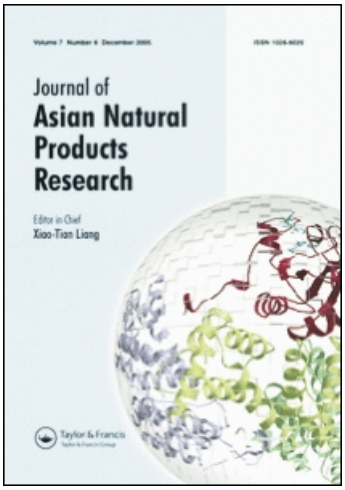

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Ginsenoside Rg1 inhibits β-secretase activity *in vitro* and protects against Aβ-induced cytotoxicity in PC12 cells

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Ginsenoside Rg1 inhibits β-secretase activity *in vitro* and protects against Aβ-induced cytotoxicity in PC12 cells

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Ginsenoside Rg1 (**1**) is a major active component of *Panax notoginseng*, a Chinese herb widely used in traditional Chinese medicine to improve learning and memory function. Increasing evidence suggests that β-amyloid peptide (Aβ) plays a central role in the pathophysiology of Alzheimer's disease (AD). To elucidate the mechanism of **1** on improving the ability of learning and memory, we investigated whether **1** could affect Aβ generation or protect Aβ-induced neurotoxicity. The results showed that **1** could inhibit β-secretase activity *in vitro* and also protect the PC12 cells against injuries caused by exposure of PC12 cells to 50 μM $A\beta_{25\text{-}35}$ for 48 h. The cell death, LDH release, NO release, ROS production, lipid peroxidation, intracellular calcium elevation, and apoptosis are associated events induced by Aβ that can be rescued by **1** in PC12 cells. In conclusion, **1** may be a promising agent for AD, and the mechanism is related to β-secretase inhibition and protection against Aβ-induced cytotoxicity.

Keywords: ginsenoside Rg1; β-secretase; β-amyloid peptide

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the central nervous system associated with progressive cognitive and memory loss. Drugs currently used to treat AD act mainly on cholinergic neurotransmission and offer only modest attenuation of the progression of symptoms. However, existing treatments cannot cure this devastating disorder. Therefore, the development and characterization of novel drugs and approaches to block key steps in the pathogenesis of AD are the primary goals in AD research [1]. AD is characterized by the presence of senile plaques, neurofibrillary tangles, and extensive neuronal loss and synaptic changes in the brain [2]. The β-amyloid peptide (Aβ) is a major component of senile plaques; excessive accumulation of Aβ in the brain has been proposed as a central event in the pathogenesis of AD. The β-secretase (BACE-1) is the key rate-limiting enzyme that initiates the formation of Aβ. So it is one of the therapeutic strategies for AD to lower the production of Aβ or attenuate the neurotoxicity of Aβ in the brain [3,4]. Although the molecular mechanism of Aβ-induced neurotoxicity is not fully understood, recent research has showed that amyloid formation causes neuronal death via a number of possible mechanisms, including Ca^{2+} homeostasis disruption, oxidative stress, excitotoxicity, energy depletion, neuroinflammation, and apoptosis [5].

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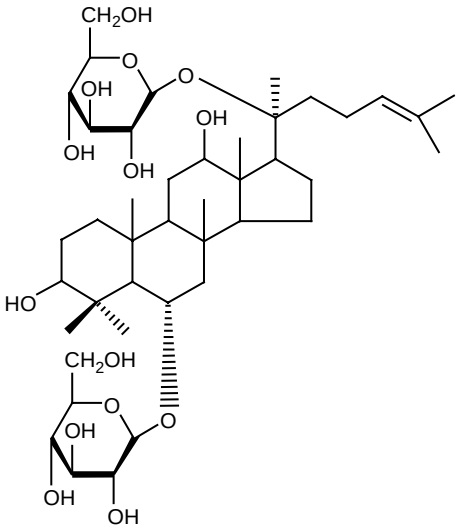

Figure 1. Chemical structure of **1**.

