

Original Article

Protective effect of Cy-3G on PC12 cells against beta-amyloid-induced apoptosis and the possible mechanism

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Abstract: Beta-amyloid protein (Abeta), a major protein component of brain senile plaques in Alzheimer's disease, is known to be directly responsible for the production of reactive oxygen species (ROS) and induction of apoptosis. In this study, the protective effect of cyanidin 3-O-glucopyranoside (Cy-3G), an anthocyanin derivate purified from fruits, on Abeta-induced rat pheochromocytoma (PC12) cultures was investigated. Although exposure of PC12 cells to 50 μ M Abeta25-35 caused significant viability loss and apoptotic rate increase, pretreatment of the cells with Cy-3G for 2 h reduced the viability loss and apoptotic rate. Cy-3G (20 μ M) significantly inhibited Abeta25-35-induced apoptosis of PC12 cells. Preincubation of the cell with Cy-3G also restored the ROS and mitochondrial membrane potential levels that had been altered as a result of Abeta25-35 treatment. Cy-3G was also found to excite the Cyt c and AIF release to cytoplasm and reduce caspase-3, caspase-8 and caspase-9 activation. Phosphorylation of JNK and P38 were also suppressed by Cy-3G. These results suggested that Cy-3G could attenuate Abeta25-35-induced PC12 cell injury and apoptosis through regulating JNK and P38 MAPK signaling. Therefore, Cy-3G may act as an intracellular ROS scavenger, and its antioxidant properties may protect against Abeta25-35-induced cell injury.

Keywords: Cyanidin 3-O-glucopyranoside, PC12 cells, apoptosis, Abeta25-35

Introduction

It is reported that the extra- and intra-neuronal aggregation and deposition of amyloid beta (Abeta) peptides play a causal role in the pathogenetic cascade leading to Alzheimer's disease (AD) [1, 2]. It is accepted that the Abeta 1-40 and Abeta 1-42 peptides, produced by the cleavage of the amyloid precursor protein, can exist in multiple aggregation forms, including soluble oligomers or protofibrils and insoluble fibrils, which are responsible for various pathological effects. Several studies suggests that the Abeta oligomers diffuse readily through the brain parenchyma and cause a selective synaptic dysfunction and/or neuronal loss in cortex and hippocampus, two stricken brain regions in AD [3]. Abeta oligomers adhere to the plasma membrane of neurons and cause lesions by a combination of radical species-initiated lipid peroxidation and formation of ion-permeable

pores which initiate a cascade of pathological processes that end with neuronal death. In particular, Abeta 25-35 is a short peptide generated by proteolysis of Abeta 1-40 [4, 5], showing neurotoxic and aggregation properties similar to full-length peptides (i.e. Abeta 1-40 and Abeta 1-42 peptides).

Several studies have highlighted that a diet rich in antioxidant components such as anthocyanins may lower age-related cognitive decline and the risk of developing neurodegenerative diseases including AD [6, 7]. Among anthocyanins, cyanidin3-O-glucoside (Cy-3G), present in colored fruit and vegetables, has recently gained attention as a neuroprotective phytochemical. In vitro studies have shown the ability of Cy-3G to counteract the neurotoxicity induced by Abeta25-35 and Abeta 1-42 peptides in SH-SY5Y cells [8-11]. In addition, Cy-3G also reduced the cerebral ischemia and age-related

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neuronal deficits in rats, suggesting the ability of Cy-3G to cross the blood brain barrier and to deliver its neuroprotective capabilities centrally [12, 13].

In this study we demonstrate the ability of Cy-3G to prevent the apoptosis of soluble Abeta 25-35 oligomers and their neurotoxicity in terms of membrane integrity loss, redox status impairment and cell death in a neuronal cell line (PC12).

Materials and methods

Cell culture

PC12 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI1640 medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and antibiotics (100 U/ml penicillin and 100 lg/ml streptomycin) at 37°C in a humid atmosphere containing 5% CO₂.

Cy-3G

Cy-3G with a purity of 98.61% was obtained from Shanghai Yuanye Medical Technological Co. Ltd. (Shanghai, China). It was dissolved in an appropriate amount of dimethylsulfoxide (DMSO) and diluted to the desired concentrations before utilization, with the final concentration of DMSO kept below 0.5%.

