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Inhibitors of amyloid β-protein aggregation mediated by GM1-containing raft-like membranes

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Abstract

The aggregation (fibril formation) of amyloid β-protein (Aβ) is considered to be a crucial step in the etiology of Alzheimer's disease (AD). The inhibition of Aβ aggregation and/or decomposition of fibrils formed in aqueous solution by small compounds have been studied extensively for the prevention and treatment of AD. However, recent studies suggest that Aβ aggregation also occurs in lipid rafts mediated by a cluster of monosialoganglioside GM1. This study examined the effects of representative compounds on Aβ aggregation and fibril destabilization in the presence of GM1-containing raft-like liposomes. Among the compounds tested, nordihydroguaiaretic acid (NDGA), rifampicin (RIF), tannic acid (TA), and quercetin (QUE) showed strong fibrillization inhibitory activity. NDGA and RIF inhibited the binding of Aβ to GM1 liposomes by competitively binding to the membranes and/or direct interaction with Aβ in solution, thus at least partly preventing fibrils from forming. Coincubation of Aβ with NDGA, RIF, and QUE in the presence of GM1 liposomes resulted in elongate particles, whereas the presence of TA yielded protofibrillar structures. TA and RIF also destabilized fibrils. The most potent NDGA prevented Aβ-induced toxicity in PC12 cells by inhibiting Aβ accumulation. Furthermore, a comparison of the inhibitory effects of various compounds between aqueous-phase and GM1-mediated aggregation of Aβ suggested that the two aggregation processes are not identical.

Keywords: Alzheimer's disease; Amyloid β-protein; Lipid raft; Monosialoganglioside GM1; Fibril formation; Protein–lipid interaction

1. Introduction

The aggregation and deposition of amyloid β-protein (Aβ) is considered to be a crucial step in the etiology of Alzheimer's disease (AD) [1–3]. A number of studies have been carried out on the mechanisms of Aβ aggregation and the characterization of Aβ aggregates in aqueous solutions mimicking biological fluids [4–9]. Accumulating evidence suggests that the most neurotoxic species are soluble oligomers acting as intermediates during the formation of aggregates (fibrils) [10–15]. Therefore, prevention of Aβ aggregation and/or decomposition of existing oligomers and fibrils by small compounds are promising strategies for the prevention and treatment of AD. Various compounds including β-sheet-binding dyes [16,17], oligopeptides [18–24], polyphenols [25–30], antibiotics [27,31,32], and anti-inflammatory drugs [33–35] have been reported to show these properties and prevent Aβ neurotoxicity.

On the other hand, membranes are suggested to play an important role in Aβ aggregation. Yanagisawa et al. discovered monosialoganglioside GM1-bound Aβ in the brains of patients with AD and suggested that GM1-bound Aβ may act as a seed for Aβ polymerization [36,37]. Recently, lipid rafts from the frontal cortex and the temporal cortex of AD brains were found

Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer's disease; ASP, acetylsalicylic acid; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; EM, electron microscopy; GM1, monosialoganglioside GM1; GT1b, trisialoganglioside GT1b; IND, indomethacin; NDGA, nordihydroguaiaretic acid; NGF, nerve growth factor; PC, egg yolk L-α-phosphatidylcholine; PG, L-α-phosphatidyl-DL-glycerol enzymatically converted from PC; QUE, quercetin; RIF, rifampicin; RUT, rutin; SM, sphingomyelin; TA, tannic acid; TC, tetracycline hydrochloride; ThT, thioflovin-T

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to contain a higher concentration of GM1 compared to the age-matched control [38]. Gangliosides including GM1 [39–41] have been shown to accelerate the rate at which soluble Aβ forms amyloid fibrils in vitro. Our group pointed out that a cholesterol-dependent cluster of GM1 formed in raft-like membranes plays a crucial role in the binding of Aβ to membranes [42]. A toxic form of Aβ is generated during the aggregation with GM1-containing raft-like liposomes, and the presence of GM1-bound Aβ in AD brains was confirmed by immunohistochemical and immunoprecipitation studies [43]. These results suggest that the membrane-mediated aggregation is also a physiologically relevant aggregation process. Recently, our group found that Aβ fibrils formed with GM1-containing raft-like liposomes have secondary structures different from those of fibrils formed in aqueous solution [44]. Therefore, the screening of compounds that can inhibit the membrane-mediated Aβ aggregation is an important research subject.

In this study, the effects of representative inhibitors on fibril formation of Aβ-(1–40) and Aβ-(1–42) in the presence of GM1-containing raft-like liposomes were examined. The compounds screened (Fig. 1) include polyphenols (quercetin QUE, tannic acid TA, nordihydroguaiaretic acid NDGA, and rutin RUT), antibiotics (rifampicin RIF and tetracycline hydrochloride TC), and anti-inflammatory drugs (indomethacin IND and acetylsalicylic acid (aspirin) ASP). NDGA, RIF, TA, and QUE exhibited potent inhibitory activities. RIF and TA also effectively degraded preformed fibrils. Furthermore, NDGA protected PC12 cells from the toxicity of Aβ-(1–42) by inhibiting the binding of the protein to the cells. The mechanisms of the inhibitory effects of these compounds will be discussed.

