Inhibition of β-amyloid peptide aggregation and neurotoxicity by α-D-mannosylglycerate, a natural extremolyte

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1. Introduction

The aggregation of soluble β-amyloid (Aβ) peptide into oligomers/fibrils is one of the key pathological features in Alzheimer’s disease (AD). The use of naturally occurring small molecules for inhibiting protein aggregation has recently attracted many interests due to their effectiveness for treating protein folding diseases such as AD, Parkinson’s, Huntington’s disease, and other amyloidosis diseases. α-D-Mannosylglycerate (MG), a natural extremolyte identified in microorganisms growing under extremely high temperatures up to 100 °C, had been shown to protect proteins against various stress conditions such as heat, freezing, thawing, and drying. Here, we report the effectiveness of MG on the suppression of Alzheimer’s Aβ aggregation and neurotoxicity to human neuroblastoma cells. According to our study – carried out by using thioflavin-T induced fluorescence, atomic force microscopy, and cell viability assay – MG had significant inhibitory effect against Aβ amyloid formation and could reduce the toxicity of amyloid aggregates to human neuroblastoma cells while MG itself was innocuous to cells. On the other hand, the structural analogs of MG such as α-D-mannosylglyceramide, mannose, methylmannoside, glycerol, showed negligible effect on Aβ aggregate formation. The results suggest that MG could be a potential drug candidate for treating Alzheimer’s disease.

Abbreviations: AD, Alzheimer’s disease; MG, α-D-mannosylglycerate; Aβ, β-amyloid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ThT, thioflavin T; AFM, atomic force microscopy; DMSO, dimethyl sulfoxide.

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is highly accelerated under stressful conditions such as high temperature, low pH, and salt conditions [4,6,19,22,28].

Recently, the use of naturally occurring osmolytes or small molecule chaperones for inhibiting protein aggregation has attracted many interests due to their effectiveness for treating amyloidosis diseases [2,8,10,12,31]. In the present work, we investigated the effect of α-D-mannosylglycerate (MG) and its structural analogs on the inhibition of Alzheimer’s Aβ aggregate formation and neurotoxicity. MG is one of the rare, natural extremolytes recently identified in hyperthermophilic microorganisms growing under extremely high temperatures, such as Pyrococcus furiosus (T_{opt} = 100 °C) and Thermococcus celer.

![Chemical structures of α-D-mannosylglycerate and its structural analogs](image)

![ThT-induced fluorescence data](image)

**Fig. 1** – (A) Chemical structures of α-D-mannosylglycerate and its structural analogs studied in this work. (B) ThT-induced fluorescence data shown in bar graph for Aβ42 (25 μM) co-incubated for 72 h with α-D-mannosylglycerate and its analogs (100 mM). Error bars were calculated with 95% confidence intervals for triplet measurements.
Hyperthermophiles, most of which are found in volcanic areas and deep-sea hydrothermal vents, had been reported to naturally accumulate uncommon osmolytes called extremolytes [17,26]. Extremolytes are believed to contribute to the survival of hyperthermophiles against thermal stress and maintain the functionality of cellular macromolecules under extreme temperatures. According to previous reports [24–26], MG not only acts as a thermo-protectant but also stabilizes proteins against other stress conditions such as freezing, thawing, and drying.

Structure-based design approaches were hampered due to the lack of structural information on soluble or aggregated Aβ peptides [7]. In this study, MG’s structural analogs such as mannosylglyceramide, mannone, methylmannoside, and glycerol (Fig. 1A) were chosen to get an insight of the structural component that helps in the disruption of amyloid formation. We found that MG strongly inhibits amyloid formation of Aβ42 and its neurotoxicity in vitro while its analogs did not indicate significant inhibition. The results suggest the possibility of using extremolytes from hyperthermophiles as potential drug candidates for diseases related to amyloidogenesis.

2. Materials and methods

2.1. Materials

Human Aβ42 peptide was purchased from rPeptide Co. (Athens, GA, USA). α-D-Mannosylglycerate and α-D-mannosylglyceramide were provided by Bitop AG (Witten, Germany). All other chemicals and reagents were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Human neuroblastoma cells (SH-SY5Y) were kindly provided by Dr. Michael Sierks at Arizona State University.

2.2. Thioflavin T (ThT)-induced fluorescence measurement

ThT-induced fluorescence was used to monitor the increase in cross-β sheet content of the amyloid samples. Measurements were carried out in a Model RF5301 spectrofluorophotometer (Shimadzu Co., Japan) according to Levine [18]. Amyloid aggregates were obtained with 25 μM Aβ42 dissolved in 10 mM HCl with 2% dimethyl sulfoxide (DMSO) at 37 °C after 3 days’ incubation in sealed glass vials (1.8 ml) to prevent any possible evaporation during incubation. Samples of volume 5 μl were mixed with ThT solution (50 μM, 1.5 ml), and the fluorescence intensity was measured at room temperature with excitation and emission wavelengths of 450 and 482 nm, respectively. Each sample was tested independently in triplicate experiments, and the average was taken. Final readings were obtained by subtracting the ThT fluorescence and were normalized against the control Aβ sample.

