

Cyanidin 3-O-glucopyranoside protects and rescues SH-SY5Y cells against amyloid-beta peptide-induced toxicity

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The amyloid- β (A β) peptide (1–42) aggregation into oligomeric and fibrillar species affects neuronal viability, having a causal role in the development of Alzheimer's disease. Among dietary anthocyanins, cyanidin 3-O-glucoside (Cy-3G) and its metabolites, such as protocatechuic acid (PA), have gained attention as potential neuroprotective agents. In this *in-vitro* study, we demonstrated that Cy-3G, but not PA, can inhibit A β _{1–42} spontaneous aggregation using thioflavin T fluorescence assay and transmission electron

microscopy. Furthermore, treatment of human neuronal SH-SY5Y cells with Cy-3G during oligomeric and fibrillar A β _{1–42} treatment prevents neuronal viability loss. These protective effects were still evident when Cy-3G treatment was initiated after the appearance of oligomeric A β _{1–42} neurotoxicity. Taken together, these results suggest that Cy-3G may protect and rescue the neuronal cells from toxicity induced by A β _{1–42}. *NeuroReport* 19:1483–1486 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: Alzheimer's disease, amyloid-beta peptide, cyanidin 3-O-glucoside, neuroprotection, protocatechuic acid

Introduction

The aggregation and deposition of amyloid- β (A β) peptide with the formation of senile plaque is considered to have a causal role in the development and progression of Alzheimer's disease (AD) [1]. The A β peptide is produced by the cleavage of the amyloid precursor protein, resulting in peptides predominantly of 40 (A β _{1–40}) or 42 (A β _{1–42}) amino acids [2]. The A β aggregation into insoluble amyloid fibrils occurs through a number of intermediate structural forms such as soluble oligomers or protofibrils. All these A β species may differentially affect neuronal function and viability *in vitro* and *in vivo* [3,4]. Common A β -neurotoxic mechanisms are membrane integrity loss, Ca⁺⁺ dyshomeostasis, reactive oxygen species (ROS) formation, and altered signaling and mitochondrial dysfunction [5]. Recent evidence suggests that soluble oligomeric A β is the main A β form that causes selective neuronal degeneration in the hippocampus and it may contribute to cognitive deficits associated with AD [6].

The main efforts for the development of AD therapies are focused on preventing A β production, aggregation, or downstream neurotoxic events [7]. Among diet components, cyanidin 3-O-glucoside (Cy-3G), an anthocyanin widely distributed among red/blue pigmented fruit and vegetables, has recently gained attention as a potential neuroprotective agent. Recent *in vivo* studies have shown that treatment with Cy-3G reduces cerebral ischemia and age-related neuronal deficits [8,9]. These findings suggest

that Cy-3G is able to cross the blood brain barrier and to counteract pathological events at the central nervous system level.

Owing to the high instability of Cy-3G at physiological pH and following its disappearance, the generated degradation products, such as protocatechuic acid (PA), however, may be responsible for the antioxidant activities and other physiological effects observed *in vivo* [10]. To date, there are no data available about the potential preventive role of Cy-3G in neurodegenerative diseases such as AD. In this *in-vitro* study, we evaluated the ability of Cy-3G and PA to inhibit A β _{1–42} spontaneous aggregation and oligomeric and fibrillar A β _{1–42}-induced toxicity in a human neuronal cell line (SH-SY5Y).

Methods

Chemicals

Cy-3G was purchased from Polyphenols AS Laboratories (Sandnes, Norway). PA, thioflavin T (Th-T) and A β _{1–42} peptide were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). All other reagents were of the highest grade of purity commercially available.

A β peptide preparation

A β _{1–42} peptides were first dissolved in hexafluoroisopropanol to 1 mg/ml, sonicated, incubated at room temperature for 24 h and lyophilized. The resulting unaggregated A β _{1–42}

film was dissolved with dimethylsulfoxide and stored at -20°C until use. The $\text{A}\beta_{1-42}$ aggregation to oligomeric and fibrillar forms were prepared as described previously by Maezawa *et al.* [11] and Dahlgren *et al.* [12], respectively. The morphology of oligomeric and fibrillar $\text{A}\beta_{1-42}$ forms obtained was checked by transmission electron microscopy (TEM).