Ginsenoside Rg1 (**1**) (Figure 1) is a major active component of *Panax notoginseng*, a Chinese herb that is widely used to enhance the central nervous system function, especially cognitive functions like learning and memory [6]. Although the cellular and molecular mechanisms that underlie the effect of **1** on memory are not fully understood, studies suggest that the improving effect of **1** on cognitive function could be related to the scavenging effects on oxygen free radicals and an enhancement of the central cholinergic function [7,8]. Apart from these neuroprotective effects, however, the protective effect of **1** against Aβ-induced neurotoxicity may be possibly involved in the beneficial effect of **1** on learning and memory. In current studies, we investigated the protective effects of **1** on Aβ-treated injuries in PC12 cells to explore its neuroprotective mechanisms in the prevention and treatment of AD.

2. Results and discussion

2.1 Effect of 1 on β-secretase activity

The results are presented in Figure 2. Ginsenoside Rg1 (**1**) inhibited the activity of β-secretase *in vitro* in a concentration-dependent manner. The IC_{50} value of Rg1 is 6.18 ± 0.96 μM.

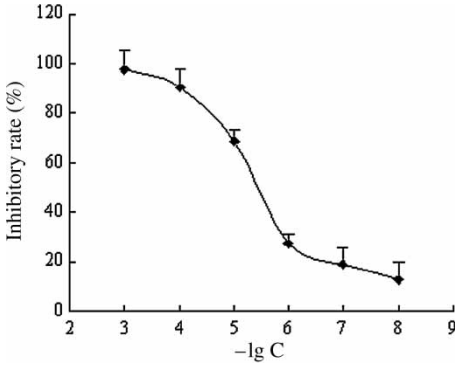

Figure 2. Effect of **1** on the activity of β-secretase. Results are expressed as mean ± SD of triplicate assays.

2.2 Toxicity of Aβ in cultured PC12 cells

The cell viability was assayed using the MTT method after incubation of PC12 cells with different concentrations of $A\beta_{25\text{-}35}$ in the range of 0–100 μM, and dose-dependent $A\beta_{25\text{-}35}$ toxicity was examined. As shown in Figure 3, exposure of PC12 cells to $A\beta_{25\text{-}35}$ for 48 h induced a decrease in the cell survival in a dose-dependent manner.

2.3 Effect of 1 on cell viability

PC12 cells were incubated with 50 μM $A\beta_{25\text{-}35}$ with/without different concentrations of **1** for 48 h. The $A\beta_{25\text{-}35}$-induced cytoxicity was evaluated by an MTT reduction assay. As shown in Figure 4, $A\beta_{25\text{-}35}$ significantly decreased the cell viability after 48 h by 68.76% ($P < 0.01$) compared with the normal cells. However, the cytotoxic effects were attenuated by the co-treatment with **1** dose-dependently; at the concentration of 1 and 10 μM, the cytotoxic effects of $A\beta_{25\text{-}35}$ on viability were significantly blocked ($P < 0.05$, $P < 0.01$).

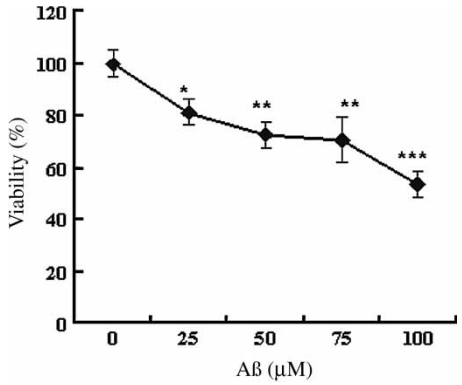

Figure 3. Concentration-dependent cytotoxicity effects of $A\beta_{25\text{-}35}$ in PC12 cells. PC12 cells were exposed to different $A\beta_{25\text{-}35}$ concentrations (0–100 μM) for 48 h. Then, cell viability was measured using the MTT assay. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. normal group.

2.4 Ginsenoside Rg1 (1) prevented Aβ-induced LDH efflux

The results (Figure 5) showed that the extracellular LDH in medium in the $A\beta_{25\text{-}35}$-treated group was significantly increased by 91.8% ($P < 0.01$) compared with that in the normal group. However, co-incubation with **1** could inhibit the excessive LDH efflux, and the effects were dose-dependent. At the concentration of 0.1, 1, and 10 μM, the LDH efflux was decreased by 21.6% ($P < 0.05$), 26.5% ($P < 0.05$), and 34.5% ($P < 0.01$), respectively, compared with the $A\beta_{25\text{-}35}$-treated group.

