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Biflavonoids Are Superior to Monoflavonoids in Inhibiting Amyloid-β Toxicity and Fibrillogenesis via Accumulation of Nontoxic Oligomer-like Structures

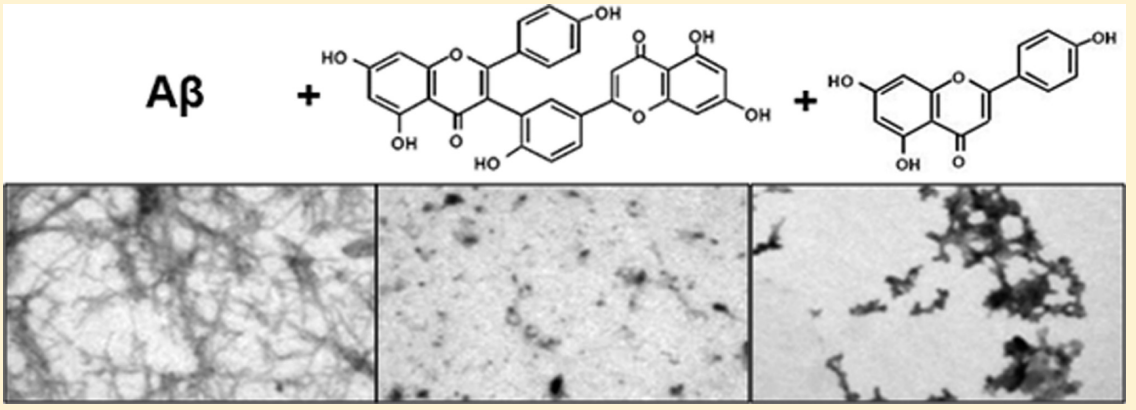
Arjun Thapa,[†,∥] Eun-Rhan Woo,[§] Eva Y. Chi,[∥] Md. Golam Sharoar,[†,⊥] Hong-Guang Jin,[§] Song Yub Shin,[†,‡] and Il-Seon Park*[,†,‡]

[†]Department of Bio-materials Engineering, [‡]Department of Cellular and Molecular Medicine, and [§]College of Pharmacy, Chosun University, Gwanju 501-759, Republic of Korea

[∥]Department of Chemical Nuclear Engineering, Center for Biomedical Engineering, University of New Mexico, Albuquerque, New Mexico 87131, United States

[⊥]Department of Genetic Engineering and Biotechnology, Rajshahi University, Rajshahi 6205, Bangladesh

ABSTRACT:



Polymerization of monomeric amyloid-β peptides (Aβ) into soluble oligomers and insoluble fibrils is one of the major pathways triggering the pathogenesis of Alzheimer's disease (AD). Using small molecules to prevent the polymerization of Aβ peptides can, therefore, be an effective therapeutic strategy for AD. In this study, we investigate the effects of mono- and biflavonoids in Aβ42-induced toxicity and fibrillogenesis and find that the biflavonoid taiwaniaflavone (TF) effectively and specifically inhibits Aβ toxicity and fibrillogenesis. Compared to TF, the monoflavonoid apigenin (AP) is less effective and less specific. Our data show that differential effects of the mono- and biflavonoids in Aβ fibrillogenesis correlate with their varying cytoprotective efficacies. We also find that other biflavonoids, namely, 2′,8″-biapigenin, amentoflavone, and sumaflavone, can also effectively inhibit Aβ toxicity and fibrillogenesis, implying that the participation of two monoflavonoids in a single biflavonoid molecule enhances their activity. Biflavonoids, while strongly inhibiting Aβ fibrillogenesis, accumulate nontoxic Aβ oligomeric structures, suggesting that these are off-pathway oligomers. Moreover, TF abrogates the toxicity of preformed Aβ oligomers and fibrils, indicating that TF and other biflavonoids may also reduce the toxicity of toxic Aβ species. Altogether, our data clearly show that biflavonoids, possibly because of the possession of two Aβ binders separated by an appropriate size linker, are likely to be promising therapeutics for suppressing Aβ toxicity.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive deposition of aggregated amyloid-β (Aβ) peptides in brain parenchyma and cerebral blood vessels.[1] Several isoforms of Aβ peptides are produced by the sequential proteolysis of the amyloid precursor protein (APP) by α-, β-, and γ-secretases.[1] However, two major isoforms of Aβ are found in AD brains, Aβ40 and Aβ42. Native Aβ peptides spontaneously undergo conformational changes to form misfolded intermediates that are rich in β-sheets. These intermediates are typically unstable and undergo further aggregation to form higher-order oligomers, protofibrils, and fibrils. Recent studies have shown that soluble Aβ oligomers and protofibrils formed during Aβ fibrillogenesis are the most toxic species and that their toxicity increases with the progression of AD.[2,3] Although the Aβ40 peptide is the most abundant isoform

