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Anti-amyloidogenic activity of tannic acid and its activity to destabilize Alzheimer's β-amyloid fibrils in vitro

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Abstract

Inhibition of the accumulation of amyloid β-peptide (Aβ) and the formation of β-amyloid fibrils (fAβ) from Aβ, as well as the destabilization of preformed fAβ in the CNS would be attractive therapeutic targets for the treatment of Alzheimer's disease (AD). We previously reported that nordihydroguaiaretic acid (NDGA) and wine-related polyphenols inhibit fAβ formation from Aβ(1–40) and Aβ(1–42) as well as destabilizing preformed fAβ(1–40) and fAβ(1–42) dose-dependently in vitro. Using fluorescence spectroscopic analysis with thioflavin T and electron microscopic studies, we examined the effects of polymeric polyphenol, tannic acid (TA) on the formation, extension, and destabilization of fAβ(1–40) and fAβ(1–42) at pH 7.5 at 37 °C in vitro. We next compared the anti-amyloidogenic activities of TA with myricetin, rifampicin, tetracycline, and NDGA. TA dose-dependently inhibited fAβ formation from Aβ(1–40) and Aβ(1–42), as well as their extension. Moreover, it dose-dependently destabilized preformed fAβs. The effective concentrations (EC_{50}) of TA for the formation, extension and destabilization of fAβs were in the order of 0–0.1 μM. Although the mechanism by which TA inhibits fAβ formation from Aβ as well as destabilizes preformed fAβ in vitro is still unclear, it could be a key molecule for the development of therapeutics for AD.
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Keywords: Alzheimer's disease; Tannic acid; β-Amyloid fibril; Thioflavin; Electron microscopy

1. Introduction

Alzheimer's disease (AD) is a common, complex, and challenging neurodegenerative disease. It is estimated to affect approximately 15 million people worldwide, and the incidence increases from 0.5% per year at age 65 years to 8% per year at age 85 years [1,2]. The neuropathological hallmarks of AD are the accumulation of extracellular amyloid plaque containing amyloid β-peptide (Aβ) and intracellular neurofibrillary tangles containing tau protein [3]. Because the β-sheet formation and aggregation of Aβ are considered to be critical events that render these peptides neurotoxic [4], many researchers favor therapeutic approaches that target the formation, deposition and clearance of Aβ from nervous tissue. Experimental therapies and clinical trials using vaccination [5–7] and nonsteroidal anti-inflammatory drugs [8,9] have been reported.

Using a nucleation-dependent polymerization model to explain the mechanism of the formation of Alzheimer's β-amyloid fibrils (fAβ) in vitro [10–13], we previously found that nordihydroguaiaretic acid (NDGA) and rifampicin (RIF) inhibit fAβ formation dose-dependently in vitro [14]. Moreover, we reported that they also destabilize fAβ(1–40) and fAβ(1–42) in a concentration-dependent manner, based on fluorescence spectroscopic analysis with thioflavin T (ThT) and electron microscopic studies [15]. The activity of NDGA to destabilize fAβs was in the order

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of: NDGA≫RIF=tetracycline (TC)>poly(vinylsulfonic acid, sodium salt)=1,3-propanedisulfonic acid, disodium salt>β-sheet breaker peptide (iAβ5)>nicotine [15,16].

Many studies have demonstrated that oxidative damage plays a central role in AD pathogenesis [17–20]. Many antioxidant compounds, such as vitamin E [21–25], NDGA [26], and nicotine [27], have been demonstrated to protect the brain from in vitro Aβ toxicity, and clinical trials to test the ability of high dose vitamin E to slow AD progression have been carried out [28–30]. Additionally, intake of wine including polyphenols has been reported to be associated with a lower risk of AD [31–34]. Tannins and related polyphenols have much stronger inhibitory effects on lipid peroxidation than vitamin E [35]. Recently, several investigators have suggested that many kinds of natural polyphenols may have neuroprotective effects both in vivo and in vitro, possibly by their abilities to scavenge reactive oxygen species [36–41]. Roth et al. [42] reported that the wine-related polyphenols, quercetin and kaempferol protect against Aβ-induced toxicity in cell cultures. Very recently, we showed that the wine-related polyphenol, myricetin (Myr), dose-dependently inhibits formation and extension of fAβ(1–40) and fAβ(1–42), as well as destabilizes preformed fAβs in vitro [43]. Here, we examined the effects of a polymeric polyphenol, tannic acid (TA), to inhibit the formation and extension of fAβ(1–40) and fAβ(1–42), as well as to destabilize fAβs at pH 7.5 at 37 °C in vitro, using fluorescence spectroscopy with ThT and electron microscopy. We also compared its anti-amyloidogenic and fibril-destabilizing effects with Myr, RIF, TC and NDGA.

