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A new purified Lawsoniaside remodels amyloid-β_{42} fibrillation into a less toxic and non-amyloidogenic pathway

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ABSTRACT

Mounting evidence indicates soluble Aβ_{42} oligomers as the most toxic species causing neuronal death which leads to the onset and progression of Alzheimer disease (AD). Recently, it has been found that neurotoxic Aβ_{42} oligomers grow from monomeric species or arise following secondary nucleation by preformed mature fibrils. Thus, the use of natural compounds such as polyphenols to hinder the growth or to remodel Aβ_{42} fibrils is one of the most promising strategies for AD treatment. In our previous study, we showed that 1, 2, 4-trihydroxynaphthalene-2-O-β-D-glucopyranoside (THNG) inhibits Aβ_{42} aggregation during the early steps of the aggregation process, inhibits its conformational change to a β-sheet-rich structure, decreases its polymerization, inhibits its fibrillogenisis and reduces oxidative stress and aggregate cytotoxicity. Here, we used different spectroscopic and cell culture methods to check the effect of THNG on fibrils disaggregation. We showed that THNG binds to mature Aβ_{42} fibrils, rearrange their secondary structure, and remodels them into non-amyloid, less toxic, species by inhibiting their interaction with the plasma membrane. Our findings reveal that THNG is a good agent to remodel amyloid fibrils and could be used as a starting molecular scaffold to design new anti-AD drugs.

1. Introduction

Misfolding, aggregation and extracellular accumulation of Aβ_{42} in neuronal tissue is considered a crucial event in the pathogenesis of AD [1]. The aggregation of Aβ_{42} and other amyloidogenic peptides/proteins into highly ordered and organized β-sheet-rich fibril is considered as the main causative factor in disease onset and progression [2–6]. Many experimental evidences have been reported, showing that oligomeric species of Aβ_{42}, arising according to the seeding-nucleation model during the early steps of aggregation are the most toxic species [7,8].

Nowadays, great interest has been raised by the use of natural molecules especially those endowed with antioxidant power such as polyphenols, vitamins [4,5,9] due to their ability to inhibit amyloid aggregation. A part from antioxidant activity, which plays a crucial in role in protein/peptide protection against oxidation, natural antioxidants can interfere with different pathways involved in amyloid formation [5]. Up until now, several antioxidants have been shown to inhibit the aggregation process; these include Warafin [10], ascorbic acid [11], vitamin K3 [12], vitamin B12 [13], oleuropein aglycone [14–16], curcumin [17], EGCG [18] and others [9]. These molecules have been reported to interfere with the accepted mechanism of amyloid aggregation (primary nucleation) and to inhibit its associated cytotoxicity.

Recently, it has been found that toxic oligomeric species of Aβ_{42} arise predominantly via a secondary nucleation model catalyzed by fibrils

Abbreviations: Aβ_{42}, β-amyloid peptide$_{42}$; AD, Alzheimer's disease; BSA, bovine serum albumin; CD, circular dichroism; CTX-B, cholera toxin subunit B; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; GM1, monosialotetrahexosylganglioside 1; HFIP, hexafluoroisopropanol; MRE, mean residue ellipticity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEM, transmission electron microscopy; THNG, 1,2,4-trihydroxynaphthalene-2-O-β-D-glucopyranoside; ThT, Thioflavine-T.

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structure [19,20]. Therefore, the use of natural compounds to destabilize and convert β-sheet-rich fibrils could be a promising strategy to inhibit the secondary nucleation mechanism leading to the decrease of the most neurotoxic species. In our previous study, we have shown that methanol extract of *Lawsonia inermis* leaves inhibits $A\beta_{42}$ aggregation and reduces the cytotoxicity and oxidative stress mediated by $A\beta_{42}$ aggregates by inhibiting the interaction of the latter with the plasma memebrane [21]. We also purified from *Lawsonia inermis* leaves a new powerful inhibitor of the early steps of $A\beta_{42}$ aggregation with powerful anti-amyloidogenic effects. The new compound was characterized by HRMS and NMR (1D, 2D) and identified as Lawoniaside (1,2,4-trihydroxynaphthalene-2-O-β-D-glucopyranoside, THNG) [22]. Recently, we have shown that THNG reduces the extent of total aggregation, delays and modifies the conformational changes, polymerization and fibrillogenesis of $A\beta_{42}$. We have also reported that THNG reduces $A\beta_{42}$ aggregate cytotoxicity by inhibiting the increase of intracellular Ca^{2+} and ROS levels in exposed cells [23].

In this study, we carried out an in vitro biophysical analysis of $A\beta_{42}$ fibrils remodeling by THNG by using Thioflavine T (ThT) assay, dynamic light scattering (DLS), circular dichroism (CD) and transmission electron microscopy (TEM). Aggregate cytotoxicity and their interaction with the plasma membrane were also checked by MTT assay, confocal microscopy and FRET analysis.

2. Materials and methods

2.1. Reagents and cells

$A\beta_{42}$ was purchased from Bachem (Bubendorf, Switzerland). Formvar and carbon-coated 400 mesh nickel grids for electron microscopy (TEM) were obtained from Agar Scientific LTD. Thioflavine T (ThT) and other chemicals and supplements were from Sigma-Aldrich. Human neuroblastoma SH-SY5Y cells were from American Type Culture Collection (ATCC).

