

Full-length article

Novel squamosamide derivative (compound FLZ) attenuates A β _{25–35}-induced toxicity in SH-SY5Y cells¹Fang FANG³, Geng-tao LIU²*Department of Pharmacology, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China***Key words**compound FLZ; β -amyloid peptide; apoptosis; oxidative stress; SH-SY5Y cells¹ Project supported by the Ministry of Science and Technology of China (No 2007CB507400).² Correspondence to Prof Geng-tao LIU.

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Abstract

Aim: The aim of the present study was to investigate the protective effect of compound *N*-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide (compound FLZ), a novel synthetic analogue of nature squamosamide, on A β _{25–35}-induced toxicity and its active mechanism in human neuroblastoma SH-SY5Y cells. **Methods:** SH-SY5Y cells were preincubated with various concentrations of compound FLZ for 30 min and then cultivated with A β _{25–35} (25 μ mol/L) for 48 h to induce neurotoxicity. Cell viability, lactate dehydrogenase (LDH) release, and the glutathione (GSH) level were determined by a biochemical analysis. The cell apoptotic ratio and intracellular reactive oxygen species (ROS) level were measured by a flow cytometry analysis. The expression of apoptosis protein (Bcl-2 and Bax) and cytochrome *c* release were assayed by the Western blot method. **Results:** The pretreatment of SH-SY5Y cells with FLZ (1 and 10 μ mol/L) markedly increased cell viability and decreased LDH release and morphological injury. Also, FLZ attenuated the A β _{25–35}-induced apoptotic cell ratio, regulated the apoptosis protein (Bcl-2 and Bax) expression, and decreased the cytochrome *c* release from mitochondria. FLZ also significantly inhibited the generation of ROS and the depletion of GSH induced by A β _{25–35} in SH-SY5Y cells. **Conclusion:** FLZ has protective action against A β _{25–35}-induced toxicity in SH-SY5Y cells, which might be mediated through its antioxidant property.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive and irreversible memory loss due to neuronal death in aged people^[1]. One of the major histopathological features of AD is the presence of senile plaques and concomitant neuronal loss in specific areas of the brain. β -Amyloid peptide (A β) is the main constituent of senile plaques and plays a critical role in the pathophysiology of AD^[2–5]. Evidence has suggested that the neurotoxic effect of A β is related to the activation of the apoptosis pathway^[6–9]. Neuronal apoptosis is the main cause of neuronal loss in patients with AD, thus, it has been proposed that neuroprotection may be a therapeutic strategy for slowing down the apoptotic process of related brain cells of AD patients^[10].

The natural squamosamide was isolated from the leaves of *Annona squamosa*. The compound FLZ is a novel synthetic cyclic derivative of natural squamosamide. Its chemical name is *N*-(2-[4-hydroxy-phenyl]-ethyl)-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide and its molecular weight is 449.5 (Figure 1). Previous studies have demonstrated that FLZ has a potent neuroprotective property against experimental Parkinsonism and memory and learning deficits in mice^[11,12]. In an *in vitro* study, FLZ was shown to protect against damage and apoptosis of primary cultured rat brain neurons, PC12, and SH-SY5Y cell lines exposed to hydrogen peroxide, glutamate, *N*-methyl-*D*-aspartate, dopamine, 1-methyl, 4-phenyl-pyridinium ion (MPP⁺), and ischemia-reoxygenation, indicating that FLZ possess a neuroprotective property^[11,13]. Based on these

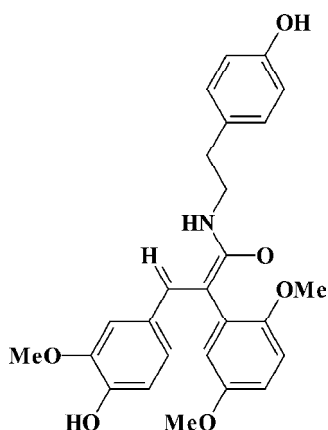


Figure 1. Chemical structure of compound FLZ. The full chemical name is *N*-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide, and its molecular weight is 449.5.

results, the objective of this paper was to study the protective effect of FLZ on $A\beta_{25-35}$ -induced toxicity in human neuroblastoma SH-SY5Y cells and its active mechanism.

Materials and methods

Materials The compound FLZ was kindly provided by Professor Xiao-tian LIANG (Department of Pharmaceutical Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China). It is a white powder with 99% purity. The compound was first dissolved in absolute ethanol and then diluted with 0.9% saline; the final ethanol concentration was <0.1% for use. $A\beta_{25-35}$ (Sigma, St Louis, MO, USA) was dissolved in sterile double-distilled water at a concentration of 1 mmol/L stock solution, was aged at 37 °C for 4 d, and then stored at -20 °C before use. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), trypsin, 2',7'-dichlorofluorescein diacetate (DCFH-DA), EDTA, propidium iodide (PI), rhodamine 123, agarose, HEPES, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), Nonidet P40, and DNase were all purchased from Sigma (USA). Dulbecco's Modified Eagle's medium (DMEM), F-12, and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). The primary mouse monoclonal antibodies Bcl-2, Bax, and cytochrome c were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were purchased from Beijing Chemical Company (Beijing, China).

