adulthood.31) Bcl-2 has been reported to directly inhibit members of the caspase family, including caspas-3 which is a potent effector of neuronal death among the identified caspases.32) In the present study, we observed that Aβ-induced apoptosis is characterized by decreased expression of the antiapoptotic Bcl-2 and Bcl-w. Furthermore, Aβ-injected rats treated with β-asarone exhibited elevated levels of antiapoptotic protein Bcl-2 and Bcl-w. These results suggested that the mitochondrial pathway of cell death might be involved in β-asarone attenuate neuronal apoptosis. Our results are in agreement with previous findings reported by Zhang et al. in PC12 cell.33)

There is very strong evidence linking the activation of JNK to neuronal loss in response to a wide array of proapoptotic stimuli in both developmental and degenerative death signaling.7 Inhibition of JNK signaling provides protection against neuronal apoptosis in multiple paradigms, including Aβ neurotoxicity. JNK signaling promotes apoptosis is linked to transcriptional regulation of many genes, including members of the Bcl-2 family.24) In addition, JNK activation is observed in cultured neurons after Aβ exposure, and its inhibition significantly attenuates Aβ toxicity. SP600125, JNK pharmacological inhibitor, effectively prevents Aβ-induced alterations of Bcl-2 family expression induced during apoptosis, indicating that this critical step in the Aβ induced-apoptosis pathway is dependent on JNK activation.34) Thus, the suppression of JNK-dependent apoptosis gene expression may be an extremely effective therapeutic strategy for preventing neuronal cell apoptosis. We observed that β-asarone significantly attenuated Aβ-induced changes in Bcl-2 and Bcl-w expression. Notably, it also significantly reduced Aβ-induced JNK phosphorylation, suggesting that β-asarone attenuation of Aβ-induced changes in Bcl-2 and Bcl-2 expression is linked to JNK pathway in rat hippocampus. These findings are consistent with previous data from the literature indicating that JNK represents a key target of various neuroprotective agents, including Estrogen.35) These data suggest that β-asarone inhibiting Aβ (25—35)-induced JNK activation may be necessary for the neuroprotective effects of β-asarone. However, the possibility that other unrelated protein kinases were responsible for mediation on β-asarone protection from Aβ-induced apoptosis cannot be entirely excluded based on the current results. JNK phosphorylation was not affected by the treatment with donepezil, but donepezil increased slightly the expression of Bcl-2 in Aβ hippocampus injection model. This is an interesting question, which warrants future investigation.
In conclusion, our results are consistent with the hypothesis that β-arosone potentially ameliorate the impairment of spatial memory, at least in part, by attenuating neuronal apoptosis in rats induced by APβ (1—42) when taken orally. The attenuation is associated with the inhibition of JNK activation, upregulation of Bcl-w, Bcl-2, and inhibition of caspase-3 activation. Our findings suggest that β-arosone, an important active principle of *Acorus tatarinowii* SCHIOTT, might be a potential drug for the AD to suppress both AD-related neuronal cell apoptosis and dysfunction of the memory system.

Acknowledgements Funding for this study was provided by the National Natural Science Foundation of China Grant No. 30873396 and the National Science Foundation for Post-doctoral Scientists of China Grant No. 20080430140.

REFERENCES