2.5 Ginsenoside Rg1 (1) prevented Aβ-induced NO production

The results are presented in Figure 6. Excessive generation of nitric oxide has been implicated in the pathogenesis of AD. Thus, we measured the NO level. Our data demonstrated that NO levels are significantly enhanced from 17.77 ± 3.27 to 27.30 ± 7.02 μM after exposure to $A\beta_{25\text{-}35}$ in PC12 cells ($P < 0.05$). Pretreatment with **1** at the concentration of 1 and 10 μM significantly decreased the NO level compared with the $A\beta_{25\text{-}35}$-treated group ($P < 0.05$).

2.6 Ginsenoside Rg1 (1) prevented Aβ-induced ROS formation

Aβ is able to promote the generation of ROS, and this may be a mechanism underlying the Aβ-induced degeneration of nerve cells. We measured the intracellular contents of ROS in PC12 cells using DCF fluorescence as an indicator

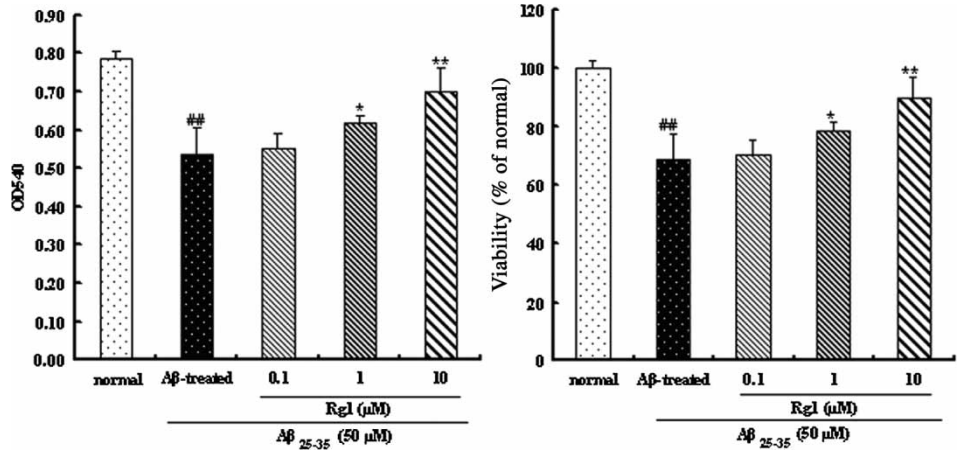

Figure 4. Effect of **1** on Aβ-induced cytotoxicity in PC12 cells. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{\#\#}P < 0.01$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Aβ-treated group.

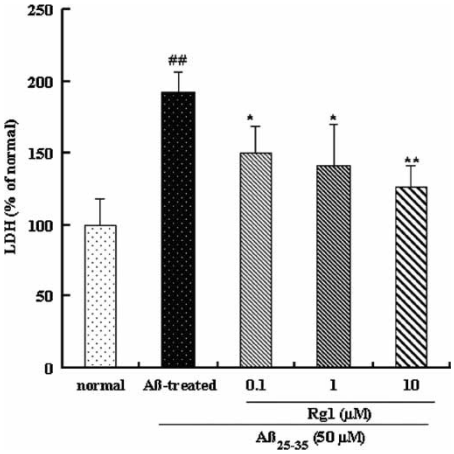

Figure 5. Effect of **1** on Aβ-induced LDH efflux in PC12 cells. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{\#\#}P < 0.01$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Aβ-treated group.