Thioflavin T fluorescence assay

To determine amyloid fibril formation, the Th-T fluorescence method was performed, as described previously [13]. Briefly, a $40\text{ }\mu\text{M}$ $\text{A}\beta_{1-42}$ solution was incubated at 37°C for different times in phosphate-buffered saline (PBS) (pH 7.4), in the absence or presence of either Cy-3G or PA. After incubation, $50\text{ }\mu\text{l}$ of solution was added to 5 mM Th-T in a final volume of 1 ml of 50 mM glycine-NaOH buffer (pH 8.5). Fluorescence was monitored at excitation and emission wavelengths of 450 and 482 nm , respectively.

Transmission electron microscopy

To analyze the morphology of aggregated $\text{A}\beta_{1-42}$ forms, the TEM was used as reported previously [14]. Briefly, aggregated $\text{A}\beta_{1-42}$ solution ($40\text{ }\mu\text{M}$) was absorbed onto formvar-carbon coated grids (200 mesh size) for 40 min and stained with 2% aqueous phosphotungstic acid solution before viewing with a Philips CM10 transmission electron microscope at 80 kV (FEI Company, Eindhoven, The Netherlands).

Cell culture and neurotoxicity assay

Human neuronal-like SH-SY5Y cells were routinely grown at 37°C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and $50\text{ }\mu\text{g/ml}$ streptomycin. To determine the neuroprotective and neurorescue effects of Cy-3G and PA, SH-SY5Y cells were seeded in 96-well plates at 3×10^4 cells/well, whereas to evaluate apoptosis and necrosis, the cells were seeded in 60-mm cultures dishes at 4×10^6 cells/dish. All experiments were performed after 24 h of incubation at 37°C in 5% CO_2 . The neuronal viability in terms of mitochondrial metabolic function was evaluated by the reduction of MTT to formazan as described previously [10]. Briefly, after removal of the treatment, SH-SY5Y cells were washed with PBS and incubated with MTT (5 mg/ml) in PBS for 2 h at 37°C in 5% CO_2 . After further washing, the formazan crystals were dissolved with isopropanol and measured (570 nm , ref. 690 nm) with a spectrophotometer. The neuronal viability was expressed as a percentage of control cells.

To determine the neuronal apoptosis and necrosis, the Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions. The annexin V binds phosphatidylserine exposed on outside of the intact membrane of apoptotic cells, whereas propidium iodide penetrates the broken membrane of necrotic cells and binds to DNA. The number of stained cells was counted by fluorescence microscopy and the values are expressed as percentages of annexin V or propidium iodide-positive cells.

Statistical analysis

Data are reported as mean \pm SD of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance and the Dunnett's post-hoc test was used. Differences were considered significant at P value

of less than 0.05 . Analyses were performed using PRISM 3 software (GraphPad Software Inc., San Diego, California, USA) on a Windows platform.

Results

We first assessed whether the Cy-3G and PA can inhibit the $\text{A}\beta_{1-42}$ peptide aggregation by using the Th-T assay. As shown in Fig. 1a, for unaggregated $\text{A}\beta_{1-42}$ peptide alone the aggregation process started after 24 h of incubation at 37°C , as shown by the time-dependent increase in the Th-T fluorescence emission. $\text{A}\beta_{1-42}$ aggregation kinetics were significantly lower in the presence of $50\text{ }\mu\text{M}$ Cy-3G, but not PA, with a maximum inhibition of 100% at 72 h . Subsequently, TEM was used to confirm the inhibitory effects of Cy-3G on $\text{A}\beta_{1-42}$ fibril formation (Fig. 1b). The number of fibrils was markedly reduced and small amorphous aggregates were occasionally observed.

We then investigated the protective effects of Cy-3G and PA against oligomeric and fibrillar $\text{A}\beta_{1-42}$ -induced neurotoxicity in terms of cell viability loss in SH-SY5Y cells by using the MTT assay. In particular, the SH-SY5Y cells were treated with Cy-3G and PA ($25\text{--}200\text{ }\mu\text{M}$) either earlier to (4 h) or during the $\text{A}\beta$ -treatment for 3 h ($10\text{ }\mu\text{M}$).