2. Materials and methods

2.1. Preparation of Aβ and fAβ solutions

Aβ(1–40) (a trifluoroacetate salt, lot number 530108, Peptide Institute, Inc., Osaka, Japan) and Aβ(1–42) (a trifluoroacetate salt, lot number 521205, Peptide Institute) were dissolved by brief vortexing in a 0.02% ammonia solution at a concentration of 500 μM (2.2 mg/ml) and 250 μM, respectively, in a 4 °C room and stored at −80 °C before assaying (fresh Aβ(1–40) and Aβ(1–42) solutions). fAβ(1–40) and fAβ(1–42) were formed from the fresh Aβ(1–40) and Aβ(1–42) solutions, respectively, sonicated, and stored at 4 °C as described elsewhere [44].

Fresh, non-aggregated fAβ(1–40) and fAβ(1–42) were obtained by extending sonicated fAβ(1–40) or fAβ(1–42) with fresh Aβ(1–40) or Aβ(1–42) solutions, respectively, just before the destabilization reaction [15,16,43]. The reaction mixture was 600 μl and contained 10 μg/ml (2.3 μM) fAβ(1–40) or fAβ(1–42), 50 μM Aβ(1–40) or Aβ(1–42), 50 mM phosphate buffer, pH 7.5, and 100 mM NaCl. Measurement of the fluorescence of ThT showed that the extension reaction proceeded to equilibrium after incubation at 37 °C for 3–6 h under non-agitated conditions. In the following experiment, the concentration of fAβ(1–40) and fAβ(1–42) in the final reaction mixture was regarded as 50 μM.

2.2. Fluorescence spectroscopy, electron microscopy, and polarized light microscopy

A fluorescence spectroscopic study was performed on a Hitachi F-2500 fluorescence spectrophotometer as described

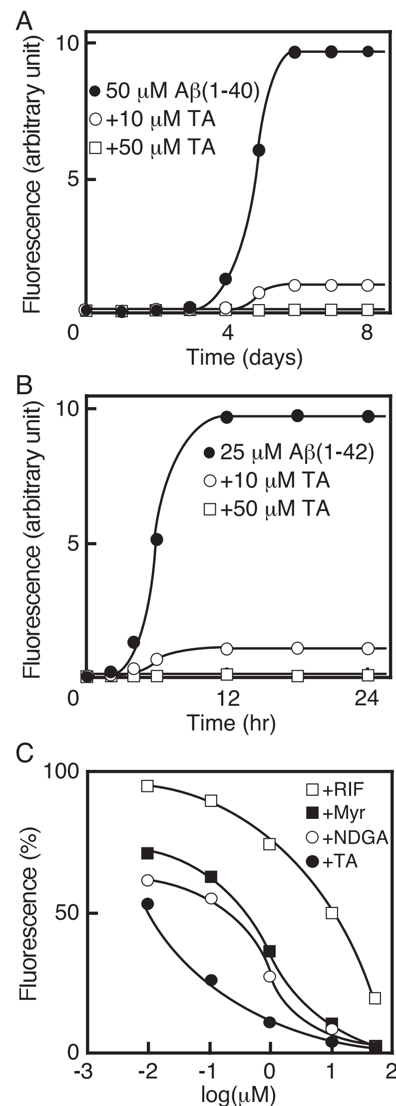

Fig. 1. Effects of TA (A, B) on the kinetics of formation of fAβ(1–40) (A) and fAβ(1–42) (B) from fresh Aβ(1–40) and Aβ(1–42), respectively. The reaction mixtures containing 50 μM Aβ(1–40) (A) or 25 μM Aβ(1–42) (B), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (●), 10 (○), or 50 μM (□) of TA (A, B) were incubated at 37 °C for the indicated times. Each figure shows a representative pattern of three independent experiments. (C) Dose-dependent inhibition of fAβ(1–40) formation from fresh Aβ(1–40). The reaction mixtures containing 50 μM Aβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM TA (●), NDGA (○), Myr (■), or RIF (□) were incubated at 37 °C for 7 days. Each point represents the mean of three independent experiments. At all points, standard errors were within symbols. The average without compounds was regarded as 100%.

elsewhere [45]. Optimum fluorescence measurements of fAβ(1–40) and fAβ(1–42) were obtained at the excitation and emission wavelengths of 445 and 490 nm, respectively, with the reaction mixture containing 5 μM ThT (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 50 mM of glycine–NaOH buffer, pH 8.5. Electron microscopic and polarized light microscopic studies of the reaction mixtures were performed as described elsewhere [44].