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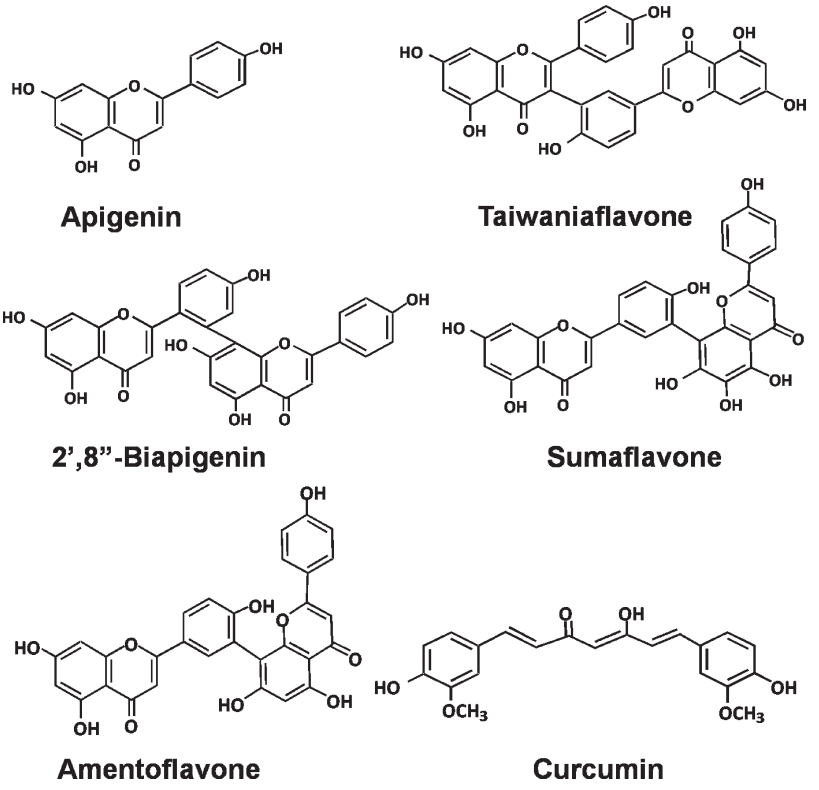

Figure 1. Chemical structures of AP, TF, 2′,8″-biapigenin, sumaflavone, amentoflavone, and curcumin.

in the brain, Aβ42 is enriched in AD brains as it progressively accumulates into extracellular senile plaques in AD brains.[4,5]

Several different therapeutic strategies have been proposed to suppress AD.[6] For example, neutralization of Aβ aggregates by specific antibodies,[7] modulation of the activity of β- or γ-secretases,[8] administration of antioxidant and anti-inflammatory compounds,[9] cholesterol lowering drugs,[10] and Cu^{2+} and Zn^{2+} chelating agents[11] are thought to reduce AD risks. In addition, small molecules that efficiently inhibit the early steps of Aβ fibrillogenesis and stabilize its nontoxic conformations have been considered as possible AD therapeutics because the self-assembly of the peptide is directly linked to the pathogenesis of AD. Recently, studies based on curcumin have shown that the two terminal phenyl groups, substituted aromatic end groups that participate in hydrogen bonding, and an optimal rigid linker length of 6−19 Å are essential structural features for the efficient inhibition of Aβ fibrillogenesis.[12] The study also suggested that a flat and planar molecule with two aromatic end groups and substituted aromatic groups can be a good ligand for Aβ and may be cytoprotective.[12−14] This conclusion was further supported by the fact that ferulic acid (structurally similar to one-half of curcumin) could not inhibit Aβ fibrillogenesis but did upon dimerization,[15,16] and the hybrid molecule of ferulic acid and styryl benzene is more effective than the individual compounds.[16] The small polyphenols, curcumin,[14] epigallocatechin gallate (EGCG),[17] and resveratrol,[18] have been found to effectively reduce Aβ toxicity in vitro and in vivo.