Cell viability

Standard tetrazolium bromide (MTT) assay was used to assess cell viability. Briefly, cells (5 × 10³ cells/well) were seeded in 96-well plates. With Cy-3G (0, 10 and 50 μM) treatment for 2 h, PC12 cells were insulted with Abeta25-35 (50 μM) for 24 h, and 50 ml MTT (Sigma) solution (2 mg/ml in PBS) was added to each well and the plates were incubated for additional 4 h at 37°C. The medium was then removed and the cells were incubated with 200 μl dimethyl sulfoxide (DMSO) in the dark for 30 min to dissolve violet crystals. The absorbance was read at 570 nm on an automatic microplate reader with DMSO as the blank. All assays were performed in quintuplicate and repeated at least three times.

Cell apoptosis assay

With Cy-3G (0, 10 and 50 μM) treatment for 2 h, Abeta25-35 (50 μM) insulted PC12 cells were

stained with annexin V-fluorescein isothiocyanate and apoptosis rates were analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

Detection of reactive oxygen species (ROS)

Detection of ROS was performed by flow cytometric analysis as described previously. In brief, cells (5 × 10⁴ cells/well) were cultured in 24-well plate with Cy-3G (0, 10 and 50 μM) treatment for 2 h, and Abeta25-35 (50 μM) insulted PC12 cells after a period of exposure (12 h). Cells were washed with PBS and resuspended in complete medium followed by incubation with 0.5 μM dihydrorhodamine 123 (Sigma) for 30 min at 37°C. ROS fluorescence intensity was determined by cytometry with excitation at 490 nm and emission at 520 nm.

Determination of mitochondrial membrane potential (MMP)

Rhodamine-123 (Rho-123) dye (Sigma) was used to detect the changes in MMP. Cells (5 × 10⁴ cells/well) were cultured in 24-well plate. After a period of exposure (24 h) with various concentrations of Cy-3G (0, 10 and 50 μM), cells were washed with PBS, incubated with Rho-123 (10 mg/mL) and subsequently subjected to flow cytometry.

Reverse transcription and real-time PCR

Total RNA was isolated using Trizol reagent (Gibco® life technology, Carlsbad, CA, USA). Reverse transcription reactions were performed as described. Real-time PCR was performed on ABI 7500 (Applied Biosystem, Foster City, CA, USA) thermal cycler. Using a standard SYBR Green PCR kit (Thermo Fisher Scientific) protocol The relative mRNA expression of target gene compared with GAPDH were calculated using the 2^{-ΔΔCt} method. The primers for each gene were listed as following: 5'-AACTG-GACTGTGGCATTGAG-3' and 5'-TGTCGGCATACT-GTTTCAGC-3' for caspase-3 (product: 186 bps); 5'-CTGGGAGAAGGAAAGTTG-3' and 5'-TTGGA-GAGTCCGAGATTG-3' for caspase-8 (product: 184 bps); 5'-CCTCACCTGCCTTATCTTG-3' and 5'-TCCCTCTTCTCCACTGTTC-3' for caspase 9 (product: 189 bps); 5'-ATCACTGCCACCCAG-AAG-3' and 5'-TCCACGACGGACACATTG-3' for GAPDH (product: 191 bps).

Western blot

Treated and untreated PC12 cells were harvest and washed twice with PBS and lysed in ice-

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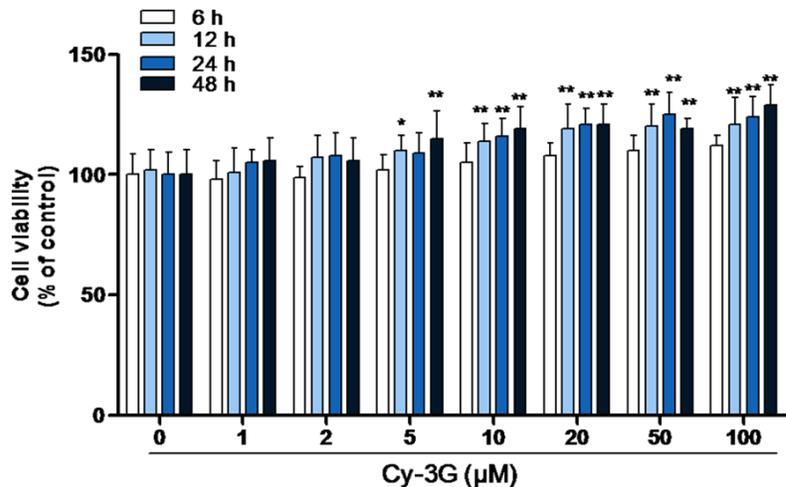