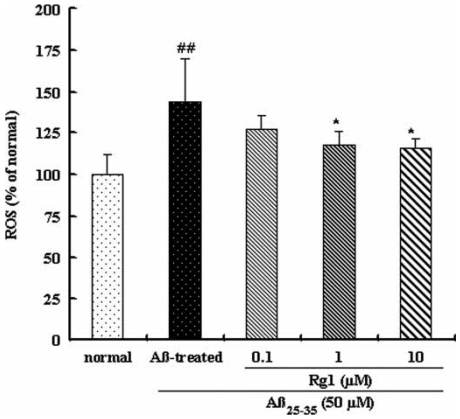

Figure 7. Scavenging effect of **1** on ROS induction by $A\beta_{25\text{-}35}$ in PC12 cells. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{\#\#}P < 0.01$ vs. normal group; $^{*}P < 0.05$ vs. Aβ-treated group.

of ROS levels. PC12 cells exposed to 50 μM $A\beta_{25\text{-}35}$ for 48 h resulted in an enhanced production of ROS compared with the normal cells. Pretreatment of cells with **1** and exposure to $A\beta_{25\text{-}35}$ for 48 h appreciably inhibited the ROS generation in a dose-dependent manner (Figure 7). Ginsenoside Rg1 (**1**) at the concentration of 0.1, 1, and 10 μM decreased the ROS production by 16.61%, 25.71% ($P < 0.05$), and 28.14% ($P < 0.05$), respectively.

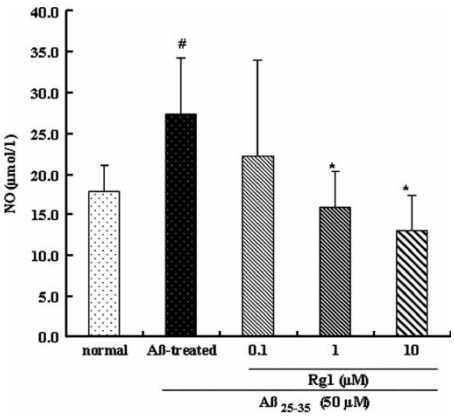

Figure 6. Effect of **1** on Aβ-induced NO production in PC12 cells. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{\#}P < 0.05$ vs. normal group; $^{*}P < 0.05$ vs. Aβ-treated group.

2.7 Ginsenoside Rg1 (1) prevented Aβ-induced lipid peroxidation

Aβ is able to promote the generation of lipid peroxidation, and this may be a mechanism underlying the Aβ-induced degeneration of nerve cells. We measured the intracellular contents of lipid peroxidation in PC12 cells using thiobarbituric acid reactive substances (TBARS) assay. The results are presented in Figure 8. The elevated MDA level of Aβ-treated PC12 cells was reduced by the addition of **1** in a dose-dependent manner.

2.8 Ginsenoside Rg1 (1) prevented Aβ-induced intracellular calcium increase

The results are presented in Figure 7. A concentration of 50 μM $A\beta_{25\text{-}35}$ markedly increased $[Ca^{2+}]_i$ in PC12 cells from 113.9 ± 15.4 to 172.2 ± 19.3 nM. However, co-incubation with **1** inhibited the rise of $[Ca^{2+}]_i$ induced by Aβ-treated in a dose-dependent manner (Figure 9).

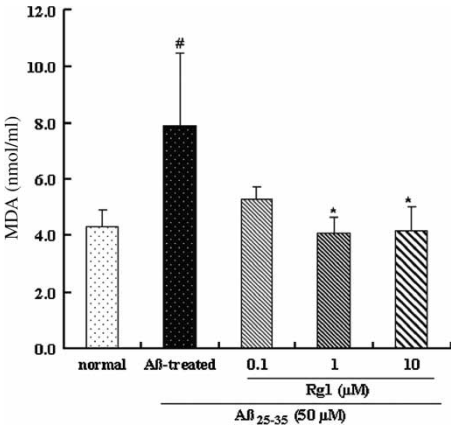

Figure 8. Effect of **1** on $A\beta_{25\text{-}35}$-induced lipid peroxidation in PC12 cells. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{\#}P < 0.05$ vs. normal group; $^{*}P < 0.05$ vs. Aβ-treated group.