The inhibitory effect of the small molecules on Aβ fibrillogenesis, however, does not guarantee that these molecules also offer cytoprotection because antifibrillogenic molecules often fail to protect cells exposed to Aβ.[19,20] For example, quercetin effectively inhibits Aβ fibril formation. However, it is less effective in ameliorating Aβ toxicity than myricetin that has an additional OH group.[19,20] On the other hand, the antifibrillogenic property of a chemical might even enhance Aβ toxicity if the toxic Aβ species accumulate while inhibiting Aβ from forming mature fibrils; the chaperone molecule clusterin is such an example.[21]

Flavonoids are naturally occurring polyphenolic compounds that have been shown to exhibit antifibrillogenic and cytoprotective properties.[14,20,22] There are at least two types of flavonoids. Monoflavonoids are planar with two terminal phenyl rings, which include myricetin, morin, quercetin, kaempferol, epicatechin, and apigenin (AP), while biflavonoids consist of two interconnected monoflavonoids (Figure 1). Cytoprotective effects of some biflavonoids and its related flavones have been previously reported,[23,24] but their antifibrillogenic activity remains unknown. In this study, we investigated the effect of biflavonoids in Aβ42-induced toxicity and fibrillogenesis and compared their efficacies to that of a monomeric flavonoid, AP. We demonstrate that the biflavonoids exert a cytoprotective effect against Aβ toxicity more efficiently and specifically than AP by potently inhibiting Aβ fibrillogenesis.

MATERIALS AND METHODS

Materials. TF (taiwaniaflavone), 2′,8″-biapigenin, amentoflavone, and sumaflavone were isolated as previously described.[24] AP, thioflavin T, staurosporine (STS), brefeldin A (BFA), etoposide (ETO), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). AlamarBlue was obtained from Biosource (Camarillo, CA). Phosphate-buffered saline (PBS) [10 mM phosphate buffer (pH 7.4), 137 mM NaCl, and 2 mM KCl] was purchased from Amresco (Solon, OH). Dulbecco's modified Eagle's medium (DMEM), Ham's F12, and fetal bovine

serum (FBS) were purchased from Welgene (Daegu, Korea). Ac-DEVD-AMC (Caspase-3/7 substrate) was from A. G. Scientific Inc. (San Diego, CA). The ECL plus Western blotting detection kit was obtained from Amersham Biosciences (Piscataway, NJ). The monoclonal antibody, 6E10, was acquired from Signet Laboratories (Dedham, MA). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Preparation of the Aβ42 Monomer, Oligomers, and Fibrils. Recombinant Aβ42 was prepared as described previously.[25] Purified Aβ42 peptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), dried under a flow of nitrogen , and further dried under vacuum for 30 min. Samples were stored at −20 °C until they were used. For toxicity and fibrillogenesis experiments, the peptide was dissolved in 0.1% NH_4OH at a concentration of 2 mg/mL followed by bath sonication for 10 min. The peptide solution was then diluted to desired concentrations with PBS. Soluble Aβ42 oligomers were prepared as previously described with slight modifications.[4] Briefly, fresh Aβ42 was dissolved in serum-free DMEM (without phenol red), vortexed for 30 s, and incubated at 4 °C for 24 h without agitation. Then, the sample was centrifuged for 15 min at 16000*g*, and the supernatant, containing soluble Aβ42 oligomers, was removed. Fresh Aβ42 or Aβ42 oligomers were further diluted in serum-free DMEM (without phenol red) prior to cellular treatment. The prepared Aβ42 oligomers did not produce thioflavin T fluorescence. To prepare Aβ42 fibrils, Aβ was incubated in PBS at 37 °C for 3 days and centrifuged at 16000*g* for 15 min.

Cell Culture and Toxicity Assay. Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 (1:1) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics in 5% CO_2 at 37 °C. For the cytotoxicity assay, cells were seeded at a density of 15000 cells/well in 96-well plates. After overnight incubation, the medium was replaced with serum-free medium and the cells were further cultured for 12 h. Then, the cells were treated with 0.5 μM Aβ42 or other cytotoxic chemicals (25 nM STS, 1 μM BFA, or 1 μM ETO) in the absence or presence of flavonoids. The final DMSO concentration in cell culture was approximately 0.2% for all experiments. Cell viability was assessed by the MTT reduction and alamarBlue assays. Briefly, 20 μL of an MTT solution (5 mg/mL) was added to each well. After 2 h, 100 μL of solubilization buffer [20% (v/v) SDS solution in 50% (v/v) DMF (pH 4.7)] was added. The absorbance was recorded at 570 nm after 12−16 h using a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA). For the alamarBlue assay, after Aβ42 treatment for 12 h, alamarBlue reagent was added to each well at a final concentration of 10%. After incubation for 4 h, fluorescence was measured at excitation and emission wavelengths of 560 and 590 nm, respectively.[26]

Caspase Activity Assay. Cell cultures and treatment were performed as described above. The cells were then washed twice with ice-cold PBS before the addition of 40 μL of cell lysis buffer [20 mM HEPES-NaOH (pH 7.0), 1 mM EDTA, 1 mM EGTA, 20 mM NaCl, 10 mM DTT, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 5 μg/mL pepstatin A, 2 μg/mL aprotinin, and 25 μg/mL ALLN]. After the sample had been incubated on ice for 20 min, 50 μL of caspase assay buffer [20 mM HEPES-NaOH (pH 7.0), 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 10 mM DTT] was added. DEVDase (Caspase-3/7-like) activity was initiated by adding 10 μM Ac-DEVD-AMC at 30 °C, and the release of AMC was monitored at excitation and emission wavelengths of 360 and 480 nm, respectively, using a microplate spectrofluorometer (Gemini-XS, Molecular Devices). Caspase activity was calculated as the initial slope of the emission intensity versus time curves.