SH-SY5Y cell cultures Human neuroblastoma SH-SY5Y cells were purchased from the Cell Center of the Institute of Basic Medical Science Research (Chinese Academy of Medical Sciences, China). The SH-SY5Y cells were cul-

tured in DMEM:F12 (1:1) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a humidified 95% oxygen and 5% CO_2 atmosphere. The medium was changed every other day. The cells were cultured for 3–4 d until a confluence of 70%–80% was achieved. The trypan blue assay was used to count the cells and evaluate their viability as a percentage of viable and non-viable cells. The cell viability was >97% prior to the following experimental procedure. The cells were plated at an appropriate density according to each experimental protocol.

Treatment of SH-SY5Y cells On the day of the experiment, the medium was removed and the cells were then cultured in the same medium without FBS and exposed to the aged peptide $A\beta_{25-35}$ for 48 h in the presence or absence of FLZ. Before adding $A\beta_{25-35}$, different concentrations of FLZ (0.1, 1, and 10 μ mol/L) were incubated with the cells for 30 min at 37 °C.

Cell viability assay After the cells were cultured with $A\beta_{25-35}$ in the presence or absence of FLZ for 48 h, the cell viability was assayed with the MTT method^[14]. Briefly, MTT was dissolved in the medium without FBS and added to cells grown in 96-well plates at a final concentration of 0.5 g/L. Following a 4-h incubation to allow its conversion into formazan crystals, the media was removed and cells were lysed with DMSO (Me_2SO) to allow the crystals to dissolve. The absorbance was read at 570 nm using a Bio-Rad 450 microplate reader (Hercules, CA, USA). The results were expressed as a percentage of MTT reduction. The absorbance of the control cells (no FLZ and no $A\beta_{25-35}$) was used as 100%.

Measurement of lactate dehydrogenase The medium was collected from the cells treated with $A\beta_{25-35}$ for 48 h in the presence or absence of FLZ and centrifuged at 14 000 \times g for 5 min. The 50 μ L of the supernatant was transferred to a tube, and the activity of lactate dehydrogenase (LDH) was determined using an LDH kit (Beijing Chemical Reagents Company, Beijing, China).

Apoptotic ratio assay by flow cytometry analysis Flow cytometry was used to assess the percentage of genomic DNA fragmentation in nuclei^[15]. The cultured cells were trypsinized and harvested, centrifuged for 5 min at 200 \times g, washed twice with phosphate-buffered saline (PBS) and fixed in 70% alcohol overnight at 4 °C. The cells were centrifuged and washed with PBS, resuspended in 0.5 mL PBS containing 50 mg/L RNase A, and incubated for 1 h at 37 °C. The samples were stained with 50 mg/L PI for 30 min at 4 °C in the dark. The flow cytometry analysis was performed by FACScan (BD Biosciences, San Jose, CA, USA).

Calculations of the percentage of apoptotic cells were based on the cumulative frequency curves of the appropriate DNA histograms. Debris was excluded from the collection of 10 000 nuclei by empirically setting the forward-scatter channel and side-scatter channel threshold levels.

Measurement of intracellular reactive oxygen species by flow cytometry analysis The production of intracellular reactive oxygen species (ROS) was determined using the fluorescent probe DCFH-DA^[16]. DCFH-DA is a membrane permeable, non-fluorescent compound. In the presence of peroxides in cells, it is converted to the fluorescent derivative dichlorofluorescein (DCF). Following treatment with A β_{25-35} (25 $\mu\text{mol/L}$) for 48 h in the presence or absence of FLZ (0.1, 1, and 10 $\mu\text{mol/L}$), the cells were rinsed with PBS and incubated with DCFH-DA (20 $\mu\text{mol/L}$, final concentration) in Me₂SO for 30 min at 37 °C. Loaded cells were washed 3 times, and the fluorescence intensity of DCF was determined by using flow cytometry (excitation=485 nm, emission=535 nm).

Measurement of intracellular glutathione The level of glutathione (GSH) was determined using *o*-phthalaldehyde (OPT)^[17]. On the day of the experiment, the cultured medium was removed, the cells were washed 3 times with PBS, and lysed by vigorous shaking in the 500 μL buffer containing 0.2% Triton-X100 and 5 mmol/L EDTA (pH =8.3). After lysis, 0.3 mL of the buffer was removed and mixed with 0.1 mL 20% trichloric acetic acid, and centrifuged at 200 \times g for 10 min. The supernatant was incubated with 50 mg/L OPT (dissolved in Me₂SO and diluted to the final concentration with PBS) for 15 min at room temperature. The fluorescence was measured at an excitation wavelength of 350 nm and emission wavelength of 420 nm. The protein content was determined by the Lowry method^[18].