2. Materials and methods

2.1. Seed-free Aβ

Human Aβ-(1–40) and Aβ-(1–42) (trifluoroacetic acid salt forms) were purchased from Peptide Institute (Minoh, Japan). The proteins were dissolved in 0.02% ammonia on ice, and any large aggregates that may act as polymerization seeds were removed by ultracentrifugation in 500 μL polyallomer tubes at 100,000×*g*, 4 °C for 3 h [45]. The protein concentration of the supernatant was determined in triplicate by Micro BCA protein assay (Pierce, Rockford, IL). The supernatant, which contained essentially monomeric proteins [42,46], was collected and stored at −80 °C until used. Just before the experiment, the stock solution was thawed and mixed with an equal volume of double concentrated buffer (20 mM Tris/300 mM NaCl/2 mM EDTA, pH 7.4).

2.2. Liposomes

GM1, trisialoganglioside GT1b (GT1b), egg yolk L-α-phosphatidylcholine (PC), L-α-phosphatidyl-DL-glycerol enzymatically converted from PC (PG), and cholesterol were purchased from Sigma (St. Louis, MO). Bovine brain sphingomyelin (SM) was obtained from Matreya (Pleasant Gap, PA). Sonicated liposomes were prepared as follows. GM1, cholesterol, and SM dissolved in a chloroform–methanol 1:1 (v/v) mixture, chloroform, and ethanol, respectively were mixed at a molar ratio of 4:3:3 and the solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under vacuum overnight, was hydrated with buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4) and vortex mixed to produce multilamellar vesicles, which were subsequently sonicated under a nitrogen atmosphere for 9 min (3 min×3 times) using a probe-type sonicator. Metal debris from the titanium tip of the probe was removed by centrifugation. Large unilamellar liposomes were prepared by the extrusion method, as reported previously [42]. The concentrations of GM1, cholesterol, and phospholipids were determined at least in triplicate by the resorcinol-hydrochloric acid method [47], the cholesterol oxidase method (free cholesterol E-test kit by Wako (Osaka, Japan) [48], and the phosphorus assay [49], respectively. The actual lipid composition was confirmed to be very close to the expected value within a 10% error [50]. Therefore, only the GM1 concentration was routinely determined in the following experiments.

2.3. Fibril formation

Aβ-(1–40) (11 μM) or Aβ-(1–42) (10 μM) was incubated with GM1-containing liposomes (15 μM GM1) in the absence or presence of various compounds for 24 h at 37 °C. RUT was purchased from Wako, and the other compounds were obtained from Nakarai Tesque (Kyoto, Japan). QUE and RIF were dissolved in dimethylsulfoxide (DMSO). The stock solutions of NDGA, IM, and ASP were prepared with ethanol. RUT, TA, and TC were soluble in buffer. The final concentration of the organic solvents was 1 v/v%. Amyloid formation was estimated by the thioflovin-T (ThT) assay [6,51]. ThT was obtained from Aldrich (Milwaukee, WI). The sample (final Aβ concentration, 0.5 μM) was added to a 5 μM ThT solution in 50 mM glycine buffer (pH 8.5). Fluorescence at 490 nm was measured at an excitation wavelength of 446 nm on a Shimadzu RF-5300 spectrofluorometer with a cuvette holder thermostatted at 25 °C. Among the compounds tested, only RIF showed absorption in this wavelength region. Therefore, the measured fluorescence intensity was corrected for inner filter effects (<15%) [52]. Blank values in the absence of Aβ were subtracted to obtain fluorescence intensities due to Aβ fibrils. A calcein solution (1 μM) in 10 mM Tris/150 mM NaCl/1 mM EDTA (pH 7.4) buffer was used as a fluorescence standard.

2.4. Fibril destabilization

Aβ-(1–40) (11 μM) was incubated with GM1-containing liposomes (15 μM GM1) at 37 °C, while amyloid formation was monitored using the ThT assay. After 9 h, various compounds dissolved in DMSO were added at a final concentration of 150 μM (1 v/v% DMSO), and the monitoring of amyloid formation was continued. The same volume of DMSO was added as a control.

2.5. Electron microscopy (EM)

Reaction mixtures were spread on carbon-coated grids, negatively stained with 1% phosphotungstic acid, pH 7.0, and examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 75 kV.

2.6. Binding of Aβ to liposomes

Aβ-(1–40) (11 μM) was incubated with GM1-containing liposomes (15 μM GM1) in the presence or absence of various compounds (150 μM) for 10 min at 37 °C. The mixtures were ultracentrifuged at 650,000×*g* for 30 min. The free concentration of Aβ in the supernatant was determined with the Beta Amyloid 1–40 Biotrak ELISA System (Amersham Biosciences, Piscataway, NJ). The bound concentration was calculated by subtracting the free concentration from the total concentration.