Thioflavin T Binding Assay for the Study of Aβ42 Fibrillogenesis. Aβ42 fibrillogenesis in PBS at 37 °C (300 μL of 20 μM Aβ) was measured without shaking. At each time point, 20 μL of the sample was mixed with 80 μL of 5 μM thioflavin T in PBS and the fluorescence was monitored immediately at excitation and emission wavelengths of 445 and 490 nm, respectively, using a microplate spectrofluorometer.[20,28] For nucleation-dependent polymerization, thioflavin T fluorescence versus time profiles show a characteristic sigmoidal shape and kinetic parameters of fibrillogenesis can be extrapolated from a semilogarithmic plot of fluorescence versus time. Briefly, values of $\log\{F(t)/[F(\infty) - F(t)]\}$ versus time were plotted, and the straight lines were fitted by $at + b$, where t is the reaction time, $F(t)$ is the fluorescence as a function of time, $F(\infty)$ is the fluorescence at the equilibrium state, and a and b are the slopes and y-intercepts of the fitted lines, respectively.[27] The rate of fluorescence increase (or rate of polymerization) is highest at the half-maximum of $F(t)$ or at time $t_{1/2}$, at which $\log\{F(t)/[F(\infty) - F(t)]\}$ equals 0. Alternatively, $t_{1/2}$ can also be solved by setting $at_{1/2} + b$ equal to 0.[27] For the fibril extension assay, seeded polymerization was performed in which Aβ42 fibrils (nuclei) were added to fresh Aβ42 and incubated in the absence or presence of flavonoids.

Circular Dichroism Spectroscopy. CD spectra of Aβ42 in solution were recorded in a 1 mm path length cuvette at a 0.5 nm interval and 1 nm resolution between 190 and 250 nm at 25 °C using a Jasco (Tokyo, Japan) spectropolarimeter. The scan rate was 50 nm/min. Five scans were recorded and averaged for each sample. Aβ spectra were determined by subtracting that of buffer (PBS containing 0.2% DMSO and 0.004% NH_4OH). The spectra of Aβ incubated with TF or AP were obtained by subtracting background spectra given by TF or AP in buffer.[25] The background was subtracted from the measurements, and average spectra were smoothed using the means-movement algorithm using the Jasco spectrum analysis program.

Transmission Electron Microscopy. A 5 μL aliquot of sample was adsorbed on a Formvar-coated 200-mesh nickel grid for 15 min and air-dried. Subsequently, the grid was negatively stained with 2% uranyl acetate for 1 min and washed with distilled water. The samples were then analyzed by TEM (H-7600, Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV at 40000-fold magnification.[14]

Detection of Aβ42 Oligomers by an Immunoblotting Assay. To detect the oligomeric forms of Aβ42, we incubated the peptide in PBS at 37 °C in the absence or presence of flavonoids. Peptide species in the reaction mixtures were cross-linked before being analyzed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) except for freshly dissolved Aβ42 (control), which was boiled for 5 min before SDS−PAGE. Briefly, glutaraldehyde was added to the sample at a final concentration of 0.01% (v/v), and the mixture was incubated for 5 min. The cross-linking reaction was then terminated with an equal volume of SDS buffer [50 mM Tris buffer (pH 6.8), 10% glycerol, 2% SDS, and 0.1% β-mercaptoethanol]. Samples were then run on a SDS−PAGE gel (16% acrylamide) without boiling and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). After being blocked with 5% milk in Tris-buffered saline containing 0.2% (v/v) Tween 20 at room temperature for 1 h, the membranes were probed with primary monoclonal