Figure 1. Effect of Cy-3G on the cell viability of PC12 cells. After PC12 cells were exposed to various concentrations of Cy-3G (0, 1, 2, 5, 10, 20, 50 and 100 µM) for 6, 12, 24 and 48 h, cell viability was determined by CCK8 assay. Data are expressed as mean \pm SD, n = 6, **P < 0.01, versus control.

cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant (20-30 µg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bedford, USA). The blots were blocked with 5% skim milk, followed by incubation with primary antibodies. Antibodies against Cyct c, AIF, caspase-3/8/9, phosphorylation (p)-P38, P38, p-JNK, JNK and GAPDH were purchased from Santa. Blots were then incubated with goat anti-mouse secondary antibody (Beyotime, Shanghai, China) or goat anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Millipore).

Separation of mitochondria and cytosol

The mitochondria/cytosol fractionation kit (Abcam) was used to prepare mitochondrial and cytosolic fractions from cells in accordance with the manufacturer's instructions. After 12 h of treatment with different concentrations of Cy-3G and Abeta25-35, the cells were harvested, washed, and centrifuged. After re-suspension with cytosolic extraction buffer, the cells were homogenized on ice with a grinder. The homogenate was subsequently centrifuged,

supernatant was collected, and the sediment was saved. Following centrifugation, supernatant was collected as cytosolic fraction and stored at -80°C. The sediment was re-suspended with mitochondrial extraction buffer, vortexed, and stored as mitochondrial fraction at -80°C.

Statistical analysis

The data were analyzed using SPSS computer software Version 16.0. The data for multiple comparisons were performed by one-way ANOVA followed by LSD t-test. A value of P <

0.05 was considered statistically significant and all results per presented as the means \pm SD.

Results

Cy-3G promoted cell viability of PC12 cells

We firstly determined the effect of Cy-3G on the cell proliferation of PC12 cells by CCK8 assay. Different doses of Cy-3G were carried out to treat cells for 6, 12, 24 and 48 h. As shown in **Figure 1**, Cy-3G could increase the cell viability in a time- and dose-dependent manner. 10 µM of Cy-3G remarkably improve the cell viability at 6, 12, 24 and 48 h compared with the untreated cells. In addition, there was no obvious difference in cell proliferation between the 50 µM and 100 µM group. As a result, 10 µM and 50 µM of Cy-3G were selected for the further study.

Cy-3G protected PC12 cells against the Abeta25-35-induced apoptosis

Abeta25-35 is a major protein component of brain senile plaques in Alzheimer's disease, which is responsible for the production of reactive oxygen species (ROS) and induction of apoptosis. As shown in **Figure 2A**, Abeta25-35 treatment for 24 h directly gave rise to cell apoptosis (34.2 \pm 4.23%) compared with the control group (2.7 \pm 0.32%). Pretreated with Cy-3G could revise the effect to a certain extent, while 10 and 50 µM of Cy-3G effectively