2.3. Polymerization assay

Polymerization of Aβ with or without fAβ added as seeds was assayed as described elsewhere [14]. Briefly, the reaction mixture contained 50 μM Aβ(1–40), or 25 or 50 μM Aβ(1–42), 0 or 10 μg/ml fAβ(1–40) or fAβ(1–42), 0–50 μM TA, Myr, RIF, TC or NDGA, 1% dimethyl sulfoxide (DMSO), 50 mM phosphate buffer, pH 7.5, and 100 mM NaCl. TA, Myr, RIF, TC, and NDGA (Sigma Chemical Co., St. Louis, MO) were first dissolved in DMSO at concentrations of 1, 10, 100 μM, 1 and 5 mM, then added to the reaction mixture to make the final concentrations 0.01, 0.1, 1, 10 and 50 μM, respectively.

Thirty-microliter aliquots of the mixture were put into oil-free PCR tubes (size: 0.5 ml, code number: 9046, Takara Shuzo Co. Ltd., Otsu, Japan). These tubes were then put into a DNA thermal cycler (PJ480, Perkin Elmer Cetus, Emeryville, California). Starting at 4 °C, the plate temperature was elevated at maximal speed, to 37 °C. Incubation times ranged between 0 and 8 days as indicated in each figure, and the reaction was stopped by placing the tubes on ice. The tubes were not agitated during the reaction. Five-microliter aliquots from each tube in triplicate were subjected to fluorescence spectroscopy and the mean of the three measurements determined. In the ThT solution, the concentration of TA, Myr, RIF, TC and NDGA examined in this study was diluted up to 1/200 of that in the reaction mixture. We confirmed that these compounds did not

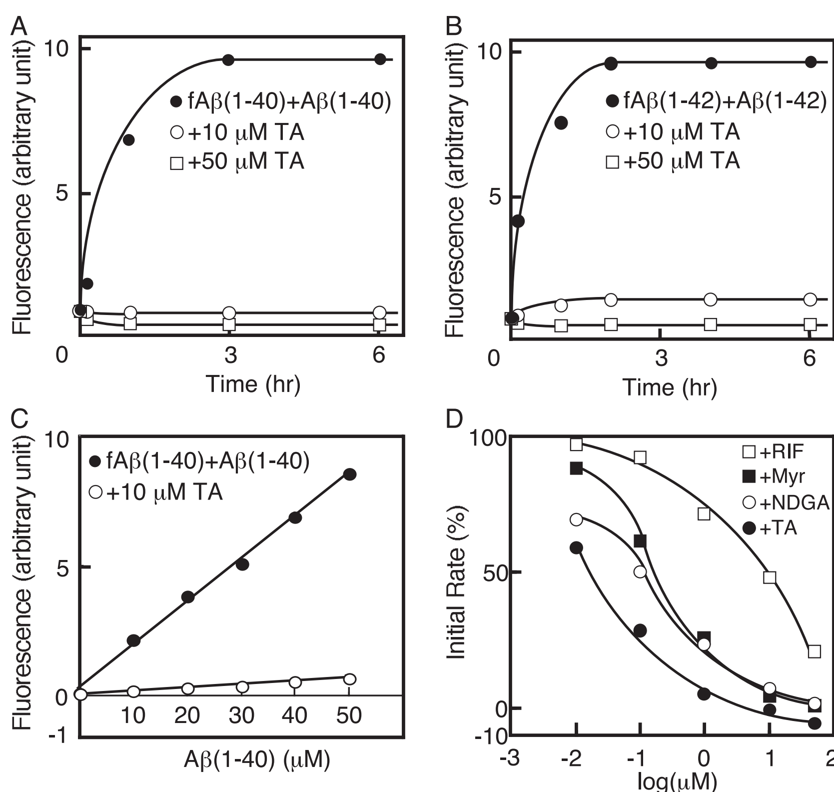

Fig. 2. Effects of TA (A, B) on the kinetics of extension of fAβ(1–40) (A) and fAβ(1–42) (B). The reaction mixtures containing 10 μg/ml (2.3 μM) sonicated fAβ(1–40) (A) or fAβ(1–42) (B), 50 μM Aβ(1–40) (A) or Aβ(1–42) (B), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (●), 10 (○), or 50 μM (□) of TA (A, B), were incubated at 37 °C for the indicated times. Each figure shows a representative pattern of three independent experiments. (C) Effect of Aβ(1–40) concentration on the initial rate of fAβ(1–40) extension in the presence (○) and absence (●) of TA. The reaction mixtures containing 10 μg/ml (2.3 μM) sonicated fAβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, 0 (●) or 10 μM (○) TA, and 0, 10, 20, 30, 40, and 50 μM Aβ(1–40) were incubated at 37 °C for 1 h. Each point represents the mean of three independent experiments. At all points, standard errors were within symbols. Linear least-square fit was performed for each straight line (R^2=1.000 and 0.991 for ● and ○, respectively). (D) Dose-dependent inhibition of fAβ(1–40) extension. The reaction mixtures containing 10 μg/ml (2.3 μM) sonicated fAβ(1–40), 50 μM Aβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM TA (●), NDGA (○), Myr (■), or RIF (□) were incubated at 37 °C for 1 h. Each point represents the mean of three independent experiments. At all points, standard errors were within symbols. The average without compounds was regarded as 100%.

quench ThT fluorescence at the diluted concentration (data not shown).