Western blot analysis of cytochrome *c*, Bax, and Bcl-2 in SH-SY5Y cells Following the treatment of A β_{25-35} , the cells were collected and washed with PBS. After centrifugation, cell lysis was carried out at 4 °C by vigorous shaking for 30 min in RIPA buffer (25 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 1 mmol/L PMSF, 1% TritonX-100, 0.5% Nonidet P40, 10 mg/L aprotinin, and 10 mg/L leupeptin). After centrifugation at 12000 \times g for 15 min, the supernatant was separated and stored at -70 °C for the measurement of Bcl-2 and Bax^[19].

The analysis of the cytochrome *c* release was performed as previously described^[20]. The cells were collected and washed twice with PBS. After centrifugation, the cell pellets were suspended in 5 mL extraction buffer (in mmol/L: HEPES 50, KCl 50, EGTA 5, MgCl₂ 2, DTT 1, and PMSF 0.1, pH=7.4), and centrifuged after 15 min on ice. The cell pellets were

resuspended in the above buffer, and homogenized in a Teflon-glass homogenizer (up and down, 50 strokes) after 45 min on ice. The homogenized buffer was transferred into the Eppendorf tube and centrifuged at 15000 \times g for 20 min at 4 °C. The supernatants were removed and stored at -70 °C until use^[20].

The protein concentration was determined by the Lowry method^[18]. After the addition of the sample loading buffer, the protein samples (equal quantity) were denaturalized by heating at 100 °C and were separated on the 12% SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane were blocked for 1 h at room temperature in fresh blocking buffer (TBST, 0.1% Tween-20 in Tris-buffered saline, pH=7.4, containing 5% nonfat dried milk) and incubated with the primary antibody (dilution: Bcl-2 and Bax 1:200, cytochrome *c* 1:1000) for 3 h at room temperature. Following 3 washes with TBST, the membrane was incubated with alkaline phosphatase-conjugated secondary antibodies in TBST for 2 h at room temperature. The membrane was washed again 3 times in TBST buffer. The protein blot was visualized by 5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt/nitro-blue tetrazolium chloride. The blot was scanned and analyzed the density using the software.

Statistical analysis All experiments were repeated at least 3 times using independent culture preparations. Quantitative data were expressed as mean \pm SD. The statistical analysis between various experimental results was performed using one-way ANOVA followed by the least significant difference test; $P < 0.05$ was considered statistically significant.

Results

Effect of FLZ on cytotoxicity induced by A β_{25-35} in SH-SY5Y cells The exposure of SH-SY5Y cells to A β_{25-35} (10~50 $\mu\text{mol/L}$) for 48 h resulted in a significant decrease of cell viability and increase of LDH release into the medium in a dose-dependent manner (Table 1). The viability of the SH-SY5Y cells treated with A β_{25-35} (25 $\mu\text{mol/L}$) for 48 h was reduced to ~70% of that of the control ($P < 0.01$), and the activity of LDH in the medium increased 1.5-fold ($P < 0.01$). Most of the SH-SY5Y cellular morphology became round in shape and aggregated together (Figure 2). The 25 $\mu\text{mol/L}$ A β_{25-35} was therefore used in the following study of the protective action of FLZ against A β_{25-35} neurotoxicity in SH-SY5Y cells.

To select a non-cytotoxic concentration of FLZ in the study of the effect of FLZ on A β_{25-35} neurotoxicity, we first evaluated the effect of various concentrations (0.1, 1, and 10 $\mu\text{mol/L}$) of FLZ itself on the cell viability of SH-SY5Y cells. FLZ at concentrations of 0.1, 1, and 10 $\mu\text{mol/L}$ showed no

Table 1. Dose-dependent effect of Aβ₂₅₋₃₅ on cell viability and LDH release in cultured SH-SY5Y cells.

Group	Cell viability (% of control)	LDH release (U/100 mL)
Control	99.81±10.51	351.83±34.46
Aβ ₂₅₋₃₅ 5 μmol/L	86.06±9.73	400.00±50.79
Aβ ₂₅₋₃₅ 10 μmol/L	80.22±4.15 ^c	414.00±42.53 ^b
Aβ ₂₅₋₃₅ 25 μmol/L	74.47±2.56 ^c	513.00±69.94 ^c
Aβ ₂₅₋₃₅ 50 μmol/L	76.45±4.01 ^c	549.00±67.49 ^c

SH-SY5Y cells were cultured with various concentrations of Aβ₂₅₋₃₅ for 48 h. Cell viability was measured by using MTT methods. LDH in the medium was determined by assay kit. Data were expressed as the mean±SD from three independent experiments. ^b*P*<0.05, ^c*P*<0.01 vs control group (without Aβ₂₅₋₃₅).