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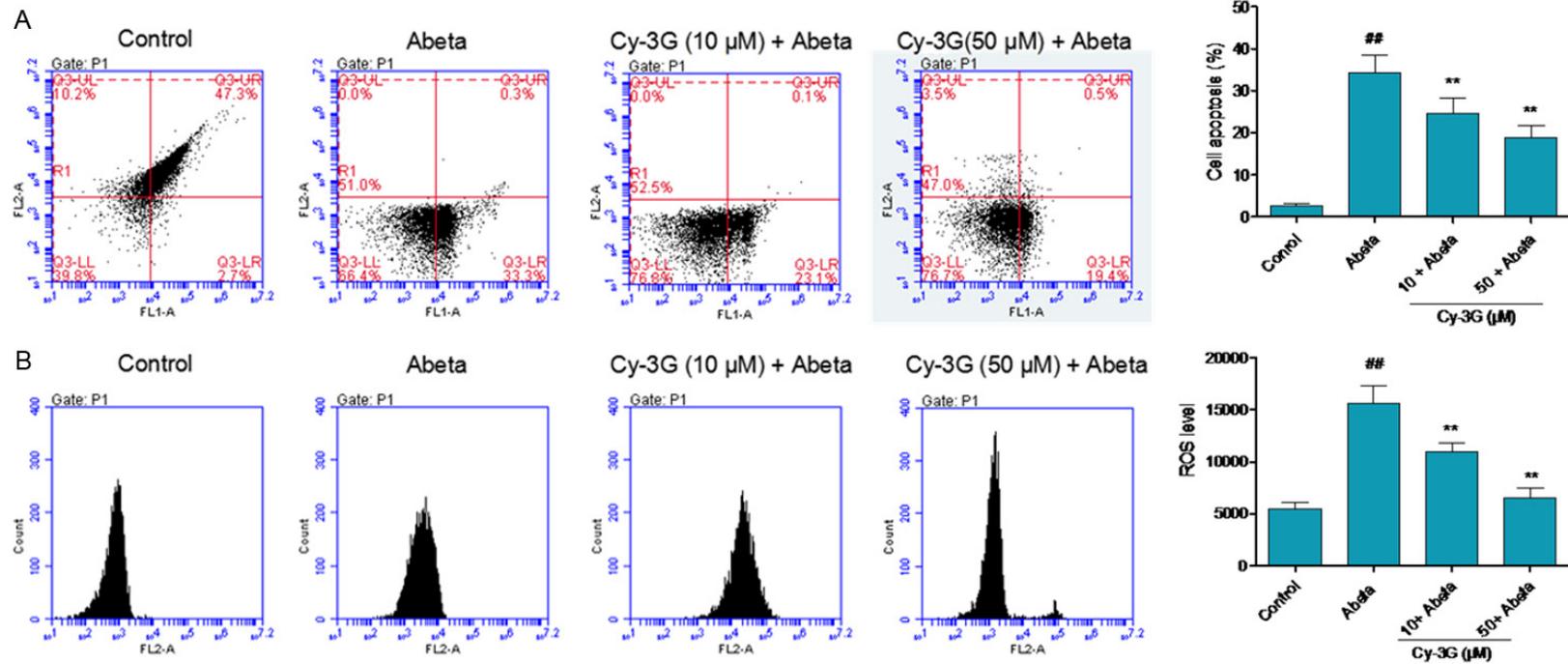


Figure 2. Protective effect of Cy-3G against Abeta25-35-induced apoptosis. A. PC12 cells treated with different dose of Cy-3G (10 and 50 μ M) for 2 h, then exposed to Abeta25-35 (50 μ M) for 24 h. Annexin V assay was used for apoptosis detection. B. FCM was used for ROS detection. Data were presented as mean \pm SD, n = 6, ^{##}*P* < 0.01, versus control, ^{**}*P* < 0.01 versus Abeta25-35 treated PC12 cells.