Initially, experiments showed that in the absence of $\text{A}\beta$, treatment of SH-SY5Y cells with Cy-3G and PA concentrations up to $200\text{ }\mu\text{M}$ for 24 h did not affect the neuronal viability (data not shown). SH-SY5Y cell treatment with Cy-3G did not prevent the decrease in neuronal viability induced by oligomeric and fibrillar $\text{A}\beta_{1-42}$ (data not shown). By contrast, the toxicity induced by both $\text{A}\beta_{1-42}$ forms was significantly reduced, in a concentration-dependent manner, by a cotreatment with 100 and $200\text{ }\mu\text{M}$ of Cy-3G; a maximum inhibition of 73 and 86% was observed against oligomeric and fibrillar $\text{A}\beta_{1-42}$ -induced toxicity, respectively (Fig. 2). In both treatments, PA did not show any protective effects against either form of $\text{A}\beta_{1-42}$ -induced neurotoxicity (data not shown).

Furthermore, to determine the Cy-3G rescue effects in terms of ability to increase the neuronal viability after the appearance of $\text{A}\beta_{1-42}$ -induced toxicity, SH-SY5Y cells were treated with $10\text{ }\mu\text{M}$ of $\text{A}\beta_{1-42}$ for 3 h , after which Cy-3G was added for 21 h . As shown in Fig. 3a, Cy-3G treatment ($100\text{--}200\text{ }\mu\text{M}$) was also able to rescue SH-SY5Y cells against the toxicity induced by oligomeric $\text{A}\beta_{1-42}$, but not fibrillar $\text{A}\beta_{1-42}$. To confirm the observed neurorescue effects of Cy-3G, we also evaluated the neurotoxicity in terms of apoptosis and necrosis. SH-SY5Y cell treatment with Cy-3G at $200\text{ }\mu\text{M}$ after oligomeric $\text{A}\beta_{1-42}$ treatment showed an inhibitory effect on oligomeric $\text{A}\beta_{1-42}$ -induced apoptosis (33%) and necrosis (73%) (Fig. 3b).

Discussion

This study provides the first evidence that Cy-3G can inhibit $\text{A}\beta_{1-42}$ spontaneous aggregation and $\text{A}\beta$ -induced neurotoxicity in SH-SY5Y cells. The treatment of SH-SY5Y cells with Cy-3G during oligomeric and fibrillar $\text{A}\beta_{1-42}$ treatment prevents neuronal viability loss. Similar protective effects were recorded when Cy-3G treatment was initiated after the appearance of oligomeric $\text{A}\beta_{1-42}$ neurotoxicity.

Taken together, these findings suggest that Cy-3G can exert neuroprotective effects with the added bonus of having an extended therapeutic time window because of its ability to rescue the $\text{A}\beta_{1-42}$ -induced neuronal viability loss.