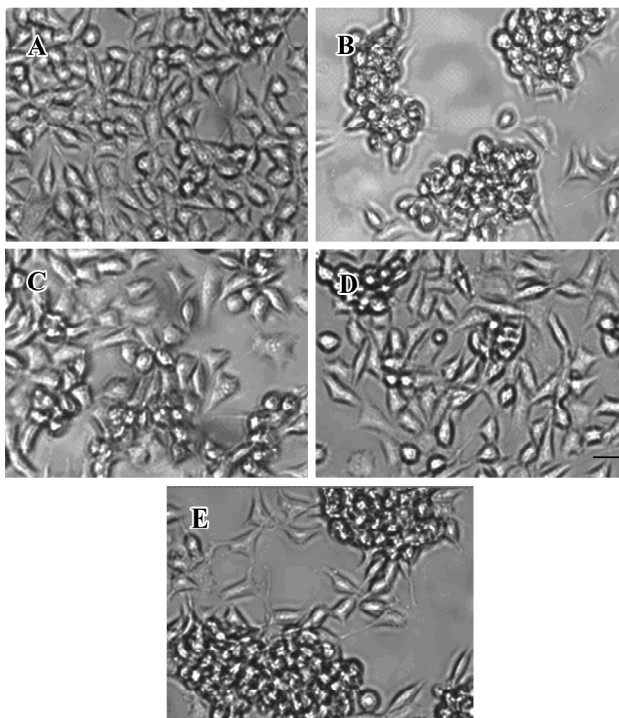


Figure 2. Phase contrast photomicrographs showing the morphology of SH-SY5Y cells exposed to Aβ₂₅₋₃₅ for 48 h in the absence or presence of various concentrations of FLZ (Bar=20 μm). A: vehicle treated cells; B: Aβ₂₅₋₃₅ (25 μmol/L) treated cells; C: FLZ (10 μmol/L) and Aβ₂₅₋₃₅ (25 μmol/L) treated cells; D: FLZ (1 μmol/L) and Aβ₂₅₋₃₅ (25 μmol/L) treated cells; E: FLZ (0.1 μmol/L) and Aβ₂₅₋₃₅ (25 μmol/L) treated cells.

apparent cytotoxicity to SH-SY5Y cells (Table 2). Pretreatment of SH-SY5Y cells with 1 and 10 μmol/L FLZ for 30 min

Table 2. Effect of compound FLZ on cell viability in cultured SH-SY5Y cells.

Group	Cell viability (% of control)
Control	102.29±2.95
FLZ (10 μmol/L)	99.27±4.33
FLZ (1 μmol/L)	98.73±8.05
FLZ (0.1 μmol/L)	100.20±9.69

SH-SY5Y cells were cultured with various concentration of compound FLZ for 48 h. Data were expressed as the mean±SD from three independent experiments. The concentrations of FLZ (0.1–10 μmol/L) used in the experiments had no toxicity to the SH-SY5Y cells.

significantly protected the cells from Aβ₂₅₋₃₅-induced cytotoxicity as demonstrated by increasing cell viability (*P*<0.01), and a concomitant reduction of LDH release (*P*<0.05) into the cultured medium (Table 3). The morphological injury of the SH-SY5Y cells were also reduced by FLZ treatment (Figure 2).

Table 3. Protective effects of FLZ on Aβ₂₅₋₃₅-induced cytotoxicity in SH-SY5Y cells. Data are expressed by the mean±SD from the four independent trials. ^c*P*<0.01 vs control group; ^e*P*<0.05, ^f*P*<0.01 vs Aβ₂₅₋₃₅ treated group.

Group	Cell viability (% of control)	LDH release (U/100 mL)
Control	100.0±3.74	322.67±29.06
Aβ ₂₅₋₃₅ (25 μmol/L)	72.7±1.51 ^c	713.78±34.05 ^c
FLZ (10 μmol/L)+ Aβ ₂₅₋₃₅ (25 μmol/L)	84.2±6.96 ^f	592.67±34.64 ^e
FLZ (1 μmol/L)+ Aβ ₂₅₋₃₅ (25 μmol/L)	83.6±6.41 ^f	616.00±24.65 ^f
FLZ (0.1 μmol/L)+ Aβ ₂₅₋₃₅ (25 μmol/L)	79.8±2.02 ^f	624.33±36.78 ^e

SH-SY5Y cells were incubated with 25 μmol/L Aβ₂₅₋₃₅ in the absence or presence of FLZ (0.1–10 μmol/L) for 48 h at 37 °C. FLZ was added to the medium 30 min before the Aβ₂₅₋₃₅ treatment.