2.7. Binding of inhibitors to liposomes

NDGA and RIF (150 μM) were incubated with GM1-containing liposomes (150 μM GM1) in the presence or absence of Aβ-(1–40) (11 μM) for 10 min at 37 °C. The mixtures were ultracentrifuged at 650,000×*g* for 30 min. For GT1b/cholesterol/SM (4:3:3), GM1/cholesterol/SM (4:3:3), SM/cholesterol (2:1), PG/PC (4:6), PC/cholesterol (2:1), or PC (500 μM) liposomes, large unilamellar vesicles and a longer ultracentrifugation time of 3 h were used, because sonicated vesicles of these lipids could not be sedimented. The free concentration of NDGA and RIF in the supernatant was determined by measuring optical density at 281 and 471 nm, respectively. The bound concentration was calculated by subtracting the free concentration from the total concentration.

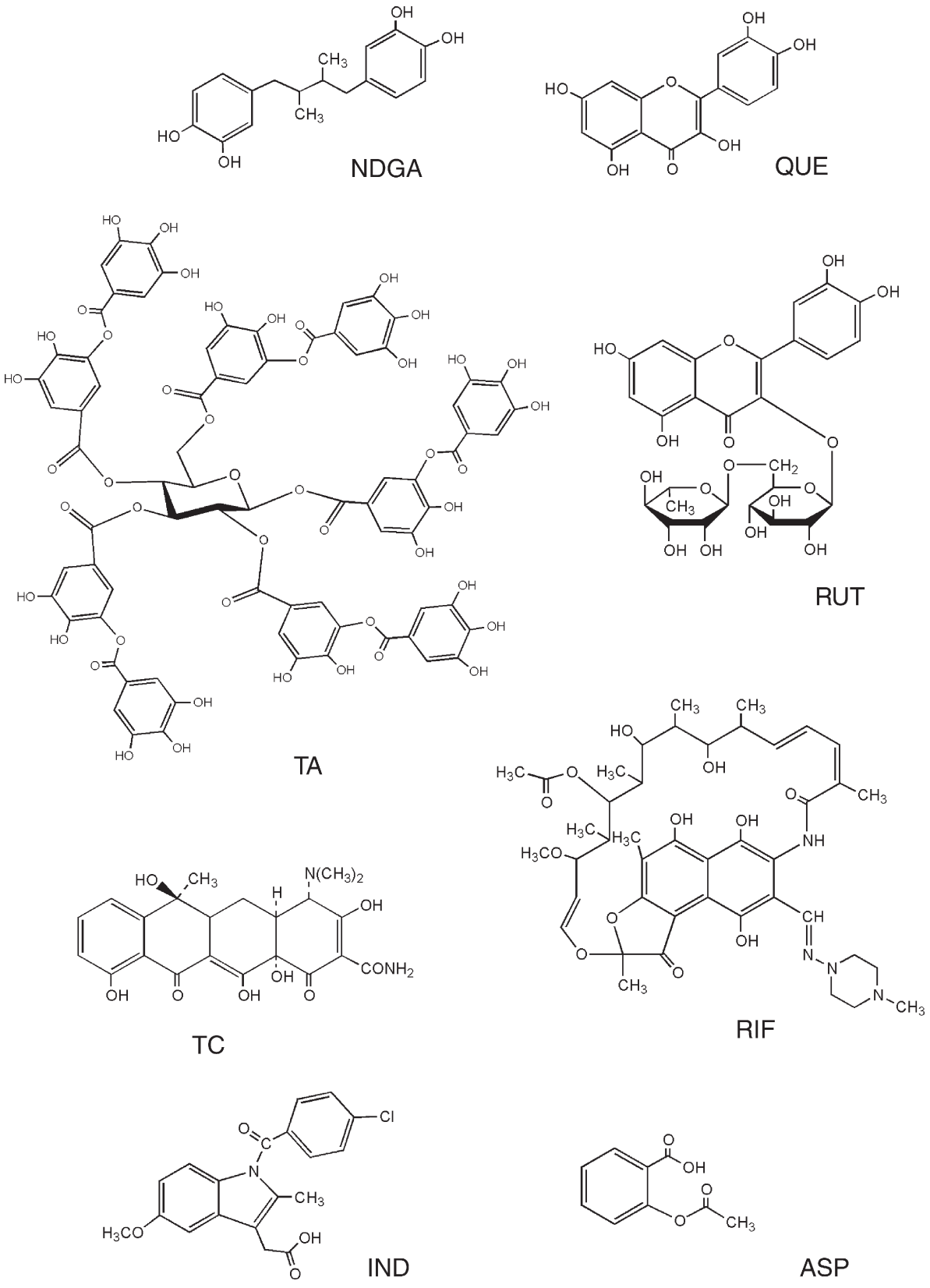

Fig. 1. Chemical structures of the compounds used in this study.

2.8. Cell culture and NGF-differentiation

Dulbecco's Modified Eagle's Medium (DMEM), horse serum, bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Rat pheochromocytoma PC12 cells were cultured in DMEM containing 5% horse serum, 10% bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C with 5% CO_2. After being plated at a density of 100,000 cells onto a poly-D-lysine-coated 35-mm glass bottom dish, cells were

incubated for 24 h at 37 °C. The cells were differentiated with 50 ng/ml of nerve growth factor (NGF; Alomone, Jerusalem, Israel) in serum-free DMEM for 6–7 days.