2.4. Measurement of fibril-destabilizing activity

Destabilization of fAβ was assayed as described elsewhere [15]. Briefly, the reaction mixture contained 25 μM fresh fAβ(1–40) or fAβ(1–42), 0–50 μM TA, Myr, RIF, TC, or NDGA, 1% DMSO, 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 1% (wt/vol) polyvinyl alcohol (Wako) to avoid the aggregation of fAβ and the adsorption of fAβ onto the inner wall of the reaction tube during the reaction. TA, Myr, RIF, TC, and NDGA dissolved in DMSO at concentrations of 1, 10, 100 μM, 1 and 5 mM were added to the reaction mixture to make the final concentrations 0.01, 0.1, 1, 10 and 50 μM, respectively.

After being mixed by pipetting, triplicate 5-μl aliquots of the reaction mixture were subjected to fluorescence spectroscopy and 30-μl aliquots were put into PCR tubes. The reaction tubes were then transferred into a DNA thermal cycler. Starting at 4 °C, the plate temperature was elevated at maximal speed to 37 °C. Incubation times ranged between 0 and 6 h as indicated in each figure, and the reaction was stopped by placing the tubes on ice. The reaction tubes were not agitated during the reaction. Five-microliter aliquots from each tube in triplicate were subjected to fluorescence spectroscopy and the mean of the three measurements was determined. At the diluted concentration, these compounds did not compete with ThT for fAβ at either 4 or 37 °C (data not shown).

2.5. Other analytical procedures

Protein concentrations of the supernatants of the reaction mixtures after centrifugation were determined by the method of Bradford [46] with a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). The Aβ(1–40) solution quantified by amino acid analysis was used as the standard. Linear least squares fit was used for statistical analysis. The effective concentration EC_{50} was defined as the concentration of TA, Myr, RIF, TC, or NDGA to inhibit the formation or extension of fAβs to 50% of the control value, or the concentration to destabilize fAβs to 50% of the control value. EC_{50} was calculated by the sigmoidal curve fitting of the data as shown in Figs. 1C, 2D and 4C, using Igor Pro ver.4 (WaveMetrics, Inc., Lake Oswego, OR, USA).

3. Results

3.1. Effects of TA on the kinetics of fAβ formation

As shown in Fig. 1A and B, when fresh Aβ(1–40) or Aβ(1–42) was incubated at 37 °C, the fluorescence of ThT followed a characteristic sigmoidal curve. This curve is consistent with the nucleation-dependent polymerization model [10,12]. fAβ(1–40) and fAβ(1–42) stained with Congo red showed typical orange-green birefringence under polarized light (data not shown). The final equilibrium level decreased after incubation of Aβ(1–40) or Aβ(1–42) with 10 and 50 μM TA (Fig. 1A and B).

As shown in Fig. 2A and B, when fresh Aβ(1–40) was incubated with fAβ(1–40) or Aβ(1–42) with fAβ(1–42), at 37 °C, the fluorescence increased hyperbolically without a lag phase and proceeded to equilibrium much more rapidly than that without seeds (compare Figs. 1 and 2). This curve is consistent with a first-order kinetic model [45]. When Aβ(1–40) and fAβ(1–40) were incubated with 10 and 50 μM TA, the final equilibrium level decreased (Fig. 2A). A similar effect of TA was observed for the extension of fAβ(1–42) (Fig. 2B). At a constant fAβ(1–40) concentration, a perfect linearity was observed between the Aβ(1–40) concentration and the initial rate of fAβ(1–40) extension both in the presence and absence of TA (Fig. 2C). This linearity is again consistent with a first-order kinetic model and indicates that at each Aβ(1–40) concentration, the net rate of fAβ(1–40) extension is the sum of the rates of polymerization and depolymerization [45,47]. In the presence of 10 μM TA, the slope of the straight line decreased to about 1/14. The interpretation of this figure implicating the