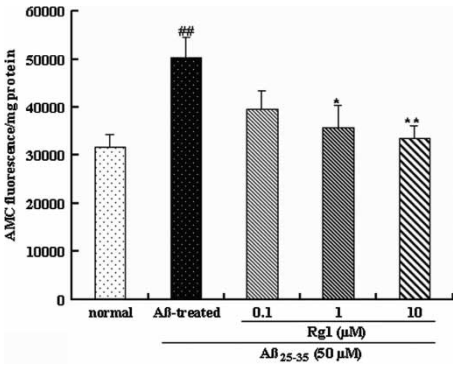

Figure 10. Effect of **1** on caspase-3 activity. The activity of caspase-3 was assessed by the fluorogenic substrate Ac-DEVD-AMC. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{\#\#}P < 0.01$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Aβ-treated group.

2.9 Effect of 1 on Aβ-induced caspase-3 activity increase in PC12 cells

As shown in Figure 10, $A\beta_{25\text{-}35}$ increased caspase-3 activity by 159.9% after treatment of PC12 cells for 48 h. Pretreatment with **1** for 1 h prior to $A\beta_{25\text{-}35}$ decreased the caspase-3 activity in PC12 cells. The inhibitory rates of **1** at the concentration of 1 and 10 μM were 29.2% ($P < 0.05$) and 33.5% ($P < 0.01$), respectively.

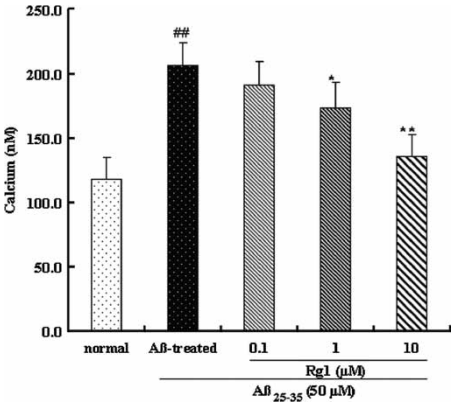

Figure 9. Ginsenoside Rg1 (**1**) prevented Aβ-induced intracellular calcium increase in PC12 cells. Values are expressed as mean ± SD. Each experiment was repeated four times. $^{\#\#}P < 0.01$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Aβ-treated group.

Recently, much of AD research has been focused on the amyloid cascade hypothesis. β-Secretase activity is the rate-limiting step in Aβ peptide production from amyloid precursor protein. Blocking the production of Aβ by specific inhibition of the key proteases is a major focus of research into AD therapy [3,4,9]. So we established a novel high-throughput screening model to find inhibitors of β-secretase using an internally quenched fluorogenic peptide substrate. This peptide substrate contains a highly fluorescent group that is efficiently quenched by resonance energy transfer to the quencher group. At the same time, the substrate contains the β-secretase cleavage site. It can be used to measure the activity of peptidases that are capable of cleaving an amide bond between the fluorescent group and the quencher group, causing an increase in fluorescence. In this study, we found that **1** inhibited β-secretase activity *in vitro*, and it may be a useful agent in AD therapy.

Ginsenosides were reported to have multiple pharmacological actions, including anti-fatigue effects, stimulatory effects on

the central nervous system, and effects on vasodilatation. Growing evidence showed that ginsenoside Rg1 could improve spatial learning and increase the hippocampal synaptophysin level in mice [10]. Studies also suggested that Rg1 had neuroprotective effects in several experimental models such as excitotoxicity insults, traumatic brain injury, and cerebral ischemia [11]. At the same time, many researchers have also found that Rg1 has protective effects in cell-damaging models [12,13]. However, the effect of Rg1 on amyloid-induced toxicity has not yet been observed. So here, we investigated the protective effect of **1** on Aβ-induced neurotoxicity.

AD is a complex disease that involves a multitude of dysfunctional processes. Oxidative damage in AD may be a direct result of Aβ. Markers of oxidative DNA damage have been localized to amyloid plaque-affected areas in the AD brain. The generation of lipid peroxidation products is associated with amyloid plaques [14]. In the present study, we demonstrated a significant increase in ROS and MDA in the PC12 cells treated with $A\beta_{25\text{-}35}$. Pretreatment with **1** ameliorates these changes in a dose-dependent manner.