2.9. Congo red staining

NGF-differentiated PC12 cells were incubated with 10 μM Aβ-(1–42) (Peptide Institute) with or without 10 μM NDGA in serum-free DMEM containing 50 ng/ml NGF for 24 h at 37 °C with 5% CO_2, and then rinsed twice with Tris-buffered saline (TBS, 150 mM NaCl and 10 mM Tris, pH 7.4). Accumulated Aβ amyloids on the cell membranes were stained with 20 μM Congo red (Nacalai Tesque, Kyoto, Japan) in TBS for 30 min at room temperature [53], and then rinsed twice with TBS. These cells were visualized using the 63× C-Apochromat objective of a Zeiss LSM 510 confocal laser scanning microscope.

2.10. Live/dead assay

NGF-differentiated PC12 cells were incubated with 10 μM Aβ-(1–42) with or without 10 μM NDGA, RIF, or TA in serum-free DMEM containing 50 ng/ml NGF for 24 h at 37 °C with 5% CO_2, and then rinsed twice with TBS. Cells were stained with 0.4 μM calcein AM and 0.4 μM EthiD-1 (Molecular Probes, Eugene, OR) in TBS for 30 min at room temperature, and then rinsed twice with TBS. The cells were visualized using the 20× Plan Apochromat objective. Calcein AM- and EthD-1-positive cells represented live and dead cells respectively.

3. Results

3.1. GM1-mediated versus aqueous-phase aggregation

The effects of various compounds on Aβ amyloid formation in the presence and absence of GM1-containing liposomes were compared. Aβ-(1–40) showed maximal ThT fluorescence intensity after a 24 h incubation at 37 °C in the presence of liposomes, whereas no ThT fluorescence was observed in its absence (data not shown), indicating GM1-bound Aβ acts as a seed for Aβ fibril formation, as reported previously [41]. The compounds screened exhibited inhibitory activities in the rank order NDGA > RIF > QUE ≈ TA > TC ≥ IND > RUT ≈ ASP (no effect) at a compound-to-protein ratio of 14 (Fig. 2A, filled bars). The effects of more potent compounds (NDGA, RIF, TA and QUE) on the aggregation of more fibrillogenic and toxic Aβ-(1–42) were also examined. These compounds also inhibited the increase in ThT fluorescence in the presence of GM1-containing liposomes (Fig. 2B). NDGA was again most potent. The inhibitory effects of these compounds on fibril formation were confirmed by EM. In the absence of any inhibitors, Aβ-(1–40) formed typical nonbranched fibrils (Fig. 3A). The presence of the most effective agent NDGA almost completely inhibited fibril formation, in accordance with the ThT assay. Small elongate particles were observed with no fibrillar structure (Fig. 3B). RIF and QUE exhibited similar effects (data not shown). The concentration dependence of each potent inhibitor is shown in Fig. 4. The 50% inhibitory compound-to-Aβ ratios were ≤0.8, ≤0.9, 1.3, and 5.5 for NDGA, TA, RIF, and QUE, respectively. TA showed a strange profile. ThT fluorescence was reduced to 20% of the control value at a ratio as low as 1.4, whereas no additional effect was observed at higher ratios. EM results showed the formation of protofibril-like structures (Fig. 3C).

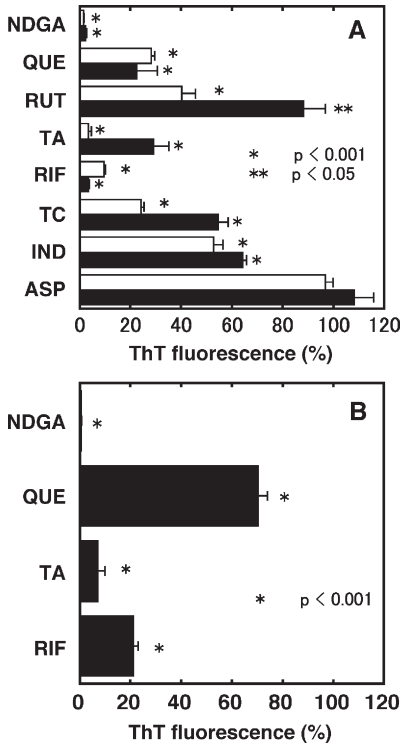

Fig. 2. Effects of various compounds on Aβ fibril formation. (A) Aβ-(1–40) (11 μM) or (B) Aβ-(1–42) (10 μM) was incubated in buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4) with various compounds (150 μM) for 24 h at 37 °C in the presence of GM1/cholesterol/SM (4:3:3) liposomes (15 μM as GM1) (filled bars) or 0.3 μM fibrils preformed in buffer (open bars) as a seed, respectively. Fibril formation was estimated by ThT assay, and the fluorescence intensity in the absence of any compounds was set to 100% (n=5).

The effects of the compounds on Aβ-(1–40) fibril formation in aqueous phase were also examined. A trace amount of preformed fibrils was added to facilitate fibril formation [41]. The rank order of the inhibitory effect was NDGA > TA > RIF > TC ≈ QUE > IND ≈ RUT > ASP (Fig. 2A, open bars), in general agreement with previous studies: NDGA and TA possess stronger activities than RIF, TC, and QUE [27,28]. RIF was more potent for GM1-mediated aggregation, whereas RUT, TA and TC were more effective for fibril formation in buffer.