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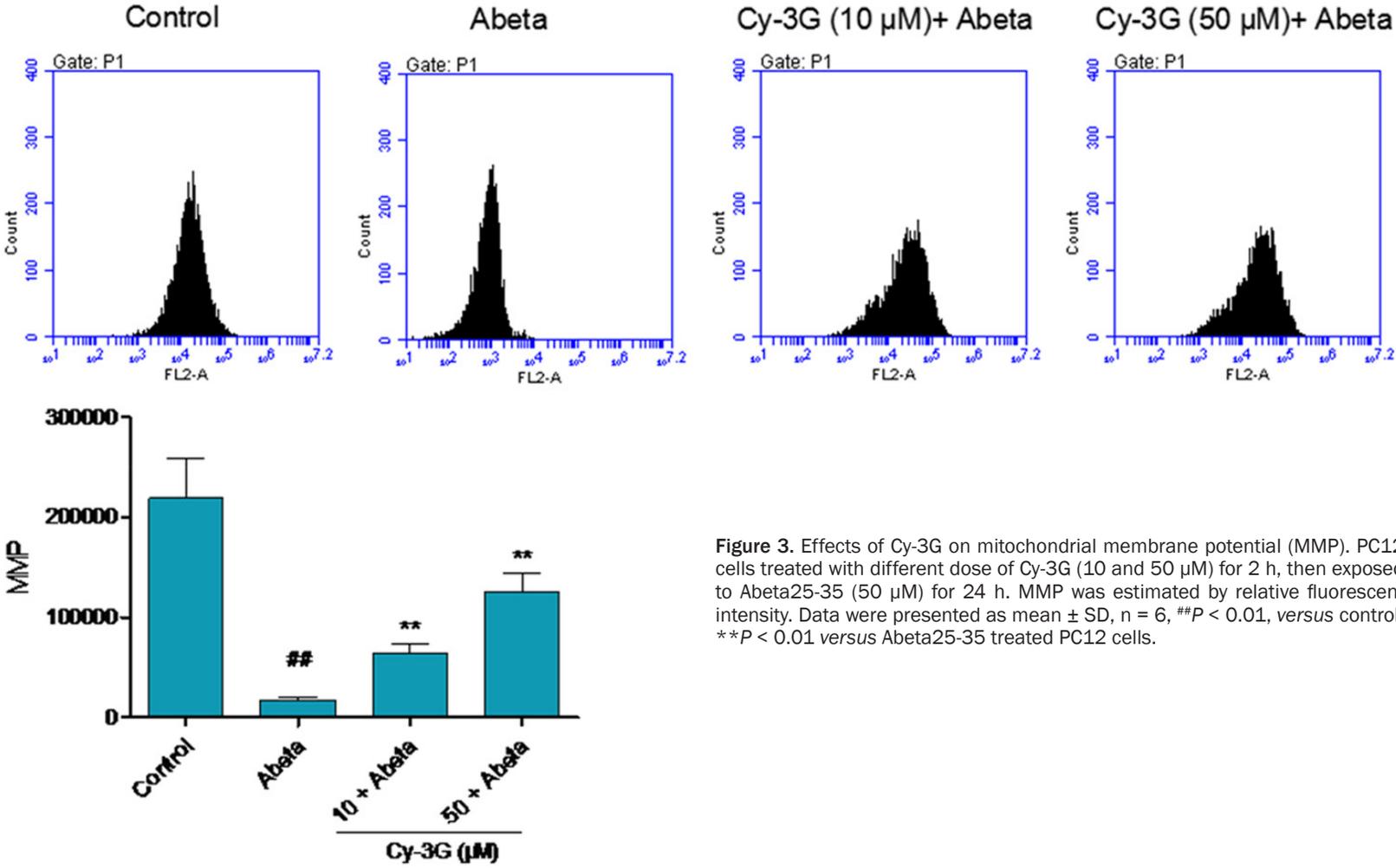


Figure 3. Effects of Cy-3G on mitochondrial membrane potential (MMP). PC12 cells treated with different dose of Cy-3G (10 and 50 μM) for 2 h, then exposed to Abeta25-35 (50 μM) for 24 h. MMP was estimated by relative fluorescent intensity. Data were presented as mean ± SD, n = 6, ##P < 0.01, versus control, **P < 0.01 versus Abeta25-35 treated PC12 cells.