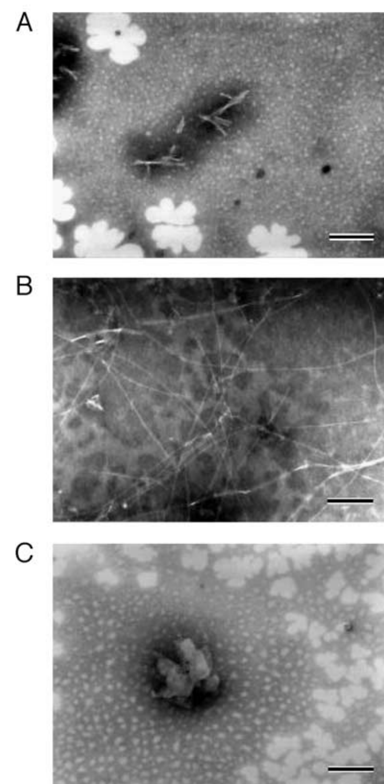

Fig. 3. Electron micrographs of extended fAβ(1–40). The reaction mixtures containing 10 μg/ml (2.3 μM) fAβ(1–40), 50 μM Aβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (B) or 50 μM TA (A, C), were incubated at 37 °C for 0 (A), or 6 h (B, C). Scale bars indicate a length of 250 nm.

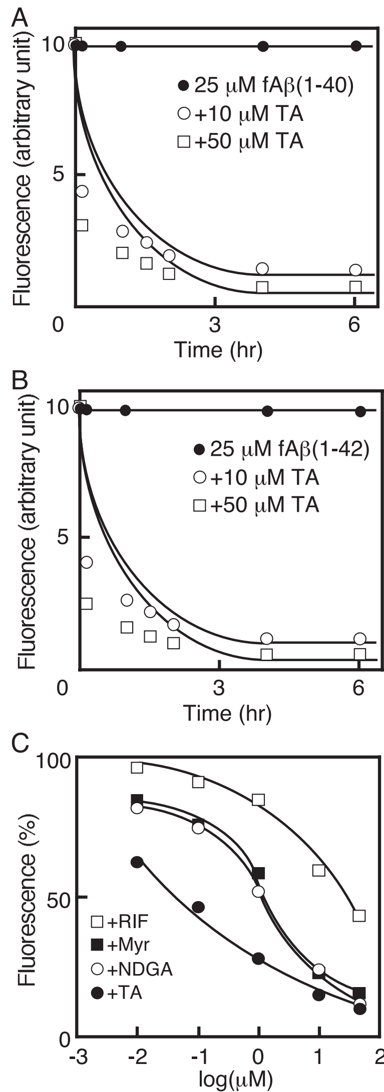

Fig. 4. Effects of TA (A, B) on the kinetics of destabilization of fAβ(1–40) (A) and fAβ(1–42) (B). The reaction mixtures containing 25 μM fAβ(1–40) (A) or fAβ(1–42) (B), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (●), 10 (○), or 50 μM (□) of TA (A, B), were incubated at 37 °C for the indicated times. Each figure shows a representative pattern of three independent experiments. (C) Dose-dependent destabilization of fAβ(1–40). The reaction mixtures containing 25 μM fAβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM TA (●), NDGA (○), Myr (■), or RIF (□) were incubated at 37 °C for 4 h. Each point represents the mean of three independent experiments. At all points, standard errors were within symbols. The average without compounds was regarded as 100%.

mechanism of the antiamyloidogenic effect of TA will be discussed later.

After incubation of fresh Aβ(1–40) with fAβ(1–40) at 37 °C, clear fibril extension was observed by electron-microscopy (Fig. 3B). However, 50 μM TA completely inhibited the extension of sonicated fAβ(1–40). On the contrary, TA seemed to destabilize seeds of fAβ(1–40) (Fig. 3A and C). TA inhibited the extension of fAβ(1–42) (data not shown).

3.2. Fibril-destabilizing assay

As shown in Fig. 4A and B, the fluorescence of ThT was almost unchanged during the incubation of fresh fAβ(1–40) or fAβ(1–42) at 37 °C without additional molecules. On the other hand, the ThT fluorescence decreased immediately after addition of TA to the reaction mixture. After incubation of 25 μM fresh fAβ(1–40) with 50 μM TA for 0.5 h, many short, sheared fibrils were observed (Fig. 5B). At 4 h, the number of fibrils was reduced markedly, and small amorphous aggregates were extensively observed (Fig. 5C). Similar morphology was observed after incubation of 25 μM fresh fAβ(1–42) with 50 μM TA (data not shown).