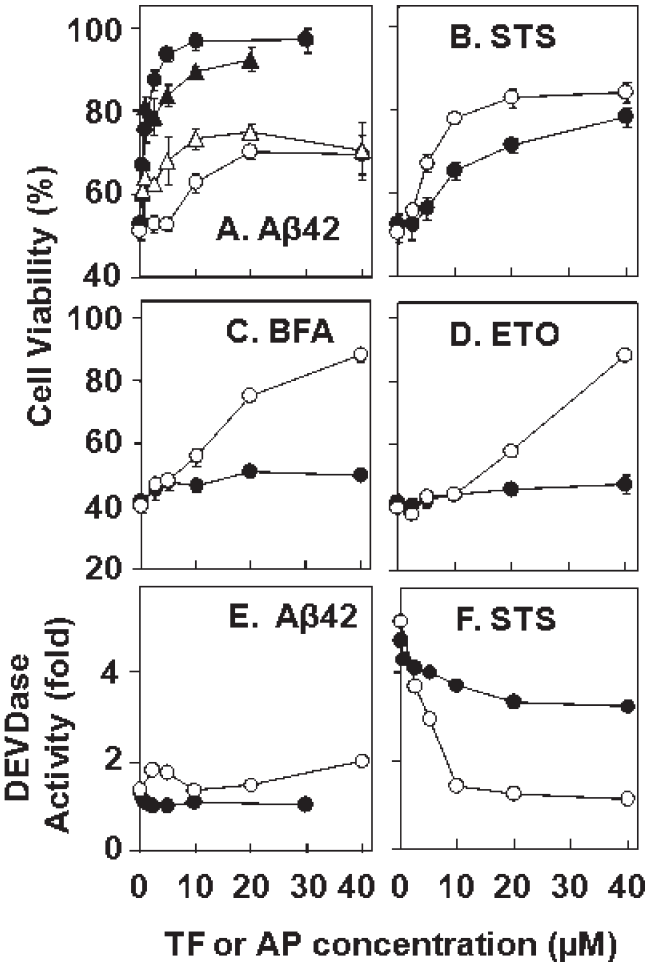

Figure 2. Effect of TF and AP on the viability and caspase activity of SH-SY5Y cells treated with Aβ42, STS, BFA, and ETO. (A−D) Cell viability was assessed by MTT assays in SH-SY5Y cells treated with 0.5 μM Aβ42 (A), 25 nM STS (B), 1 μM BFA (C), and 1 μM ETO (D) in the presence of TF (●) or AP (○) for 12 h (Aβ42 and STS) or 24 h (BFA and ETO). The viablility was further assessed by an alamarBlue assay for panel A (data for samples of TF and AP denoted with ▲ and Δ, respectively). Error bars indicate the standard deviation of triplicate independent experiments. (E and F) DEVDase (caspase-3/7-like) activity in cells treated with 0.5 μM Aβ42 (E) or 25 nM STS (F) in the presence of TF (●) or AP (○) monitored using 10 μM Ac-DEVD-AMC after incubation for 12 h.

antibody 6E10 and the anti-mouse horseradish peroxidase-conjugated secondary antibodies.[14] The blots were visualized using the ECL plus reagent kit.

RESULTS

TF Specifically and Potently Inhibits Aβ Toxicity. The chemical structures of TF and other biflavonoids are shown in Figure 1. Note that TF, isolated from *Selaginella tamariscina*, is composed of two APs connected by a 3,3‴ linkage.[24] First, we compared the cytoprotective effects of TF and AP on Aβ42-induced toxicity in cultured SH-SY5Y cells using the MTT and alamarBlue assays.[28] Only ~1 μM TF was necessary to increase the cell viability by 50% (EC_{50}) (filled symbols in Figure 2A), while more than 10 times more AP was required to obtain a similar level of rescuing effect (empty symbols in Figure 2A).

We also examined the effect of TF or AP on cell death induced by other known cytotoxic agents such as STS (a protein kinase inhibitor), BFA (an inhibitor of transport of protein from ER to Golgi), and ETO (a topoisomerase II inhibitor). TF was less effective than AP at protecting cells from STS-induced cell death (filled circles in Figure 2B). The observed EC_{50} of AP on STS-exposed cells was >5 μM, and AP was not able to completely suppress toxicity at concentrations up to 40 μM (empty circles in Figure 2B). Moreover, TF was barely effective at rescuing BFA- and ETO-treated cells (filled circles in Figure 2C,D). On the other hand, TF had a marginal effect on STS-induced caspase-3/7 activation (DEVDase), which is a hallmark of apoptosis, whereas AP effectively inhibited the activation (Figure 2F). Known as a protein kinase inhibitor, STS induces mitochondrion-mediated apoptosis in which caspases play important roles in signal transduction,[29] implying that the suppressive effect of AP on the cell death may be due to an inhibitory effect on a step of the caspase activating pathway, although the exact mechanism remains to be elucidated. Note that the treatment of Aβ42 induced only a low level of caspase-3/7 activity, and consequently, the effects of the flavonoids on the activity were difficult to detect (Figure 2E). All these clearly suggest that TF is highly effective at rescuing cells from Aβ42-induced toxicity, whereas AP is only moderately effective. In contrast to TF, AP demonstrated a broad spectrum, nonspecific cytoprotection against several toxic insults, including Aβ.