NO is an enzymatic product of nitric oxide synthase. In addition to its vasoactive and immunological properties, NO has significant neurophysiological functions. However, NO can also be neurotoxic primarily due to its free radical properties, and it has been implicated in neurodegenerative diseases [15]. In the present study, we demonstrated a significant increase in NO in the PC12 cells treated with $A\beta_{25\text{-}35}$. Co-treatment with **1** ameliorated these changes in a dose-dependent manner. These results suggested that Aβ induced PC12 cells to release NO, potent sources of oxidative stress known to occur in AD.

A mechanism of Aβ toxicity is Aβ-mediated membrane disruption. Poorly selective Aβ channels formed in the plasma membrane of neuronal cells can contribute directly to neuronal failure by destroying the membrane potential, and by allowing increased Ca^{2+} flux directly through the Aβ channels, or indirectly by triggering increased Ca^{2+} flux through voltage-sensitive calcium channels [16]. The deposit of β-amyloid protein is thought to be toxic to neurons possibly via induction of intracellular calcium. Our results also suggested that **1** abolished Aβ-induced cell death and calcium increase in PC12 cells, indicating that calcium elevation is an associated phenomenon induced by Aβ that can be rescued by **1**.

Caspase has been proposed to play a pivotal role in apoptosis. The caspase family consists of more than 10 homologs. Among the members of caspases, caspase-3 has been suggested to play an important role in several models of apoptosis [17]. In AD brains, the protein level of caspase-3 was also increased [18]. These observations prompted us to investigate the possible involvement of caspase-3 activity in Aβ-induced apoptosis in PC12 cells. The results of the present study showed that caspase-3 activity significantly increased in Aβ-induced PC12 cells, and that treatment with **1** significantly prevented Aβ-induced apoptosis.

In conclusion, our results confirm that **1** provides a combination of anti-amyloid, anti-oxidative, and anti-apoptotic effects. Thus, this study provides evidence of multiple pathways in Aβ-induced death of PC12 cells, and **1** plays an important role in combating AD and other neurodegenerative disorders through anti-amyloid neurotoxicity.

3. Experimental

3.1 Chemicals and reagents

Rg1 was obtained from the Shanghai Winherb Medical Science Co. Ltd (Shanghai, China). RPMI-1640 was a Gibco product (Grand Island, NY, USA). β-Amyloid peptide ($A\beta_{25\text{-}35}$), MTT, and fura-2/AM were purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant human

BACE-1, fluorogenic peptide substrate IV, and fluorogenic substrate Ac-DEVD-AMC were purchased from R&D Company (New York, NY, USA). 2,7-Dichlorodihydrofluorescein diacetate was the product of Molecular Probes (Eugene, OR, USA). The LDH, NO, and MDA assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

3.2 *β-Secretase activity assay* in vitro

β-Secretase assay was performed according to previously reported methods [3,4,19]. Briefly, the assays were performed in 384-well black plates using Fluostar Galaxy (Offenburg, Germany). Different concentrations of Rg1 and the substrate were incubated with β-secretase in an assay buffer (100 mM sodium acetate, pH 4.0). The hydrolysis of the substrate was followed at 37°C for 10 min, by measuring the accompanying increase in fluorescence. Readings at the excitation wavelength of 320 nm and the emission wavelength of 405 nm were taken every 60 s for 20 cycles. The activity of β-secretase was expressed as relative fluorescence units per min.

3.3 *Cell culture and treatment*

PC12 cells have been widely employed as a neuronal cell model and a large number of studies were generated. The cells were grown in RPMI-1640 supplemented with 10% horse serum, 5% fetal bovine serum, and 100 unit/ml penicillin–streptomycin. Conditions were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2–3 days. The cells were plated onto 96-well plates at a density of 2×10^5 cells/ml. After 80% confluence, the cells were incubated in a serum-free RPMI-1640 medium containing different concentrations of $A\beta_{25\text{-}35}$ (37°C for 7 days) from 0 to 100 μM.

Rg1 were added to the wells at different concentrations using PBS for controls. After 1 h, $A\beta_{25\text{-}35}$ was added to the wells at a final concentration of 50 μM. The cells were incubated for 48 h at 37°C.