After incubation with 50 μM TA for 4 h, fAβ(1–40) and fAβ(1–42) were not stained with Congo red as strongly as fresh fAβ(1–40) and fAβ(1–42) (data not shown). However, they all showed orange-green birefringence under polarized light (data not shown). This means that a significant amount of intact fAβ(1–40) and fAβ(1–42) still remains in the mixture after the reaction. When the protein concentration of the supernatant after centrifugation at 4 °C for 2 h at $1.6\times10^4 g$ was measured by the Bradford assay, no proteins were detected in the supernatant in any case (data not shown). This implies that although TA could destabilize fAβ(1–40) and fAβ(1–42) to visible aggregates (Fig. 5C),

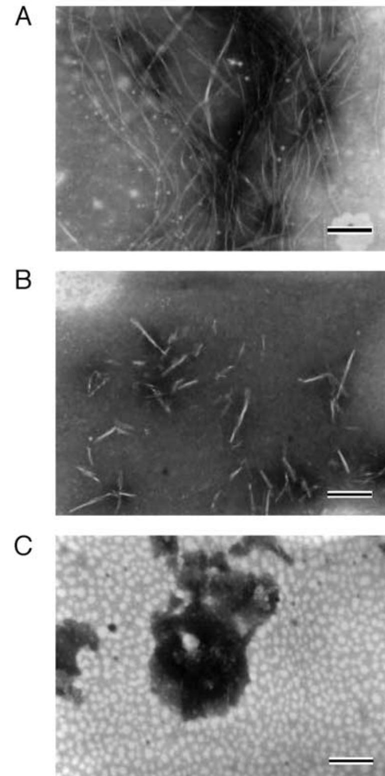

Fig. 5. Electron micrographs of destabilized fAβ(1–40). The reaction mixture containing 25 μM fAβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 50 μM TA was incubated at 37 °C for 0 (A), 0.5 (B) or 4 h (C). Scale bars indicate a length of 250 nm.

Table 1
The effective concentrations (EC_{50})[a] of TA, Myr, RIF, TC and NDGA for the formation, extension and destabilization of fAβ(1–40) and fAβ(1–42)

Compounds	Formation[b]		Extension[c]		Destabilization[d]	
	FAβ(1–40)	fAβ(1–42)	fAβ(1–40)	fAβ(1–42)	fAβ(1–40)	fAβ(1–42)
TA	0.012 μM	0.022 μM	0.023 μM	0.011 μM	0.065 μM	0.026 μM
Myr	0.34	0.43	0.20	0.13	1.5	0.58
RIF	9.7	9.1	7.7	10	22	31
TC	10	10	8.0	16	23	45
NDGA	0.17	0.87	0.10	0.096	1.2	0.86

[a] EC_{50} (μM) were defined as the concentrations of TA, Myr, RIF, TC or NDGA to inhibit the formation or extension of fAβs to 50% of the control value, or the concentrations to destabilize fAβs to 50% of the control value. EC_{50} were calculated by the sigmoidal curve fitting of the data as shown in (Figs. 1C, 2D and 4C), using Igor Pro ver.4 (WaveMetrics).

[b] The reaction mixtures containing 50 μM Aβ(1–40) or 25 μM Aβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 or 50 μM TA, Myr, RIF, TC or NDGA were incubated at 37 °C for 7 days and 24 h, respectively.

[c] The reaction mixtures containing 10 μg/ml (2.3 μM) sonicated fAβ(1–40) or fAβ(1–42), 50 μM Aβ(1–40) or Aβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 or 50 μM TA, Myr, RIF, TC or NDGA were incubated at 37 °C for 1 h.

[d] The reaction mixtures containing 25 μM fAβ(1–40) or fAβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 or 50 μM TA, Myr, RIF, TC or NDGA were incubated at 37 °C for 4 h.

they could not depolymerize fAβ(1–40) and fAβ(1–42) to the monomers or oligomers of Aβ(1–40) and Aβ(1–42).

3.3. Comparison of the activity of TA, Myr, RIF, TC, and NDGA

As shown in (Figs. 1C, 2D and 4C), TA, Myr, RIF and NDGA dose-dependently inhibited the formation and extension of fAβs, as well as dose-dependently destabilized preformed fAβs. We calculated EC_{50}, the concentrations of TA, Myr, RIF, TC, and NDGA to inhibit the formation or extension of fAβs to 50% of the control value, or their concentrations to destabilize fAβs to 50% of the control value, by the sigmoidal curve fitting of the data as shown in (Figs. 1C, 2D and 4C); (Table 1). In all molecules examined, EC_{50} to inhibit the formation or extension of fAβs was similar to EC_{50} to destabilize fAβs. All data presented in Table 1 may indicate that the anti-amyloidogenic activity of the molecules in this study may be in the order of: TA≫NDGA=Myr≫RIF=TC.