Kinetic Studies of Aβ Fibrillogenesis and Fibril Destabilization in the Presence of TF or AP. Many polyphenolic compounds are known to influence Aβ fibrillogenesis by inhibiting or accelerating Aβ polymerization.[30] We examined and compared the effect of TF or AP in the polymerization of Aβ42 with a thioflavin T fluorescence assay. In these experiments, 20 μM Aβ42 was used because it is above the reported critical micelle concentration of the peptide[31] and forms fibrils in a reasonable time frame such that its aggregation kinetics can be easily captured. Figure 3A shows the fluorescence profiles of Aβ42 aggregation with increasing TF concentrations. The profiles exhibit the characteristic sigmoidal shape, expected for a nucleation-dependent polymerization mechanism,[20,27] where fluorescence plateaued after 6 h. The final fluorescence level, indicative of the amount of mature fibrils formed, decreased with an increasing TF concentration (Figure 3A). The addition of AP also decreased thioflavin T fluorescence levels, but less effectively compared to addition of TF (Figure 3B). Semilogarithmic plots of thioflavin T fluorescence versus time gave a linear relationship for the indicated time (Figure 3C,D). From these plots, $t_{1/2}$ values were obtained (see Materials and Methods), which gives us information about the kinetics of the nucleation step during the early stage of fibrillogenesis.[27] It is expected that the $t_{1/2}$ value would increase if the flavonoids inhibited the nucleation process, the rate-determining step of polymerization. For Aβ incubated with TF, $t_{1/2}$ values were 55 ± 2, 62 ± 1, 64 ± 1, and 70 ± 1 min (average ± standard deviation) for 0, 0.5, 1, and 2 μM TF, respectively. For Aβ incubated with AP, $t_{1/2}$ values were 58 ± 2, 65 ± 2, and 73 ± 2 min for 1.25, 2.5, and 5 μM AP, respectively. The small changes in $t_{1/2}$ values with either TF or AP indicated that flavonoids marginally act on the nucleation step.[27] We also noticed the disappearance of the linearity at the higher concentrations of flavonoids or upon longer incubation times (Figure 3C,D), implying the involvement of unidentified processes and, thus, rendering the logistic equation invalid.