Protective effect of Cy-3G on PC12 cells

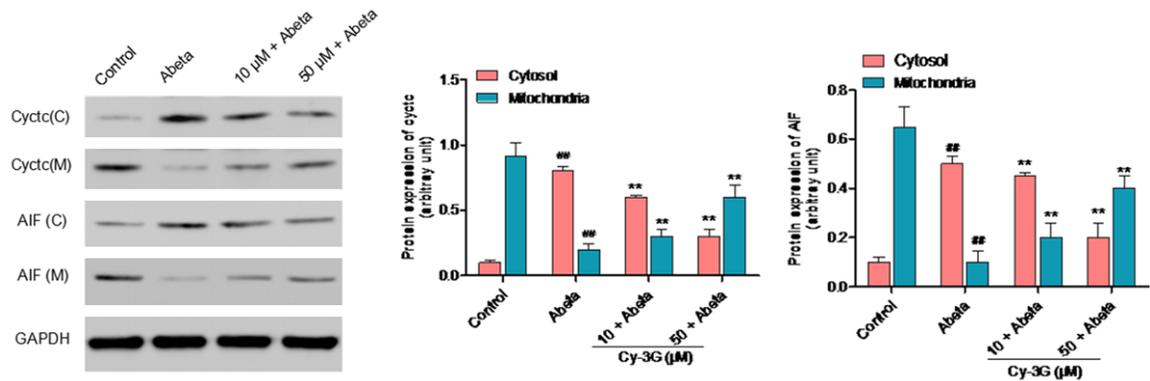


Figure 4. Effects of Cy-3G on the protein expression of cytochrome c and AIF. PC12 cells treated with different dose of Cy-3G (10 and 50 μM) for 2 h, then exposed to Abeta25-35 (50 μM) for 6 h. GAPDH were used as loading control. The cytosol and mitochondria of cells were prepared and the protein expression of cytochrome c and apoptosis-inducing factor (AIF) were analyzed by western blot, respectively. Data were presented as mean \pm SD, $n = 6$, $##P < 0.01$, versus control, $**P < 0.01$ versus Abeta25-35 treated PC12 cells.

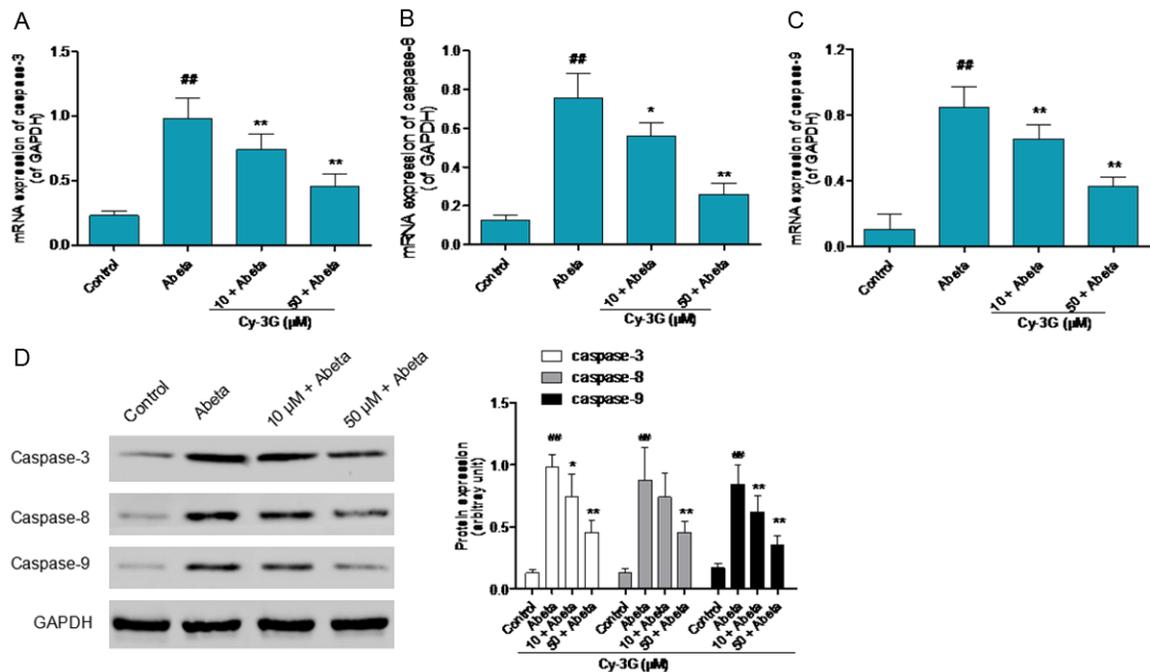


Figure 5. Effects of Cy-3G on caspase-3/-8/-9 expression. A-C. PC12 cells treated with different dose of Cy-3G (10 and 50 μM) for 2 h, then exposed to Abeta25-35 (50 μM) for 12 h. mRNA expression of caspase-3/-8/-9 was estimated by RT-PCR. D. PC12 cells treated with different dose of Cy-3G (10 and 50 μM) for 2 h, then exposed to Abeta25-35 (50 μM) for 24 h. Protein level of caspase-3/-8/-9 was identified by western blot. GAPDH were used as loading control. Data were presented as mean \pm SD, $n = 6$, $##P < 0.01$, versus control, $*P < 0.05$, $**P < 0.01$ versus Abeta25-35 treated PC12 cells.

reduced cell apoptosis to $24.5 \pm 3.65\%$ and $18.8 \pm 2.87\%$ respectively.

Overproduction of ROS in cells is the main cause of mitochondrial damage and cell apoptosis, and we identified the ROS level as previously described. In **Figure 2B**, ROS production in Abeta25-35 treated cells was significantly increased in comparison with the control group.