4. Discussion

Recently, our systematic in vitro study indicated that the overall activity of the anti-amyloidogenic molecules was in the order of: NDGA≫RIF=TC>poly(vinylsulfonic acid, sodium salt)=1,3-propanedisulfonic acid, disodium salt>iAβ5>nicotine [15,16]. NDGA is smaller than RIF, and has two *ortho*-dihydroxyphenyl rings symmetrically bound by a short carbohydrate chain. This compact and symmetric structure might be quite suitable for specifically binding to free Aβ and subsequently inhibiting the polymerization of Aβ into fAβ [15]. Alternatively, this structure might be suitable for specific binding to fAβ and subsequent destabilization of the β-sheet rich conformation of Aβ molecules in fAβ [15]. Very recently, we revealed that the anti-amyloidogenic and fibril-destabilizing activity of NDGA and wine-related polyphenols may be in the order of: NDGA=Myr=morin=quercetin>kaempferol>(+)-catechin=(−)-epicatechin [43]. We speculated that the difference in the three-dimensional structure and the numbers of hydroxyl groups of these polyphenols would affect greatly the anti-amyloidogenic and fibril-destabilizing activity [43]. In the present study, we found that the polymeric polyphenol, TA, dose-dependently inhibits fAβ formation from fresh Aβ, as well as destabilizes preformed fAβ in vitro. The anti-amyloidogenic and fibril-destabilizing activity of molecules examined in this study may be in the order of: TA≫NDGA=Myr≫RIF=TC (see Table 1). TA is a polymer of phenolcarboxylic acid, gallic acid which is a part of Myr [48], and has much more hydroxyl groups than Myr (Fig. 6). Tomiyama et al. [49,50] suggested that RIF binds to Aβ by hydrophobic interactions between its lipophilic ansa chain and the hydrophobic region of Aβ, thus blocking the association between Aβ molecules that lead to fAβ formation. The anti-amyloidogenic activity of TCs, small-molecule anionic sulfonates or sulfates, melatonin, iAβ5 and nicotine may also be related to the propensity to bind to the specific sites of Aβ [51–55]. Interestingly, TA, wine-related polyphenols, NDGA, RIF, melatonin and nicotine have all been reported to have antioxidant activity [26,35,38,48,50,53,56]. Thus, TA with potent antioxidant motifs could bind specifically to Aβ and/or fAβ, inhibit fAβ formation and/or destabilize preformed fAβ through mechanisms yet unknown. Further studies, such as nuclear magnetic resonance experiments, are essential to reveal the exact structure–activity relationships for these compounds which exhibit anti-amyloidogenic and fibril-destabilizing effects in vitro.

TA did not extend the length of the lag phase in the formation fAβs from Aβs (Fig. 1). Moreover, it did not extend the time to proceed to equilibrium in the extension reaction (Fig. 2). These results are in sharp contrast to those of apolipoprotein E (apoE), in which apoE extends both the length of the lag phase and the time to proceed to