Effect of FLZ on apoptosis in SH-SY5Y cells induced by Aβ₂₅₋₃₅ The hypodiploid sub-G₁ peak is regarded as apoptotic cells. The results of the flow cytometry assay showed that the apoptotic ratio of the Aβ₂₅₋₃₅-treated SH-SY5Y cells markedly increased as compared with that of untreated cells. FLZ (1 and 10 μmol/L) treatment significantly decreased the apoptotic cell accumulation in the sub-G₁ peak in comparison with the Aβ₂₅₋₃₅-treated SH-SY5Y cells, indicating that FLZ attenuated Aβ-induced apoptosis (Figure 3).

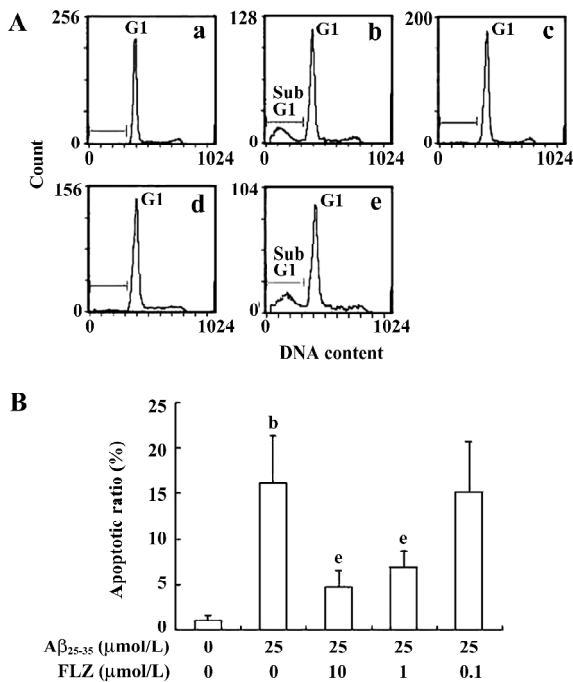


Figure 3. Effect of FLZ on apoptosis induced by Aβ₂₅₋₃₅ in SH-SY5Y cells. (A) By flow cytometry. a: vehicle treated cells; b: Aβ₂₅₋₃₅ (25 μmol/L) treated cells; c, d, e: FLZ (10, 1, and 0.1 μmol/L) plus Aβ₂₅₋₃₅ (25 μmol/L) treated cells, respectively. (B) The apoptotic ratio was quantitatively determined by flow cytometry. SH-SY5Y cells were cultivated with 25 μmol/L Aβ₂₅₋₃₅ in the absence or presence of FLZ (0.1–10 μmol/L) for 48 h at 37 °C. FLZ was added to the medium 30 min before the Aβ₂₅₋₃₅ treatment. Data are expressed by the mean±SD from the three independent trials. ^b*P*<0.05 vs vehicle treated cells, ^e*P*<0.05 vs Aβ₂₅₋₃₅ treated group.

Effect of FLZ on ROS production and the GSH level induced by Aβ₂₅₋₃₅ in SH-SY5Y cells Hydrogen peroxide was reported to mediate Aβ neurotoxicity. In our experiments, the exposure of SH-SY5Y cells to Aβ₂₅₋₃₅ (25 μmol/L) induced a 2-fold increase in DCF fluorescence intensity, indicating that Aβ₂₅₋₃₅ stimulated the production of ROS. The count of the high fluorescence intensity of the Aβ₂₅₋₃₅-treated cells was also more than that of the untreated cells. The pretreatment of FLZ (10 μmol/L) almost completely inhibited the increase in DCF fluorescence in the SH-SY5Y cells (Figure 4).

In contrast to the increase of ROS, the intracellular GSH level decreased dramatically in the SH-SY5Y cells after exposure to Aβ₂₅₋₃₅ (25 μmol/L) for 48 h. The pretreatment of FLZ significantly attenuated the decrease of the GSH level induced by 25 μmol/L Aβ₂₅₋₃₅ (Table 4).

Effect of FLZ on cytochrome c release, Bax, and Bcl-2 protein expressions in SH-SY5Y cells treated with Aβ₂₅₋₃₅