Cy-3G pretreatment with 10 and 50 μM effectively reduced the soaring ROS level in PC12 cells.

Effect of Cy-3G on the MMP of PC12 cells

Rhodamine 123 is a cell-permeant, cationic, green fluorescent dye that readily accumulates in the mitochondrial matrix due to charge and

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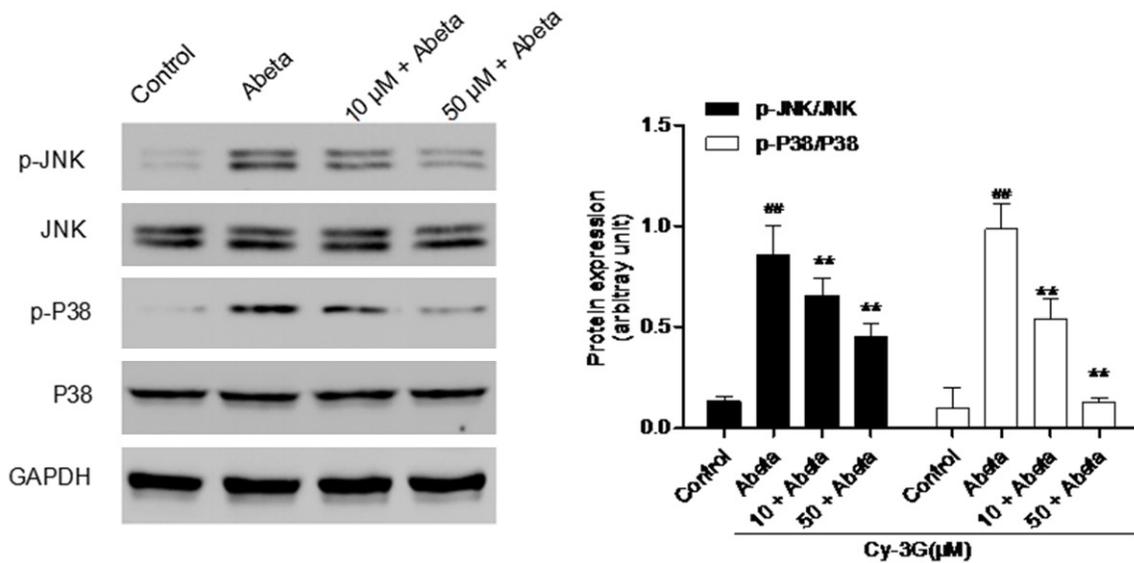


Figure 6. Effects of Cy-3G on phosphorylation of JNK and P38 MAPK signaling. PC12 cells treated with different dose of Cy-3G (10 and 50 μM) for 2 h, then exposed to Abeta25-35 (50 μM) for 6 h. Phosphorylation of JNK and P38 was evaluated by western blot analysis. GAPDH were used as loading control. Data were presented as mean \pm SD, n = 6, ^{##} $P < 0.01$, versus control, ^{**} $P < 0.01$ versus Abeta25-35 treated PC12 cells.

solubility in both the inner mitochondrial membrane and matrix space. The fluorescent intensity (FI) for rhodamine 123 is positively proportional to the mitochondrial membrane potential (MMP) [14]. The **Figure 3** showed that Abeta25-35 treatment for 24 h notably decreased the MMP compared with the control group, while Cy-3G pretreatment effectively increased the MMP.

Cy-3G suppressed the release of cytochrome c and AIF

Mitochondria are closely responsible with the cell intrinsic death pathway. MMP dissipation is the key event of mitochondria-dependent apoptosis, which is followed by the release of mitochondrial cytochrome c and AIF into cytosol. Release of cytochrome c and AIF were determined by western blot analysis. As a result, the expression of cytosol cytochrome c and AIF in displayed a higher level in the Abeta25-35 treatment cells compared with the untreated PC12 cells, but was remarkably decreased by Cy-3G treatment for 12 h (**Figure 4**).