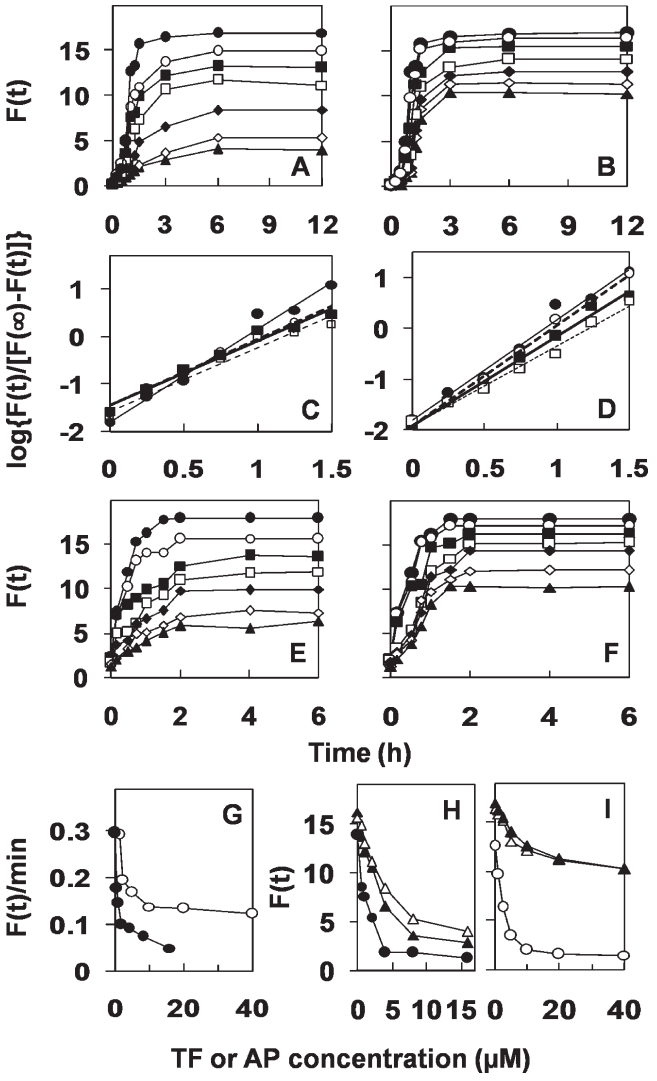

Figure 3. Effect of TF and AP on the fibrillogenesis of Aβ42. Formation of fibrils was assessed via a thioflavin T binding assay. (A and B) Fluorescence intensity of 20 μM Aβ42 incubated in PBS at 37 °C in the presence of 0 (●), 0.5 (○), 1 (■), 2 (□), 4 (◆), 8 (◇), and 16 μM TF (▲) or 0 (●), 1.25 (○), 2.5 (■), 5 (□), 10 (◆), 20 (◇), and 40 μM AP (▲) for 0−12 h. (C and D) Logarithmic plot of $\{F(t)/[F(\infty) - F(t)]\}$ calculated from data in panels A and B as described in Materials and Methods. A linear relationship was observed at the indicated time and concentrations [correlation coefficients, R^2, of 0.99, 0.98, 0.99, and 0.98 for 0 (●), 0.5 (○), 1 (■), and 2 μM TF (□) or 0.99, 0.99, 0.99, and 0.99 for 0 (●), 1.25 (○) 2.5 (■), and 5 μM AP (□), respectively]. (E and F) Results of the fibril extension assay. Aβ42 fibrils (nuclei) (1.1 μM) were mixed with fresh Aβ42 (20 μM) and incubated in the presence of 0 (●), 0.5 (○), 1 (■), 2 (□), 4 (◆), 8 (◇), and 16 μM TF (▲) or 0 (●), 1.25 (○), 2.5 (■), 5 (□), 10 (◆), 20 (◇), and 40 μM AP (▲) for 0−6 h. (G) Initial rates of Aβ42 nucleation-dependent polymerization in the presence of TF (●) or AP (○). The rates were determined by calculating the slope of early linear portions in panels E and F. (H and I) Differential effects of TF (H) or AP (I) on Aβ42 polymerization according to the incubation times, 1 h (● and ○), 3 h (▲), and 12 h (△). These were redrawn using data from panels A and B. $F(t)$, the fluorescence at time t, was normalized by subtracting the background.

Next, we compared the effect of TF or AP on Aβ fibril extension by measuring the thioflavin T fluorescence of fresh Aβ samples seeded with preformed fibrils (nuclei). As shown in panels E and F of Figure 3, the fluorescence increased without a lag phase to give hyperbolic curves and reached a plateau in ~2 h.[20,27] The addition of TF or AP led to concentration-dependent decreases of the final amount of fibrils formed. Consistent with our previous observation, TF was more effective at retarding fibril elongation than AP (Figure 3E,F). To further elucidate the difference between TF and AP in fibril extension, the initial rates of Aβ fibril extension, the slopes of the linear portion of the fluorescence profiles, were extrapolated and compared. TF and AP both decreased the rate of Aβ fibril extension, with TF being the more effective inhibitor (Figure 3G). TF and AP did not significantly impact fibril nucleation during the lag phase (Figure 3C,D), but once the rapid Aβ elongation phase was initiated, AP inhibited Aβ fibrillogenesis less efficiently (Figure 3G), which resulted in a large difference in the final level of fibrils formed (Figure 3H,I). Our data clearly demonstrated that the effects of the two flavonoids on fibril formation may stem from the different effects they exert on Aβ fibril extension.

The initial rates of fibril extension in the absence or presence of TF were linearly dependent on Aβ42 concentration (Figure 4A). The linear relationship indicates that fibril extension is a first-order reaction with respect to Aβ concentration. The net rate of fibril extension has contributions from both polymerization and depolymerization.[32] To further elucidate whether the observed reduced fibril growth rate by flavonoids is due to a decreased level of polymerization or an increased level of depolymerization, the effect of the flavonoids on the disaggregation of preformed Aβ42 fibrils was examined. As shown in panels B and C of Figure 4, the fluorescence of preformed fibrils rapidly decreased with increasing flavonoid concentration, indicating that incubating fibrils with flavonoids caused the fibrils to disaggregate. Again, TF was more effective than AP at disaggregating fibrils. The fibril disaggregation was examined by TEM. As shown in Figure 4D, Aβ42 prepared by incubation for 3 days was predominantly composed of fibrils. The fibrils were converted to smaller aggregates in the presence of TF; a large aggregate was detected in the presence of AP, but its shape was altered greatly (Figure 4D). It appears that TF really disaggregates the fibrils, while AP may transform it to another form whose molecular details remain to be elucidated. The IC_{50} values of TF, AP, and other biflavonoids for Aβ42 polymerization, extension, and disaggregation are summarized in Table 1. Approximately 10 times more AP than TF was needed to inhibit fibril formation or to induce fibril disaggregation or transformation to the same degree (Table 1). Taken together, our data clearly show that TF is more antifibrillogenic than AP.