3.2. Effects of compounds on binding of Aβ to liposomes

To obtain insights into the mechanism of prevention of GM1-mediated fibrillization, we determined the effects of NDGA, RIF, QUE, and TA on the binding of Aβ-(1–40) to GM1-containing liposomes. In the absence of any inhibitors, about 60% of Aβ was bound to the liposomes under the conditions examined (Fig. 5). The presence of NDGA and RIF significantly reduced the binding to the membranes. In contrast, QUE and TA did not affect the binding.

3.3. Binding of inhibitors to liposomes

The above results suggest that NDGA and RIF may bind to GM1-liposomes, covering the binding sites for Aβ. Therefore, the binding of these compounds to the liposomes was

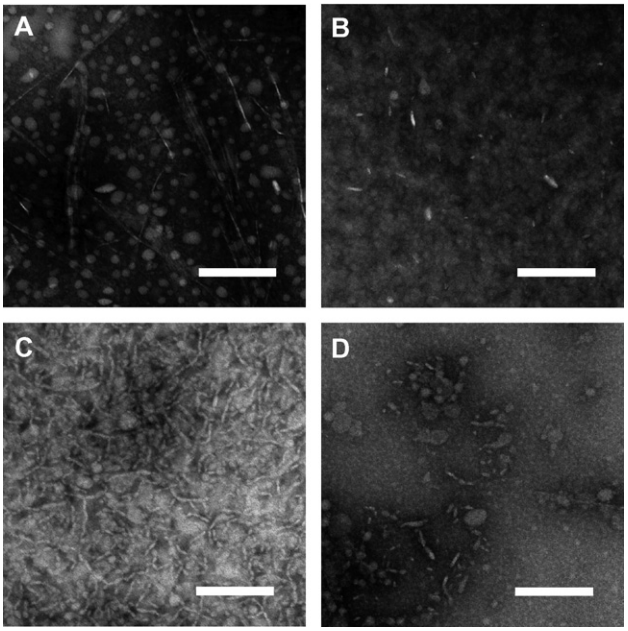

Fig. 3. Negative stain transmission EM. Aβ-(1–40) (11 μM) was incubated in buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4) with various compounds (150 μM) for 18 h at 37 °C in the presence of GM1-containing liposomes (15 μM as GM1). (A) No compound (control). (B) With NDGA. (C) With TA. The fibril destabilization by TA is shown in D. Aβ was incubated in the buffer with the GM1-containing liposomes for 18 h at 37 °C. TA was subsequently added, and the incubation was continued for an additional 24 h. Liposomes (20–50 nm) are seen in all micrograms. Bars represent 200 nm.

examined (Fig. 6A). Approximately 50% of NDGA and 20% of RIF were bound to the membranes, respectively. The coexistence of Aβ-(1–40) competitively inhibited the binding of these compounds.

To get insights into the mechanism of membrane binding of these compounds, the binding to liposomes of various lipid compositions was investigated (Fig. 6B). NDGA (filled bars) bound to zwitterionic PC more strongly than to GM1-liposomes, whereas RIF (open bars) showed little affinity for PC, SM/cholesterol and negatively charged PG/PC bilayers. The weak affinity for these membranes was not due to larger liposome

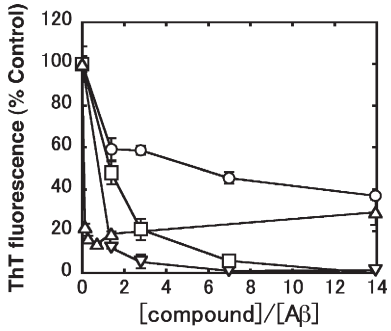

Fig. 4. The concentration dependence of fibrillation inhibitory effects. Fibril formation experiments were performed as in Fig. 2A at different compound-to-protein ratios in the presence of GM1 liposomes ($n=5$). The Aβ-(1–40) concentration was kept at 11 μM. Compounds: ○, QUE; □, RIF; △, TA; ▽, NDGA.

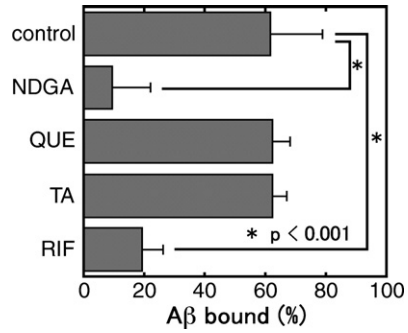

Fig. 5. Inhibition of Aβ binding to GM1 liposomes by various compounds. Aβ-(1–40) (11 μM) was incubated in buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4) with various compounds (150 μM) for 10 min at 37 °C in the presence of GM1-containing liposomes (150 μM as GM1). After ultracentrifugation of the mixtures, the free concentration of Aβ in the supernatant was determined by ELISA ($n=6$).

sizes. RIF was incubated with smaller sonicated vesicles, and the mixtures were separated by gel filtration. RIF was not detected in the vesicle fractions (data not shown). In contrast, RIF also tightly bound to GT1b-containing raft-like membranes (Fig. 6B).