3.4 *MTT assay*

Cell viability was assessed by the MTT assay as described previously [20]. The assay was initiated by removing the culture medium and adding MTT (0.5 mg/ml). Following 4 h incubation at 37°C, the medium was aspirated, and 100 μl DMSO was added to lyse the cells and dissolve the formazan crystals. The absorbance was recorded at 540 nm in a microplate reader [21]. Cell viability was expressed as a percentage of the absorption in the untreated cultures (100%).

3.5 *LDH efflux assay*

LDH efflux assay was carried out using the LDH assay kit according to the manufacturer's instruction. As a quantitative measure of cellular toxicity, the LDH efflux was estimated in 10 μl of culture medium. The activity of LDH was determined spectrophotometrically.

3.6 *NO assay*

NO production by PC12 cells was measured by Griess assay. Briefly, PC12 cells were treated as described previously. One hundred microliters of conditioned medium were collected after 48 h of stimulus. NO production was measured using the NO assay kit according to the manufacturer's instructions.

3.7 *Intracellular ROS production determination*

For the detection of intracellular ROS, differentiated cell cultures grown on 35 mm dishes were incubated with 5 μM 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) for 30 min at 37°C in differentiating medium, followed by a 30 min incubation in differentiating medium alone. After chilling in ice, the cells were washed in ice-cold PBS, mechanically

detached and resuspended in PBS. The fluorescence formed by the reaction of DCF-DA with ROS was quantified using a fluorescence plate reader (Fluostar Galaxy, Germany) [22].

3.8 Measurement of lipid peroxide levels

The organism produces oxygen free radical through an enzyme and a non-enzyme system. The latter can induce lipid peroxidation by attacking polyunsaturated fatty acid in the biological membrane and then form lipid peroxide, injuring the cell and tissue [23]. Lipid peroxidation in the PC12 cells was determined by measuring the formation of TBARS using the MDA assay kit according to the manufacturer's instructions.

3.9 Measurement of $[Ca^{2+}]_i$ increase induced by Aβ

The $[Ca^{2+}]_i$ content was determined by the modification of the previous method [24,25]. Briefly, PC12 cells were collected and loaded by fura-2/AM at the final concentration of 5 μM in Hanks solution at 37°C in dark condition for 40 min. After being loaded with fura-2/AM, the cells were centrifuged at 200*g* for 5 min twice and resuspended in Hanks solution containing 0.2% bovine serum albumin. The fura-2/AM-loaded cells were incubated with 0.1, 1, and 10 μM Rg1 at 37°C for 10 min, and $A\beta_{25\text{-}35}$ at the final concentration of 50 μM was added and mixed for 5 min. The fluorescence was measured by Fluostar Galaxy at λ_{ex} 340 nm and 380 nm, λ_{em} 520 nm. The excitation wavelength of fura-2/AM was 380 nm. After combining with calcium, the maximal excitation wavelength was 340 nm. Triton X-100 was added to measure the maximal fluorescene value and EGTA was added to measure the minimal fluorescene value. $[Ca^{2+}]_i$ was calculated according to the formula: $[Ca^{2+}]_i = Kd \times (R - R_{min})/(R_{max} - R) \times (Ff2/Fb2)$, where $R = F340/F380$ and $Kd = 224$ nmol/l.

3.10 Caspase-3 activity detection

Caspase-3 activity was performed according to previously reported methods [26,27] with modification. Briefly, after treatment, the PC12 cells were lysed in buffer (1% Triton X-100, 0.32 mol/l sucrose, 5 mmol/l EDTA, 1 mmol/l phenylmethysulfonyl fluoride, 1 mg/l aprotinin, 1 mg/l leupeptin, 2 mmol/l dithiothreitol, and 10 mmol/l Tris–HCl, pH 8.0) for 15 min at 4°C and followed by centrifugation at 12 000*g* for 10 min. Caspase-3 activity was tested in the resulting supernatants by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC with an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

3.11 Statistical analysis

The results are presented as mean ± SD. Statistical evaluation was conducted by ANOVA for comparisons among groups, followed by Student's *t*-test for analysis of significance. The differences were considered to be significant at $P < 0.05$.

Acknowledgements

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