2.3. Preparation of SH-SY5Y cell samples for cytotoxicity tests

SH-SY5Y cells were cultured in minimal essential medium (MEM) and Ham’s modification of F-12 (Irvine Scientific, Santa Ana, CA, USA) each constituting 40, 18% fetal bovine serum (FBS), 1% l-glutamine (3.6 mM), and 1% penicillin/streptomycin antibiotics (Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37 °C in a humidified incubator under 95% air and 5% CO₂. For the cytotoxicity assay, cells were incubated for 24 h to a density of 10⁶ cells/well in 6-well polystyrene plates. Amyloid seed samples (25 μM) without/with MG or mannosylglyceramide (25 μM, 1 mM, 10 mM, and 100 mM) were prepared by pre-incubation in a solution (10 mM HCl, 2% DMSO) for 24 h at 37 °C, followed by 10 times dilution with Tris–HCl buffer (pH 7.4, 20 mM). Then, each sample (10 μl) was added to the wells containing the cell culture (90 μl), resulting in a final concentrations of Aβ42 at 0.25 μM. Samples were further incubated for 48 h at 37 °C. The effect of MG or mannosylglyceramide alone on the cell viability was tested in the absence of Aβ42.
2.4. MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure the cell cytotoxicity. To each SH-SY5Y cell sample (100 µl) obtained above, 5 mg/ml MTT (10 µl) was added, and cell survival was determined according to Shearman et al. [29]. The cells were incubated with MTT for 3 h at 37 °C. The samples were then centrifuged to remove the medium, and 100 µl of 2-propanol containing 0.1N HCl was added to the wells to solubilize the formazan crystals. The absorbances of the samples were read using a Victor Wallac multiwell assay plate reader at 560 nm (Gaithersburg, MD). Each experiment was performed twice with each individual experiment having four replicate wells for each sample and control. Cell toxicity was calculated relative to control cells untreated with peptides and solutes. The error bars were calculated with a 95% confidence level. Statistical analysis was performed by means of one-way analysis of variance (ANOVA). A \( p \)-value less than or equal to 0.05 was considered statistically significant.

2.5. Atomic force microscopy (AFM)

Amyloid formation was visualized with Nanoscope III scanning probe workstation (Digital Instruments Inc., CA, USA) according to Stine et al. [30]. AFM probes were from (Veeco Metrology, LLC, Santa Barbara, CA) with resonant frequency of 306–444 kHz. The spring constant of the cantilever having the AFM tip was 12–103 N/m, and the length and the width of the cantilever were 140–180 and 48–52 µm. Samples of volume 5–10 µl were transferred onto freshly cleaved mica (Ted Pella Inc., Redding, CA) for 60 s at ambient conditions. Samples were then washed twice with 50 µl of deionized water and blow-dried with nitrogen gas. Images were obtained in tapping mode in air at a scan frequency of 1–2 Hz. Two different samples were imaged in each case with each image having a scan area of 5 µm \( \times \) 5 µm. Nanoscope software (Digital Instruments Inc.) was used to flatten the images, and the final data was presented in height mode. SPIP software (Image Metrology, Denmark) was used to observe the histogram profiles of each sample.

3. Results

The effect of MG and its structural analogs on Aβ aggregation was observed by ThT-induced fluorescence assay and AFM image analysis. ThT had been known to associate rapidly with amyloid aggregates, giving rise to a new emission maximum at around 482 nm [18]. We used ThT fluorescence assay to measure the extent of suppression of amyloid formation after 72 h incubation upon the addition of MG and its analogs (100 mM) to Aβ42 samples (25 µM), respectively. As shown in Fig. 1B, samples containing MG showed negligible fluorescence increase compared to control Aβ42 sample, suggesting high inhibition effect on amyloid formation. On the contrary, the samples added with the structural analogs exhibited high fluorescence, indicating no inhibition effect. Among the chemicals, we observed the concentration dependency of MG and \( \alpha\)-D-mannosylglyceramide as shown in Fig. 2. Samples containing 10 and 100 mM of MG, respectively, showed higher inhibitory effect compared to those with 25 µM and 1 mM of MG. In particular, the 100 mM concentration sample almost suppressed the amyloid formation and also prolonged the lag time for the formation of amyloid oligomers (Fig. 2A). In contrast, \( \alpha\)-D-mannosylglyceramide did not affect the

![Fig. 3 – AFM images of Aβ42 (25 µM) samples incubated with control, \( \alpha\)-mannosylglycerate, \( \alpha\)-mannosylglyceramide, mannose, glycerol and methylmannoside at 100 mM concentration. The images were taken at saturation phase of amyloid formation in tapping mode. Pictures represent a scan area of 5 µm \( \times \) 5 µm of each sample.](attachment:image.png)
ThT-induced fluorescence even at 100 mM concentrations (Fig. 2B). When we incubated a fresh Aβ42 solution added with potassium chloride salt up to 100 mM as a control experiment, we could not observe any effect of potassium chloride on amyloid aggregate formation (data not shown).