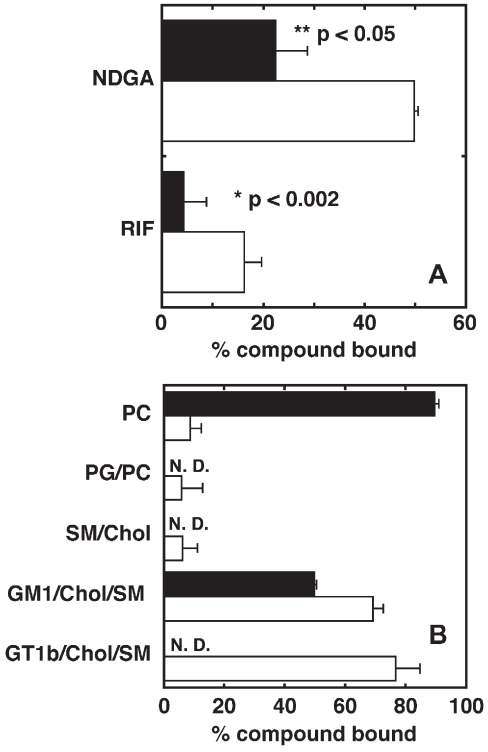

Fig. 6. Competitive binding of Aβ and compounds to GM1 liposomes. (A) NDGA or RIF (150 μM) was incubated in buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4) with GM1-containing liposomes (150 μM as GM1) for 10 min at 37 °C in the presence (filled bars) or absence (open bars) of 11 μM Aβ-(1–40). After ultracentrifugation of the mixtures, the free concentration of each compound in the supernatant was determined by optical density ($n=2$ for NDGA and $n=4$ for RIF). (B) NDGA (filled bars) or RIF (open bars) (150 μM) was incubated in the buffer with 375 μM GT1b/cholesterol/SM (4:3:3), GM1/cholesterol/SM (4:3:3), SM/cholesterol (2:1), PG/PC (4:6), PC/cholesterol (2:1), or PC (500 μM) liposomes for 10 min at 37 °C. The binding of the compounds to the liposomes was determined as (A). N. D.: not determined.

3.4. Fibril destabilization

If these compounds also possess the ability to destabilize existing fibrils, it would be beneficial for therapeutic purposes. Aβ was incubated with GM1-liposomes, while ThT fluorescence was monitored (Fig. 7). After a 7 h lag, the fluorescence intensity abruptly increased, reaching a maximal value within 3 h. The addition of compounds at 9 h, where the ThT value reached 40% of the maximal value, reduced the fluorescence, indicating the possibility that these compounds destabilize fibrils. The rank order of effectiveness was NDGA > TA > RIF > QUE. After a 24 h coincubation, the mixtures were examined by EM. The RIF-treated sample contained elongate particules similar to Fig. 3B (data not shown). The addition of TA yielded short sheared fibrils and small aggregates (Fig. 3D). Despite almost the nullification of ThT fluorescence, the NDGA-treated specimen contained a lot of fibrils similar to those in Fig. 3A (data not shown).

3.5. Cell study

The protective effects of the potent inhibitors NDGA, TA and RIF on Aβ-induced cytotoxicity were examined by use of NGF-differentiated PC12 cells as a neuronal model. More toxic Aβ-(1–42) was used for this purpose instead of Aβ-(1–40). Aβ-(1–42) (10 μM) was incubated with cells in the absence or presence of 10 μM inhibitors for 24 h at 37 °C. The cell viability was determined by the live/dead assay (Fig. 8A). The most potent inhibitor, NDGA, significantly prevented Aβ-induced cytotoxicity, whereas RIF had no inhibitory effect. The incubation with TA alone without Aβ changed the cell morphology (Fig. 8B), indicating cytotoxicity, which was not reflected by cell viability (data not shown).

To confirm that NDGA protected cells from Aβ by blocking the binding of the protein to the cells, the cellular accumulation of Aβ-(1–42) was visualized with the amyloid-specific dye Congo red [53]. We have recently shown that this dye can be utilized to stain amyloids of native Aβ-(1–42) formed on living cell membranes (Wakabayashi, M. and Matsuzaki, K., unpublished work). In the absence of NDGA, cells were stained by the dye (Fig. 9A), suggesting Aβ accumulated and formed

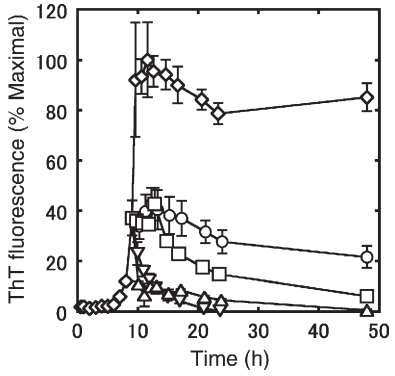

Fig. 7. Fibril destabilization by various compounds. Aβ-(1–40) (11 μM) was incubated in buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4) with GM1-containing liposomes (15 μM GM1) at 37 °C, while amyloid formation was monitored with the ThT assay. After 9 h, various compounds dissolved in DMSO were added at a final concentration of 150 μM (1 v/v% DMSO), and amyloid formation was continued to be monitored ($n=5$). The same volume of DMSO was added as a control. Compounds: ◇, none (control); ○, QUE; □, RIF; △, TA; ▽, NDGA.