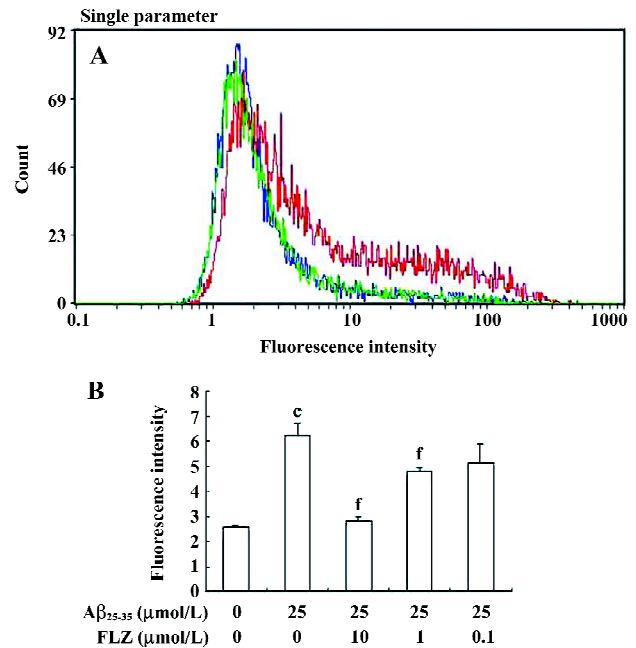


Figure 4. Effect of FLZ on ROS generation in SH-SY5Y cells treated with Aβ₂₅₋₃₅. FLZ was added to the medium 30 min before Aβ₂₅₋₃₅ (25 μmol/L), and then the cells were cultured for 48 h. The fluorescence intensity was measured by using the DCHF-DA probe with flow cytometry. At least the 10000 cells were detected. A: Results are plotted as log fluorescence intensity versus relative cell number. Curve 1 (Red): Aβ₂₅₋₃₅ treated cells, Curve 2 (blue): untreated cells, Curve 3 (green): FLZ (10 μmol/L) and Aβ₂₅₋₃₅ treated cells. B: Quantitative analysis of ROS in SH-SY5Y cells was determined by flow cytometry. Data are expressed as the mean±SD from three experiments. ^c*P*<0.01 vs untreated cells, ^f*P*<0.01 vs Aβ₂₅₋₃₅ treated cells.

Table 4. Effect of FLZ on the intracellular GSH level in SH-SY5Y cells induced by Aβ₂₅₋₃₅.

Group	GSH Level (% of Control)
Control	100.00±21.64
Aβ ₂₅₋₃₅ (25 μmol/L)	58.02±15.14 ^b
FLZ (10 μmol/L)+Aβ ₂₅₋₃₅ (25 μmol/L)	111.94±5.33 ^f
FLZ (1 μmol/L)+Aβ ₂₅₋₃₅ (25 μmol/L)	110.78±10.67 ^f
FLZ (0.1 μmol/L)+Aβ ₂₅₋₃₅ (25 μmol/L)	78.04±3.95 ^e

GSH level in SH-SY5Y cells was measured using OPT fluorescence after exposure to 25 μmol/L Aβ₂₅₋₃₅ for 48 h as described in Materials and Methods. FLZ was added to the medium 30 min before the Aβ₂₅₋₃₅ treatment. The values are expressed as a percentage of the OPT fluorescence of control condition. Data was shown as the mean±SD. ^b*P*<0.05 vs control group; ^e*P*<0.05, ^f*P*<0.01 vs Aβ₂₅₋₃₅ treated cells.

The Western blot analysis showed that the treatment of SH-SY5Y cells with Aβ₂₅₋₃₅ resulted in an increase of the pro-apoptotic Bax protein expression, while the expression of the anti-apoptosis Bcl-2 protein decreased. When the immunoblots were quantified by densitometry analysis, the ratio of Bax to Bcl-2 in the Aβ₂₅₋₃₅-treated cells significantly increased as compared with the untreated cells. The pre-treatment of SH-SY5Y cells with 1 and 10 μmol/L FLZ reversed the alternations of Bax and Bcl-2 expressions induced by Aβ₂₅₋₃₅, and substantially reduced the ratio of Bax/Bcl-2 (Figure 5). The effect of FLZ at 0.1 μmol/L concentration based on the above criteria was weak.

Aβ₂₅₋₃₅ caused an increase of cytochrome *c* release from the mitochondria of SH-SY5Y cells. FLZ at concentrations of 1 and 10 μmol/L effectively blocked cytochrome *c* release from mitochondria of SH-SY5Y cells induced by Aβ₂₅₋₃₅ (Figure 5).

Discussion

The accumulation of plaques containing Aβ in the brain is an invariant feature of AD pathology, and abundant evidence suggests that Aβ contributes to the etiology of AD^[21]. Neuronal apoptosis was observed in human AD brains^[22,23]. Several investigators reported that Aβ induced apoptosis in multiple cell types *in vitro*^[19,24-26]. Aβ₂₅₋₃₅ is considered to be the shorter toxic fragment exerting neurotoxic effects similar with Aβ_{1-40/42}, such as learning and memory impairment, neuronal apoptosis, cholinergic dysfunction, and oxidative stress^[27-29], so Aβ₂₅₋₃₅ is usually used to establish the *in vitro* model of AD for the study of the neurotoxic properties of Aβ and for drug screening. The human dopaminergic neuroblastoma cell line SH-SY5Y is widely applied in different neurochemical research. Aβ was uptaken rapidly