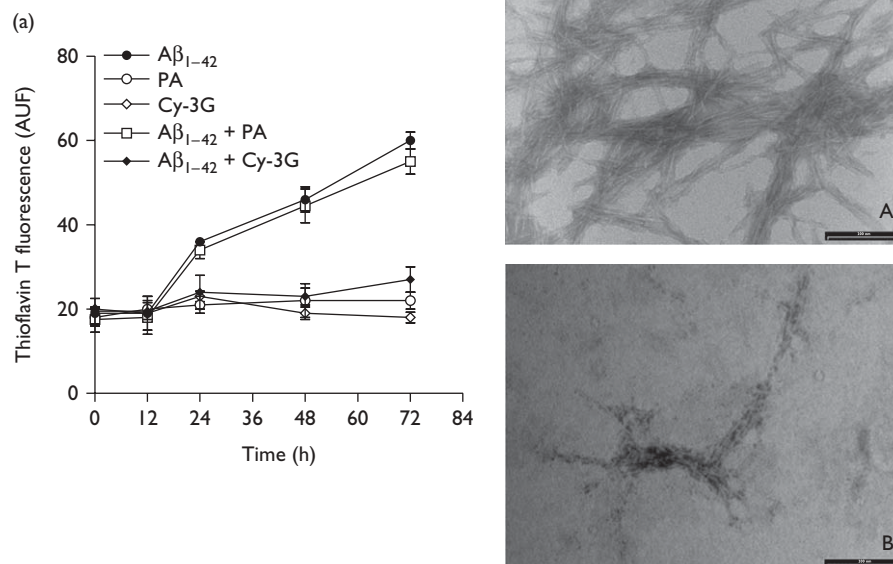


Fig. 1 Cyanidin 3-*O*-glucoside (Cy-3G), but not protocatechuic acid (PA), inhibits Aβ₁₋₄₂ spontaneous aggregation. (a) The solution of unaggregated Aβ₁₋₄₂ (40 μM) was incubated at 37°C in the absence or presence of either Cy-3G or PA (50 μM) for the indicated times. After incubation, the solutions were added to thioflavin T and the fluorescence was monitored. Values are reported as mean ± SD of three independent experiments. (b) Electron micrographs of unaggregated Aβ₁₋₄₂ (40 μM) incubated for 5 days at 37°C in the absence (A) or presence (B) of Cy-3G (50 μM). Magnification: × 46 000; scale bar=500 nm.

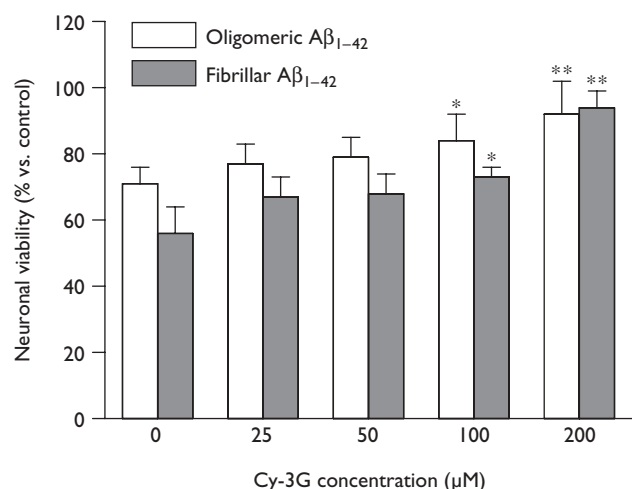


Fig. 2 Cyanidin 3-*O*-glucoside (Cy-3G) protects SH-SY5Y cells against oligomeric and fibrillar Aβ₁₋₄₂-induced toxicity. SH-SY5Y cells were treated with various concentrations of Cy-3G during oligomeric and fibrillar Aβ₁₋₄₂ treatment for 3 h (10 μM). Neuronal viability was determined by MTT assay and the results are expressed as percentage of control cells. Values are reported as mean ± SD of three independent experiments (**P* < 0.05, ***P* < 0.01 vs. untreated samples; analysis of variance with Dunnett's post-hoc test).

Regarding the Cy-3G antiaggregating effects, it is plausible that Cy-3G interacts directly with Aβ₁₋₄₂. In particular, it has a polyphenol structure composed of phenolic rings, which appear to be quite suitable for specific aromatic interactions with aromatic residues of Aβ₁₋₄₂ [15]. The aromatic residues, such as phenylalanine and tyrosine, are relatively abundant in amyloidogenic sequences and they are

critical for Aβ aggregation [16]. In addition, these interactions could also be reinforced by the H-bond between the -OH groups of Cy-3G and donor/acceptor groups of Aβ.

Further, the neuroprotective and neurorescue effects of Cy-3G could also be ascribed to its predominantly membrane incorporation [17] and its capacity to block the interaction of Aβ₁₋₄₂ with the neuronal plasma membrane. In this regard, recent studies have suggested that the soluble oligomeric Aβ₁₋₄₂ adheres to plasma and intracellular membranes and causes lesions by a combination of radical-induced lipid peroxidation and formation of ion-permeable pores, a cascade of toxic events leading to neuronal death [18]. We recently demonstrated that treatment of SH-SY5Y cells with Cy-3G, but not PA, inhibits oxidative stress-induced ROS formation at membrane level [10]. Therefore, it is plausible that Cy-3G prevents the oligomeric Aβ₁₋₄₂-induced neuronal membrane lipid peroxidation and destabilization. Recent studies have shown that merely altering the membrane lipid composition and fluidity protects neuronal cells from Aβ-mediated toxicity [19].