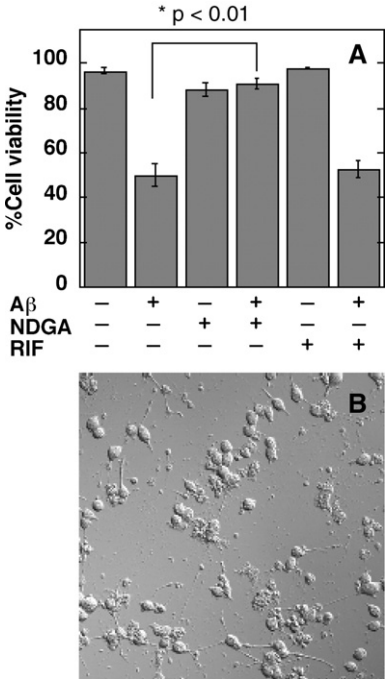

Fig. 8. Cytotoxicity. Inhibitors (10 μM) were incubated with NGF-differentiated PC12 cells in the absence or presence of 10 μM Aβ-(1–42) for 24 h at 37 °C. (A) Cytotoxicity was determined by the live/dead assay ($n=3$). (B) A DIC image in the presence of TA only without the protein.

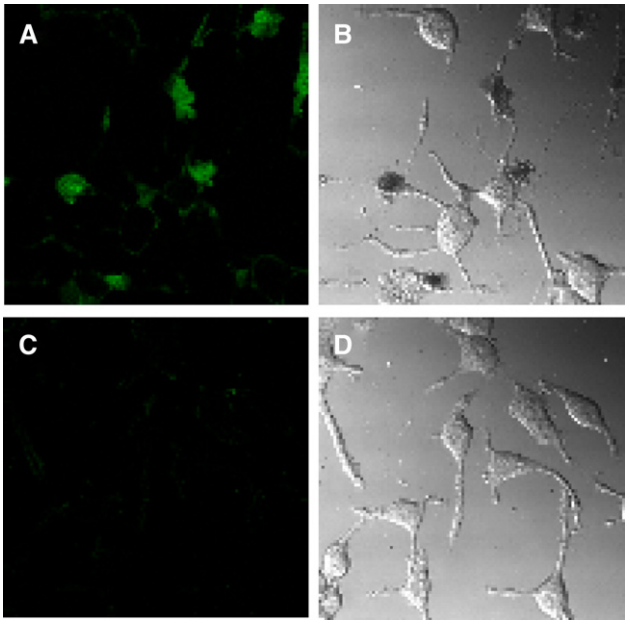

Fig. 9. Inhibition of Aβ accumulation in PC12 cells by NDGA. Aβ-(1–42) (10 μM) was incubated with NGF-differentiated PC12 cells in the absence (A and B) or presence (C and D) of 10 μM NDGA for 24 h at 37 °C. Amyloids were stained by Congo red (A and C). B and D show DIC images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amyloids on the cells. The coincubation with equimolar NDGA almost completely inhibited amyloid formation (Fig. 9C).

4. Discussion

4.1. GM1-mediated versus aqueous-phase aggregation

This study examined for the first time the effects of various compounds on Aβ fibril formation mediated by GM1. The present data suggest that Aβ fibrils formed in GM1-containing membranes are not identical to fibrils formed in aqueous solution, in accordance with our previous infrared spectroscopic study [44]. The order of fibrillization inhibitory effects of various compounds was different in both cases (Fig. 2A). Notably, TA, which is known to be one of the most potent inhibitors of aqueous-phase fibril formation of Aβ [27], was less effective for membrane-phase aggregation. In solution, the addition of an equimolar amount of TA completely inhibited the ThT fluorescence increase, and EM showed that TA destabilizes seeds of fibrillar Aβ [27]. In contrast, the maximal inhibition of ThT fluorescence development by TA was ~80% in the presence of GM1 liposomes (Fig. 4), and protofibrillar structures were generated (Fig. 3C). ThT molecules bound to the structures appear to be weakly fluorescent. In supporting this interpretation, we observed that fluorescence intensity of ThT bound to protofibrils of Aβ-(1–40) formed in solution was also 4-fold weaker than that bound to mature fibrils (Naiki et al., unpublished observation).

The fibril destabilization study also supports the above conclusion. The addition of NDGA hardly affected the morphology of fibrils preformed in membranes as examined by EM (data not shown), whereas this compound breaks down fibrils formed in solution into amorphous aggregates [26]. Strangely, the addition of NDGA almost nullified ThT fluorescence (Fig. 7), suggesting that NDGA possesses a higher affinity for fibrils formed in membranes than ThT. In contrast, RIF destabilized preformed fibrils into elongate particles even in the presence of fibrils (data not shown), probably because binding of the more bulky structure (Fig. 1) is advantageous for breaking down preformed fibrils.

Aβ fibrillization in solution proceeds in the nucleation-dependent polymerization model characterized by the presence of a lag phase [6,7]. Hayashi et al. reported that the lag phase disappeared in the presence of GM1-containing liposomes (500 μM as GM1, [Aβ]=50 μM), concluding that GM1-bound Aβ serves as a seed [43]. However, the present results (Fig. 7) indicate that the situation is more complicated. The reduction of liposome concentration resulted in the appearance of a lag phase, suggesting that the membrane binding and subsequent conformational change, which is a fast process, is not enough for the seed formation.