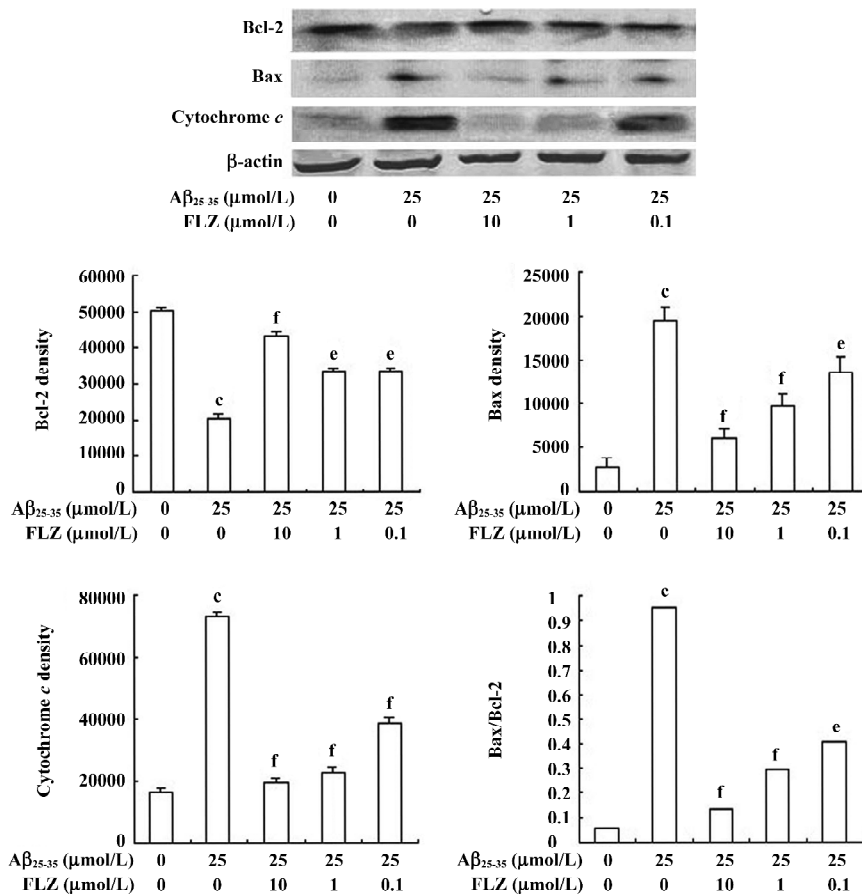


Figure 5. Effects of FLZ on the expression of Bcl-2, Bax, and cytochrome *c* release from mitochondria induced by Aβ₂₅₋₃₅ in SH-SY5Y cells. SH-SY5Y cells were exposed to 25 μmol/L Aβ₂₅₋₃₅ for 48 h in the presence or absence of indicated concentrations of FLZ, and harvested for Western-blot analysis. FLZ was added to the medium 30 min before the Aβ₂₅₋₃₅ treatment. Data were shown as the mean±SD from three experiments. ^b*P*<0.05, ^c*P*<0.01 vs untreated cells, ^e*P*<0.05, ^f*P*<0.01 vs Aβ₂₅₋₃₅ treated cells. The ratio of Bax/Bcl-2 was calculated by the mean of Bax divided the mean of Bcl-2.

into the SH-SY5Y cells and reserved for several days^[30]. Some evidence has been accumulated that suggests that A β_{25-35} induces neurotoxic effects in SH-SY5Y cells similar to the pathological changes of neurons in the mouse brain^[24-26]. The results of the present paper also indicated that the exposure of SH-SY5Y cells to A β_{25-35} (25 $\mu\text{mol/L}$) for 48 h displayed remarkable injuries. The cell viability decreased and LDH release from the cells increased in a dose-dependent manner. Most of the cells became round in shape and aggregated together. The cell apoptotic ratio also increased significantly. When the SH-SY5Y cells were precultured with FLZ (1 and 10 $\mu\text{mol/L}$) for 30 min, all of the above A β_{25-35} -induced injuries were significantly reduced. The cell viability, LDH release, and apoptotic ratio were all improved. The morphology of the FLZ-treated cells were close to that of the control cells. These results suggest that FLZ has protective action against A β_{25-35} -induced neurocytotoxicity.