2.2. Purification of THNG

THNG was isolated from *Lawsonia inermis* leaves using a bio-guided fractionation and purification, as previously described [21]. The chemical structure of THNG was determined by High resolution Mass spectrometry (HRMS), NMR (1D, 2D) and thin layer chromatography (TLC), as previously described [21].

2.3. Preparation of $A\beta_{42}$ fibrils

The film of $A\beta_{42}$ was treated as previously described with minor modifications [24]. Peptide solutions were prepared by dissolving lyophilized $A\beta_{42}$ in 100% hexafluoroisopropanol (HFIP) to a 1.0 mM final concentration. After HFIP evaporation under vacuum, the samples were stored at −20 °C until use. A 500 μM alkaline solution of the monomeric peptide was obtained after sonication for 5 min in a CH_3CN/Na_2CO_3 (300 μM)/NaOH (250 mM) mixture (48.3/48.3/3.4: v/v/v), then, the solution was diluted to 100 μM with a H_2O/Na_2CO_3 (300 μM)/NaOH (250 mM) mixture (48.3/48.3/3.4: v/v/v). The working $A\beta_{42}$ aggregate solution (25 μM in all experiments, monomeric peptide concentration) was obtained by dilution in 10 mM phosphate buffer, pH 7.7, containing 11 mM NaCl.

2.4. ThT assay

$A\beta_{42}$ fibrillogenesis was evaluated by ThT assay, as previously described, with minor modifications [25]. Soluble $A\beta_{42}$ (25 μM) was incubated alone for 7 d at 37 °C. Then, 10 μl of THNG (75 μM) was added to preformed fibrils for 30 min or 3 h. At the end of the incubation, the samples ($A\beta_{42}$ pre-incubated for 7 d or $A\beta_{42}$ pre-incubated for 7 d and treated with 75 μM THNG for 30 min or 3 h) were mixed with 350 μl of 25 μM ThT in 25 mM K_2PO_4. Blanks were prepared similarly but without $A\beta_{42}$. Sample florescence was measured using a Perkin Elmer LS55 spectrofluorimeter equipped with a thermostated cell-holder attached to a Haake F8 water bath. Excitation and emission wavelengths were 440 nm and 485 nm, respectively. The fluorescence values were calculated by subtracting the fluorescence of the ThT blank. Percent inhibition of ThT binding was calculated using the following formula: $I\ (\%) = [(F_0 - F_1) / F_0] * 100$, where F_0 and F_1 are the florescence at 485 nm of untreated or THNG-treated $A\beta_{42}$ fibrils, respectively.

2.5. Dynamic light scattering

Size distribution analysis of $A\beta_{42}$ fibrils, untreated or treated with 75 μM THNG for 30 min or 3 h was carried out at 25 °C using a Malvern Zetasizer Nano S dynamic light scattering (DLS) device (Malvern, Worcestershire, UK) on 25 μM $A\beta_{42}$ samples. Each sample was analyzed considering the refraction index and viscosity of its dispersant. A 10 mm reduced volume plastic cell was used for readings.

2.6. Far-UV circular dichroism

The spectra of $A\beta_{42}$ fibrils untreated or treated with 75 μM THNG for 30 min or 3 h were recorded at 25 °C (0.5 nm data pitch, 200 nm/min speed, 2 s response) with 3 accumulations. CD measurements were made on 120 μl aliquots withdrawn from the reaction mixture using a 1.0 mm path length CD cuvette (Hellma). The spectra were acquired by using a JASCO J-810 spectropolarimeter equipped with a thermostated cell-holder attached to a thermo Haake C25P water bath.

2.7. Transmission electron microscopy analysis

3.0 μl samples of $A\beta_{42}$ fibrils untreated or treated with 75 μM THNG for 30 min or 3 h were spotted onto a Formvar and carbon-coated nickel grid and negatively stained with 1.0% (w/v) uranyl acetate. The grids were air-dried and examined using a JEM 1010 Transmission electron microscope at 50 kV excitation voltage.

2.8. Cell culture and MTT assay

Human neuroblastoma SH-SY5Y cells were grown in complete culture medium containing a DMEM/Ham's nutrient mixture F-12 (1:1) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich), glutamine and antibiotics (penicillin and streptomycin) and maintained at 37 °C under 5% CO_2.

Cell protection by THNG against $A\beta_{42}$ fibrils toxicity was determined by the MTT assay. Briefly, SH-SY5Y cells were seeded into 96-well culture plates at a density of 10^4 cells/well and allowed to attach for 24 h. $A\beta_{42}$ fibrils (25 μM) untreated or treated with 75 μM THNG for 30 min or 3 h were diluted in the culture medium containing 5.0% FCS and administered to the cells for 24 h. Then, the culture medium was removed and the MTT solution (0.5 mg/ml) was added to each well and allowed to incubate in the dark at 37 °C for 4 h. At the end of the incubation, the cells were lysed using DMSO (100%). The amount of formazan produced was determined by measuring the absorbance at 595 nm using a Microplate reader (Biorad).

2.9. Confocal immunofluorescence

SH-SY5Y cells grown on glass coverslips were incubated for 24 h with 25 μM $A\beta_{42}$ fibrils untreated or treated with 75 μM THNG for 30 min or 3 h and then washed with PBS. Cell membrane labeling was performed by incubating the cells with 10 ng/ml CTX-B Alexa488 (against GM1) in complete medium for 10 min at room temperature. Then, the cells were fixed in 2.0% buffered paraformaldehyde for 10 min and permeabilized by treatment with a 1:1 acetone/ethanol solution for 4.0 min at room temperature, washed with PBS and blocked