4.2. Mechanism of inhibition of fibrillization in GM1 liposomes

The four more potent inhibitors can be classified into several groups (Fig. 10). NDGA and RIF inhibit fibril formation by two different mechanisms, which makes these compounds significantly active (Fig. 4). First, these compounds prevent the binding of Aβ to GM1 liposomes by competitively binding to the membranes (Figs. 5 and 6). NDGA also showed a high affinity for PC liposomes (Fig. 6B), suggesting that the driving force behind the membrane binding of NDGA is hydrophobic interaction. Since Aβ has the hydrophobic C-terminal part as well as several aromatic amino acid residues, NDGA may also directly interact with Aβ in solution, preventing the binding of the protein to membranes. Indeed, preliminary NMR measurements suggested this possibility, although the direct interaction will be diminished at lower Aβ concentrations at which liposome and cell studies were performed (unpublished work). Interestingly, QUE with a structure similar to NDGA, in that two phenol rings are separated by several carbon atoms, loses membrane affinity. The presence of additional oxygen atoms makes the molecule more polar than NDGA, preventing membrane binding. The weak toxicity of NDGA itself (Fig. 8A) may be related to the 'non-specific binding' of the compound to membranes. In contrast, RIF specifically binds to ganglioside-containing membranes (Fig. 6B). The compound showed marginal affinity for zwitterionic PC, negatively charged PG/PC, and raft-like SM/cholesterol bilayers. The presence of multiple OH groups (Fig. 1) may be advantageous for the specific interaction with gangliosides, which also possess a number of OH groups. The second mechanism for the inhibition of fibril formation involves the formation of elongate particles (Fig. 3B).

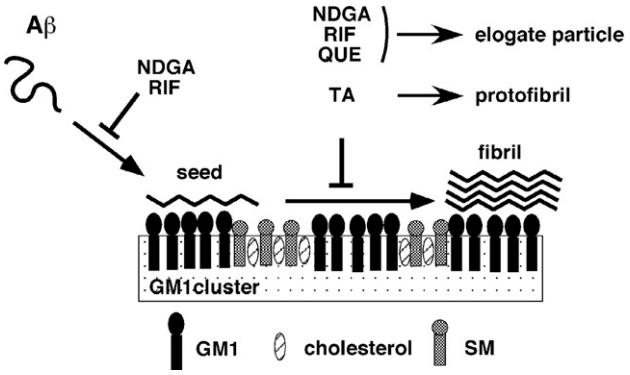

Fig. 10. Mechanisms of the inhibition of Aβ fibrillation by various compounds in lipid rafts. Aβ binds to a GM1 cluster, forming a β-sheet structure that acts as a seed for fibrillation. NDGA and RIF block the binding process by competitively binding to the membrane and/or direct interaction with Aβ in solution. These compounds as well as QUE inhibit the subsequent fibrillation process by forming elongate particles. The presence of TA leads to the formation of protofibrils.

On the other hand, QUE and TA block Aβ aggregation without affecting the membrane binding of the protein (Fig. 5). QUE forms elongate particles with Aβ similarly to NDGA and RIF, whereas TA induces protofibrillar structures (Fig. 3C). The formation of elongate particles may be indicative of specific interaction between the compounds and a conformationally altered form (probably β-sheet) of Aβ either formed during the aggregation process or in the fibrils. Krebs et al. recently proposed that Th-T binds to channel-like crevices that run along the length of β-sheets [54]. Relatively compact planar structures may be important for the formation of elongate particles (Fig. 1).

4.3. Cell study

We have recently confirmed that Aβ binds to and accumulates on GM1-rich domains on cell membranes, guaranteeing the relevance of our liposomal model system [55]. Equimolar NDGA almost completely inhibited Aβ-induced cytotoxicity (Fig. 8). Although oxidative stress has been suggested to be involved in Aβ-induced cytotoxicity [56–58], the cytoprotective effect of NDGA is not due to the antioxidant activity of the compound but due to the inhibition of the fibril formation of Aβ on cells (Fig. 9), in accordance with the liposomal study. Polyphenols, such as (−)-epigallocatechin gallate [59] and curcumin [30], are reported to cross the blood–brain barrier. Taking the small molecular size into consideration, NDGA appears to be a promising leading compound as a preventive drug for AD.

In contrast to NDGA, RIF and TA were disappointing drug candidates. Equimolar RIF had no effect on Aβ-induced cytotoxicity (Fig. 8). Tomiyama et al. reported that RIF completely protected NGF-differentiated PC12 cells from cytotoxicity triggered by Aβ-(1–40) preaggregated in solution [31]. However, they used a 50-fold excess of the compound. TA itself showed cytotoxicity (Fig. 8B), although it is reported to be nontoxic at a dietary concentration of up to 5% [60].

In summary, NDGA, RIF, and TA are potent inhibitors of both the solution-phase and membrane-mediated formation of Aβ fibrils in vitro, although the individual inhibitory mechanisms are different. Although TA and RIF possess effective destabilizing activities for fibrils formed in membranes, our cell study showed that NDGA is a promising candidate as a lead compound for AD therapy.

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