According to AFM analysis of each samples, control sample without any additive contained high density of typical unbranched, Aβ42 amyloid fibrils as shown in Fig. 3. The sample with MG contained few aggregates with no fibrils, while the samples with MG’s structural analogs had large amount of aggregates and a high content of fibers (Fig. 3). According to AFM histogram profile analysis, the sample with MG had the aggregates with the average height of 2.11 nm, while aggregates contained in the samples with α-α-mannosylglyceramide, mannose, methylmannoside, and glycerol had the average height of 6.31, 5.52, 5.11, and 4.34 nm, respectively. The ThT data and AFM images indicate the high inhibition effect of MG and the non-inhibitory effect of structural analogs.

In order to observe the effect of MG and α-α-mannosylglyceramide on the cytotoxicity of amyloid formation, we carried out MTT assay using human neuroblastoma cells (SH-SYSY). In accordance with ThT and AFM data, the samples with MG exhibited increased cell survival rate as shown in Fig. 4A, compared to the α-α-mannosylglyceramide samples where there were negligible changes in MTT activity compared to the

Fig. 4 – Dose–response of SH-SYSY cell death induced by Aβ42 (25 μM) seed samples with (A) α-α-mannosylglycerate and (B) α-α-mannosylglyceramide. The effect of MG or mannosylglyceramide alone on the cell viability was observed in the absence of Aβ42. Data shown as percentage of control values from two independent experiments with each experimental value being the average of four trials.
samples containing Aβ42 alone (Fig. 4B). It is noteworthy that MG itself exhibited no toxicity to the cells even at 100 nM concentrations. Those data indicate that MG can suppress the Aβ42-induced toxicity, which implies the possibility of using MG as a drug candidate for treating AD. Considering that physiological Aβ concentration in human cerebral cortex is in the range of several nM [27], the amount of MG needed for therapeutic purpose will be in much lower concentrations than those used in this work. It needs to be seen in future whether MG can efficiently penetrate the blood brain barrier in order to use MG for treating Alzheimer’s disease patients.

4. Discussion

According to our results, the inhibition effect of MG compared to its structural analogs may be due to its carboxyl group in the ring, as that is the only specific group lacking in the analogs that have had shown no inhibitory effect (Fig. 1A). α-D-Mannosylglyceramide has a chemical structure very close to MG except for an amide group instead of a carboxyl group in the ring branch, but did not suppress Aβ42 aggregation and cytotoxicity to human neuroblastoma cells. Mannose and methylmannoside, which have ring structure similar to MG but have different side groups, did not exhibit any inhibition either. According to previous reports [1,16,17], protein stability could be enhanced by the addition of molecules such as extremolytes, amino acids, and polyols, and the solutes typically contain multiple hydroxyl and/or carboxyl group. It was proved that the stabilizing efficiency generally increases with the number of hydroxyl and/or carboxyl group due to the increased surface free energy, which results in more compact configuration of proteins (i.e. preferential hydration mechanism) [3,13]. Thus, the differential effects between MG and α-D-mannosylglyceramide may be originated from the increased surface free energy due to the carboxyl group on MG. Stabilization of protein by preferential hydration hypothesis is based on the free energy change upon transition of protein configuration. Preferential hydration hypothesis alone may not provide sufficient explanation for the inhibitory effect of MG on Aβ aggregation since Aβ peptide is natively unfolded [14]. According to Jarrett et al. [11], the aggregation of Aβ peptides into amyloid fibrils was driven by the hydrophobic interactions between highly apolar residues. Based on the observation, attempts had been made to inhibit Aβ fibrillation by covering the “hot spots” responsible for Aβ fibrillation. For example, Tjernberg et al. found that penta peptides such as KLVFF, which consist of central hydrophobic region (16–20) of Aβ, exhibits high affinity to Aβ and thus prevents the aggregation of Aβ by hampering the hydrophobic interactions [32]. Beside the hydrophobic forces, electrostatic interactions between residues also had a significant effect on the formation of Aβ aggregates according to previous studies performed by NMR and computational modeling [14,15,20]. In the present work, the differential inhibitory effect between MG and α-D-mannosylglyceramide was thought to be in part resulted from the differences in electrostatic interactions of MG and α-D-mannosylglyceramide with charged residues of Aβ. According to literatures [14,15,20], positively charged Lys(28) within Aβ peptide has a critical role in stabilizing the β-turn and thus inducing the growth of amyloid fibrils due to its intermolecular electrostatic interactions with a negatively charged Glu(22) or Asp(23). Based on the previous works and our results, we speculate that the electrostatic interaction between MG and Lys(28) may cause the inhibition of Aβ aggregation, and further verification of this proposed mechanism is awaited. To conclude, it is suggested that naturally occurring hyperthermophilic osmolytes, especially with structural functionality similar to MG, could be potential drug candidates or lead compounds against Aβ aggregation associated with Alzheimer’s disease.

REFERENCES