It is very important to study the mechanism by which FLZ exerts its protective action against A β_{25-35} -induced neurocytotoxicity. Although which signaling pathway mediated A β -induced neurotoxicity is not fully defined, oxidative stress has been proposed to play a key role^[31-33]. A β stimulates the production of ROS by a direct or indirect pathway^[31-34]. Several investigators have demonstrated that ROS is involved in the apoptotic mechanism of A β -mediated neurotoxicity and may contribute to the increase in the apoptotic processes found in AD^[25,29,31-33]. The production of ROS can occur very early and cause damage to cardinal cellular components, such as lipid, protein, and nuclei acids, resulting in cell death by modes of apoptosis or necrosis. The high metabolic rate, a low concentration of GSH and the antioxidant enzyme catalase, and the large proportion of polyunsaturated fatty acids in the brain make brain tissue particularly vulnerable to oxidative damage^[7]. Some studies have reported that free-radical scavengers or antioxidants, such as melatonin, EGb-761, vitamin E, and estrogen could attenuate the A β -induced apoptosis and neurocytotoxicity^[25,29,31,35]. Some antioxidants were reported to be effective in the treatment of mild-to moderate dementia of AD patients^[36,37]. It seems reasonable that antioxidants will play an important role in the search of drugs as pharmacotherapy of AD. Data from this present study showed that 25 $\mu\text{mol/L}$ A β_{25-35} resulted in a significant increase of the ROS level in SH-SY5Y cells. This result is consistent with previous descriptions of the A β -mediated generation of ROS. In addition, the authors found that GSH, the most abundant antioxidant in cells, was depleted by the addition of A β_{25-35} . The results suggested that oxidative stress was involved in A β -induced toxicity in SH-SY5Y cells.

As major sources of ROS, mitochondrial structures are exposed to high concentrations of ROS and might therefore be particularly susceptible to oxidative injury. It was reported that mitochondrial damage plays a pivotal role in cell apoptosis^[7]. The present results show that A β_{25-35} induced mitochondrial dysfunction in the MTT analysis, because the MTT underwent conversion of the yellow MTT to purple formazan crystals by mitochondrial succinate dehydrogenase in viable cells, which primarily reflects the mitochondrial metabolic capacity of viable cells and the intracellular redox state^[14]. Overproduction of ROS induced the opening of the mitochondrial permeability pore and caused the mitochondrial intermembrane space soluble protein (cytochrome *c*, apoptosis-inducing factor) release into the cytoplasm^[38]. In most pathways of apoptosis, the release of mitochondrial cytochrome *c* and apoptosis-inducing factor are also key events in initiating the cascade of reactions leading to apoptotic cell death^[39]. The release of cytochrome *c* is clearly regulated by the pro- and anti-apoptotic proteins of the Bcl-2 family (Bax, bak, bad, bim, and bid as pro-, and bcl-2 and bcl-x_L as anti-apoptotic proteins). Bax promotes the release of cytochrome *c* from the mitochondria, and Bcl-2 inhibits the release of cytochrome *c*. The relative ratio of pro-apoptotic and anti-apoptotic proteins is important to determine cell survival or death^[40-42]. The overexpression of Bcl-2 or Bcl-x_L can inhibit free-radical generation and protects cells from apoptosis induced by various stimuli^[43,44]. In the present study, A β_{25-35} treatment decreased the expression of Bcl-2, increased the expression of Bax, and promoted the cytochrome *c* release from mitochondria. The cell apoptotic ratio also significantly increased. In the previous study at our laboratory, A β_{25-35} -induced cell apoptosis was also identified by other methods, such as the DNA ladder (data not shown).

In the present study, the pretreatment of FLZ significantly inhibited the increase of ROS generation and the decrease of GSH content in SH-SY5Y cells. In our previous study, FLZ was shown to inhibit microsomal lipid peroxidation induced by Fe²⁺-cysteine, and also to scavenge oxygen free radicals, indicating that FLZ has an antioxidant property^[45]. Moreover, H₂O₂ is a donor of hydroxyl radical that has been reported to mediate A β protein toxicity^[46]. The pretreatment of FLZ inhibited the H₂O₂-induced apoptosis of cells^[11]. The pretreatment of FLZ also protected PC12/SH-SY5Y cells against dopamine/MPP⁺-induced apoptosis through inhibiting cytochrome *c* release and caspase 3 activation^[11,13]. The present study further confirmed that FLZ reduced the A β -induced relative ratio of Bax and the Bcl-2 protein by increasing Bcl-2 and decreasing the

Bax expressions; and also decreased the cell apoptotic ratio of SH-SY5Y cells. Taken together, it appears that the protective action of FLZ against A β ₂₅₋₃₅ neuron toxicity may be in part due to its antioxidant property.

There is debate as to whether treatment with antioxidants might theoretically act to prevent propagation of tissue damage and improve both survival and neurological outcomes^[48]. The great pharmacological disadvantage of most antioxidants is their very limited passage through the blood-brain barrier. Therefore, antioxidants with much better blood-brain barrier permeability are required for their potential application in treating neurodegenerative diseases, such as AD^[47]. Fortunately, the results of a pharmacokinetic study of FLZ in rats indicated that the oral administration of FLZ penetrates through the blood-brain barrier very well (data to be published).

Because apoptosis is the main cause in neurodegeneration, such as AD and Parkinson's disease (PD), and oxidative stress is an early event in the apoptosis process, it appears that FLZ is a novel neuroprotectant to protect against oxidative injury and apoptosis, and a good candidate for neurodegeneration therapy. Further pharmacological and toxicological studies on FLZ are in progress.

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