Suppression of caspase-3/8/9 by Cy-3G

We further evaluated the mRNA expression of caspase-8/3/9 by FQRT-PCR assay after 12 h of Abeta25-35 treatment. Abeta25-35 increased the mRNA expression of caspase-8/3/9

compared with the untreated cells, while Cy-3G effectively reduced the expression of caspase-8/3/9 dose-dependently (**Figure 5A, 5B** and **5D**). We also identified the protein level of caspase-8/3/9 by western blot assay after 24 h of Abeta25-35 treatment. As shown in **Figure 5D**, Abeta25-35 increased the protein level of caspase-8/3/9 in comparison with the control group, while Cy-3G pretreatment significantly reduced the expression of caspase-8/3/9 in a dose-dependent manner.

Regulation on JNK and P38 MAPK signaling pathway

Cell apoptosis is closely associated with activation of JNK and P38 MAPK signaling pathways, which is characterized by increased protein phosphorylation of P38 MAPK and/or JNK. Therefore, we detected their phosphorylation levels by western blot analysis following 6 h of treatment with Abeta25-35. As shown in **Figure 6**, phosphorylation of JNK and P38 MAPK was facilitated by Abeta25-35. Cy-3G could remarkably decrease the phosphorylation of JNK and P38 MAPK.

Discussion

Although several in vitro studies have shown the ability of Cy-3G to counteract the neurotoxicity induced by H_2O_2 and Abeta1-42 peptides

in SH-SY5Y cells [20, 21]. In addition, Cy-3G also reduced the cerebral ischemia and age-related neuronal deficits in rats, suggesting the ability of Cy-3G to cross the blood brain barrier and to deliver its neuroprotective capabilities centrally.

In the present study, we investigated for the first time the anti-apoptosis activity of Cy-3G, a natural compound isolated from colored fruit and vegetables, on nervous PC12 cells, and elucidated the molecular mechanism involved. As a result, Cy-3G promoted cell viability significantly and in a time and dose dependent manner. Annexin V/PI staining displayed that Cy-3G pretreatment for 12 h decreased the apoptotic percentage and ROS level of Abeta25-35 treated cells in a dose-dependent manner. These results show that Cy-3G evidently decrease apoptosis in PC12 cells. It is generally recognized that the signal transduction of apoptosis in cells is closely associated with MAPKs pathways in response to extracellular stimuli, including small molecular compounds [15, 16]. The MAPK system is composed of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and P38 MAP kinase. ERK are widely expressed protein kinases (intracellular signaling molecules) involved in functions including the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells. JNK and P38 MAPK pathways can be activated by genotoxic agents and cytokine-mediating stress response, causing growth inhibition and apoptosis in cells. According to the evidence mentioned above, P38 MAPK and JNK pathways play a crucial role in apoptotic signal transduction of cells. Thus their phosphorylated proteins were detected by western blot assay in order to understand which signaling pathway(s) is/are activated. As a result, Cy-3G significantly decreased the phosphorylation levels of both P38 MAPK and JNK in Abeta25-35 treated cells, indicating simultaneous block of these two signaling pathways. Decreased protein expression of activated caspase-3/-8/-9 indicated that Cy-3G suppressed caspase-dependent apoptosis, which can be activated by the mitochondrial death pathway or/and the death receptor pathway. Mitochondria are active participants in programmed cell death [17]. The mitochondrial death pathway is frequently activated by ROS overproduction [18, 19], concomitantly with a

decrease in mitochondrial membrane potential (MMP) due to the loss of integrity in the outer mitochondrial membrane.

Activation of the intrinsic mitochondrial pathway can elicit the release of several apoptotic factors, such as cytochrome c and AIF [20]. Released cytochrome c facilitates the formation of the apoptosome containing adaptor Apaf-1 and another initiator caspase-9 in the presence of dATP, and subsequently caspase-3 is activated to result in cell apoptosis [21]. AIF can elicit cell apoptosis independent of activation of caspase 3 [14]. In the current study, we observed that Cy-3G remarkably inhibited the release of cytochrome c and AIF from mitochondria to cytosol, suppressed the protein expression of caspase-9, indicating that Cy-3G attenuated apoptosis of Abeta25-35 treated PC12 cells via the mitochondrial death pathway.

In summary, Cy-3G inhibited the protein phosphorylation levels of p38 MAPK and JNK, decreased the ROS level, and attenuated the release of cytochrome c and AIF from mitochondria, decreased caspase-9 and caspase-3, and thus protected PC12 cells against Abeta25-35 induced apoptosis. Cy-3G may be a good and useful natural drug for AD therapy.

Disclosure of conflict of interest

None.

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