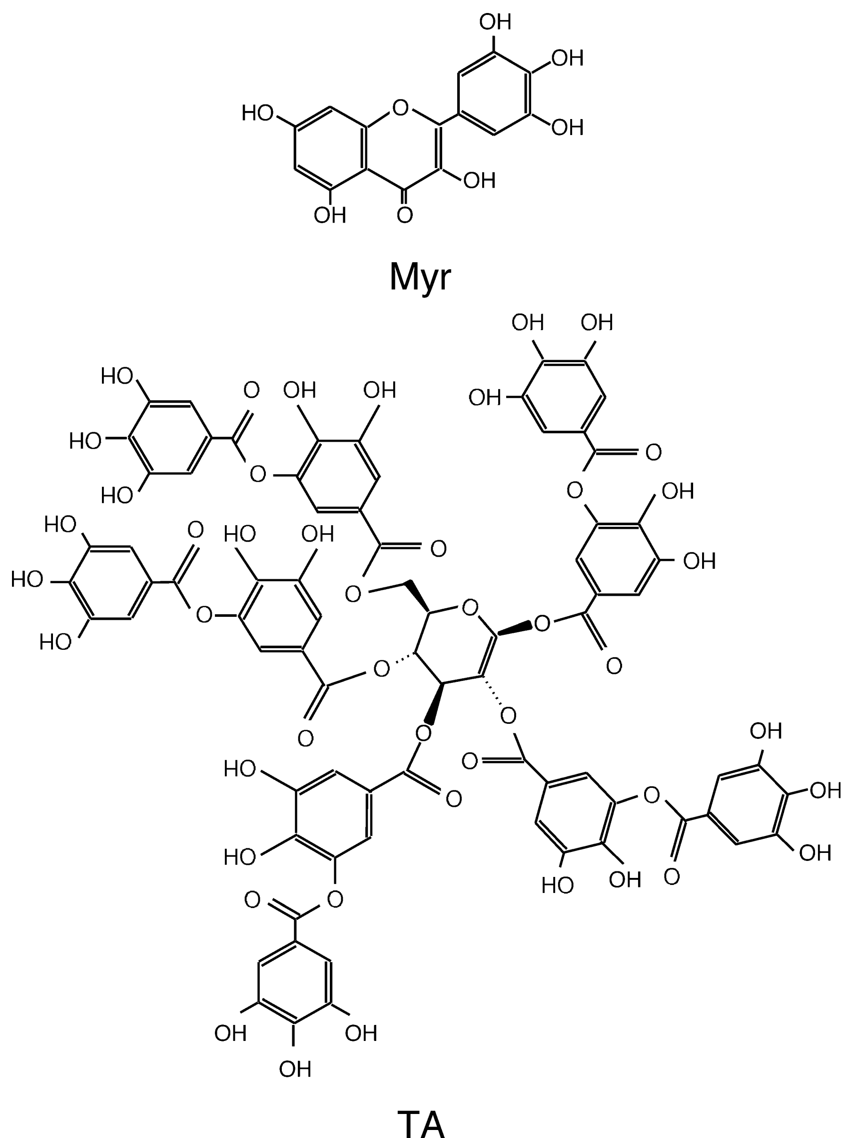

Fig. 6. Structure of Myr and TA.

equilibrium in a dose-dependent manner [14]. Although apoE was suggested to inhibit the formation of fAβs in vitro, by making a complex with Aβs, thus eliminating free Aβs from the reaction mixture [12,14], TA could inhibit the formation of fAβs by different mechanisms. As shown in Fig. 2C, the extension of fAβ(1–40) followed a first-order kinetic model even in the presence of TA. The net rate of fAβ(1–40) extension is the sum of the rates of polymerization and depolymerization [45,47]. Thus, one possible explanation for the finding in Fig. 2C may be that TA could bind to the ends of extending fAβ(1–40) and increase the rate of depolymerization by destabilizing the conformation of Aβ(1–40) which has just been incorporated into the fibril ends. Alternatively, TA would bind to Aβ(1–40) and consequently decrease the rate of polymerization. Previously, Harper et al. [57–59] analyzed the process of in vitro Aβ assembly using an atomic force microscope at a fine resolution. They reported that protofibrils, transient species of Aβ assembly, were formed during the first week of incubation of Aβ40 before mature fibrils were generated. This model of Aβ assembly was supported by a recent study of Nichols et al. [60]. In the present study, we examined the inhibition of both the formation fAβs from Aβs and the fAβs extension in the presence of TA. In the EM examination of the present study, we observed mature fibrils in Aβ(1–40) solutions incubated with fAβ(1–40) at 50 μM and 37 °C for 6 h, but protofibrils were hardly recognized (Fig. 3B). This discrepancy is likely to mainly stem from the differences in incubation period and peptide concentrations. Further studies are essential to clarify whether TA inhibits formation of protofibrils in vitro.

Tannins (commonly referred to as TA) are water-soluble polyphenols which differ from most other natural phenolic compounds in their ability to precipitate proteins such as gelatin from solution [61]. This property (sometimes called astringency) is the reason for their past and present use in

the tanning of animal skins [61]. Tannins are commonly found in a large array of higher plant species of both herbaceous and woody types [62]. They can accumulate in large amounts (often more than 10% of the dry weight) in particular organs or tissues which can be almost any plant part: bark, wood, leaves, fruits or roots [62]. The biological activities of tannins include marked anti-tumor, anti-viral, inhibition of lipid peroxidation and plasmin activity, mediation of DNA nicking, amelioration of renal failure, and several others [35,63–67]. The polymeric tannins such as TA are generally stronger against the radicals than small molecule polyphenols, such as catechin, quercetin, and kaempferol [48]. Moreover, a novel type of TA, Pistafolia A, was reported to prevent against oxidative neuronal cell damage [68]. In this study, we showed that TA dose-dependently inhibits fAβ formation from fresh Aβ, as well as destabilizes preformed fAβ in vitro. Moreover TA exhibited much higher anti-amyloidogenic and fibril-destabilizing effects than the others, such as NDGA, Myr, RIF, and TC. Previously, cell culture experiments with human embryonic kidney (HEK) 293 cells indicated that fAβ destabilized by NDGA or Myr might be less toxic than intact fAβ [15,43]. Thus, TA could prevent the development of AD, not only through scavenging reactive oxygen species, but also through directly inhibiting the deposition of fAβ in the brain. Joslyn and Glick [69] have shown that TA is nontoxic at a dietary concentration of 5% or less. Although the exact mechanism of anti-amyloidogenic activity of TA is unclear, these structurally similar compounds could be key molecules for the development of therapeutics for AD and other human amyloidoses.

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