In contrast to Cy-3G, the lack of antiaggregating and neuroprotective effects of PA observed can be ascribed to its structural properties, such as phenol ring alone and low number of -OH groups that do not allow efficient interaction with Aβ [20], and to its inability to counteract oxidative damage at neuronal membrane level [10].

Finally, besides the antioxidant and structural properties of Cy-3G underlying its neuroprotective action, other cellular mechanisms may also be involved in the neurorescue effects of Cy-3G [21]. A recent in-vivo study demonstrated that supplementation of flavonoids, including the anthocyanin, could induce the main neuronal survival pathways such as phosphatidylinositol 3-kinase/AKT and extracellular signal-regulated kinase at brain level [22].

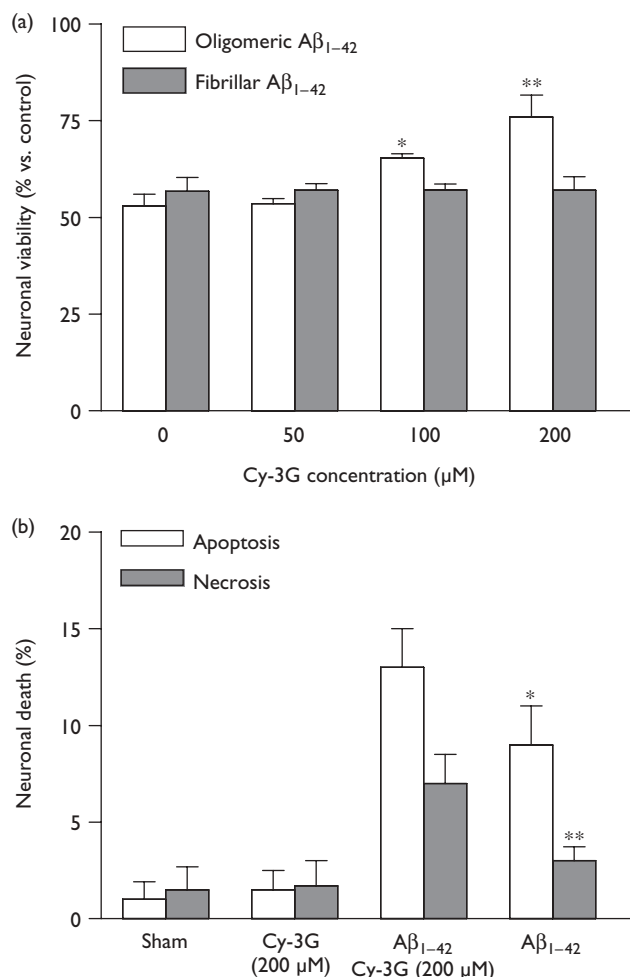


Fig. 3 Cyanidin 3-O-glucoside (Cy-3G) rescues SH-SY5Y cells against oligomeric Aβ₁₋₄₂-induced, but not fibrillar Aβ₁₋₄₂-induced toxicity. SH-SY5Y cells were treated with 10 μM of Aβ peptides for 3 h, after which Cy-3G was added at various concentrations for 21 h. (a) Neuronal viability was determined by MTT assay and the results are expressed as percentage of control cells; (b) neuronal death was determined with annexin V and propidium iodide and the results are expressed as percentage of apoptotic and necrotic cells. Values are reported as mean ± SD of three independent experiments (**P* < 0.05, ***P* < 0.01 vs. untreated samples; analysis of variance with Dunnett's post-hoc test).

Taken together, these findings are particularly relevant, as Cy-3G is able to quickly reach the central nervous system and localize in brain regions, such as the cortex and hippocampus, [8] usually impaired by soluble oligomeric Aβ in patients with AD.

Conclusion

In this study, we showed that Cy-3G can inhibit Aβ₁₋₄₂ spontaneous aggregation and Aβ₁₋₄₂-induced neurotoxicity in SH-SY5Y cells. Our findings suggest that Cy-3G and other anthocyanins may provide an effective therapeutic strategy for the treatment of AD.

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