TF Inhibits the Structural Transformation of Aβ42. To improve our understanding of the inhibitory role of TF or AP in Aβ fibrillogenesis, we measured the secondary structures of Aβ42 in the absence or presence of TF or AP by CD; 20 μM Aβ42 was used in these experiments. Freshly prepared Aβ42 in PBS exhibited strong negative ellipticity at ~195 nm (Figure 5A,B), indicative of a largely unfolded, presumably monomeric state with random coil properties. Upon incubation at 37 °C, the peptide exhibited changes in the far-UV CD spectrum and reached a maximal negative ellipticity at 217 nm after 12 h (Figure 5A,B). These changes arise from the formation of β-sheet structures in the peptides.[25] The random coil to β-sheet transition was not observed in the presence of TF at 20 μM Aβ where the peptide remained as a random coil during incubation for up to 12 h (Figure 5A). TF thus effectively suppressed the formation of β-sheet structures of Aβ peptides. In contrast, AP was largely ineffective at inhibiting the formation of β-sheets (Figure 5B),

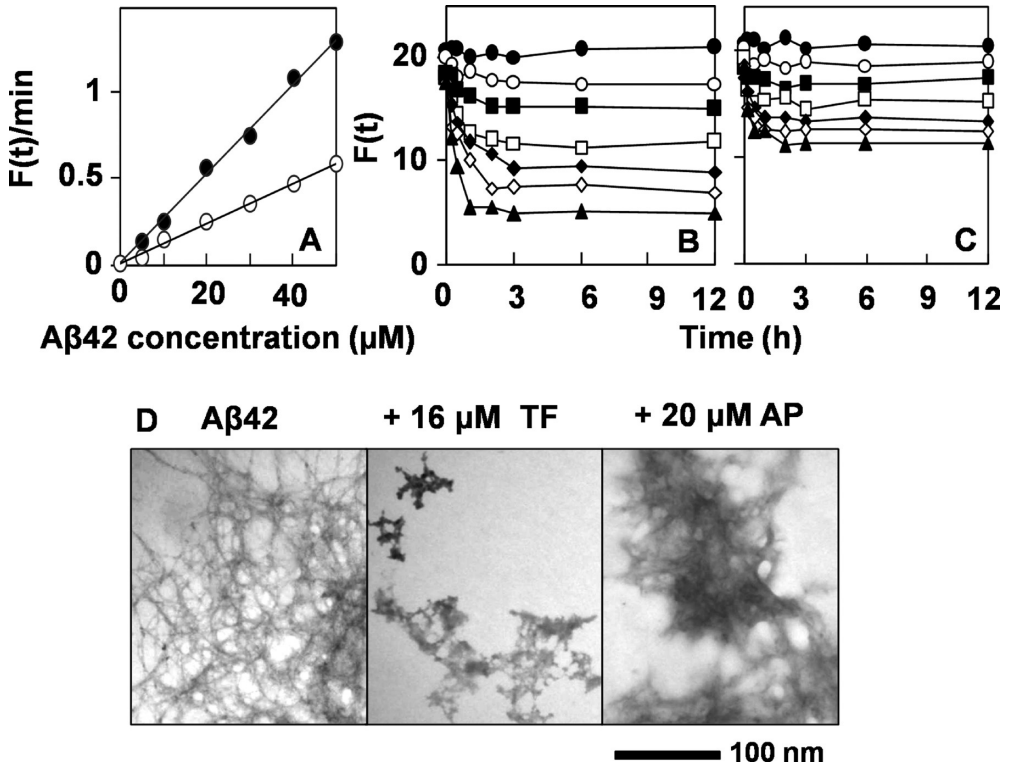

Figure 4. Effect of TF on Aβ42 fibril extension and disaggregation of Aβ fibrils by TF and AP. (A) The initial rate of Aβ42 fibril extension was determined as described in the legend of Figure 3G with varying concentrations of Aβ42 in the absence (●) or presence (○) of 8 μM TF. A linear relationship with a positive slope was found (R^2 = 0.99 for both data sets). (B and C) Destabilization of preformed Aβ42 fibrils. Fibrils (20 μM) were incubated in the presence of 0 (●), 1 (○), 2 (■), 4 (□), 8 (◆), 16 (◇), and 20 μM TF (▲) or 0 (●), 1.25 (○), 2.5 (■), 5 (□), 10 (◆), 20 (◇), and 40 μM AP (▲) for 0−12 h. Then, amounts of fibrils were measured as described for the legend of Figure 3A. $F(t)$, the fluorescence at time t, was normalized by subtracting the background. (D) TEM images of fibrillar Aβ42 (0.5 μM) incubated either alone or in the presence of TF or AP in PBS at 37 °C for 12 h. The scale bar is shown at the bottom.

which is consistent with its poor antifibrillogenic activity compared to that of TF.

TF Accumulates Oligomeric Aβ Structures. Aβ fibrillogenesis (Figures 3 and 4) and structural analysis data (Figure 5) clearly showed that TF potently inhibits Aβ42 fibrillogenesis and the formation of β-sheet structures. In addition, TF is also effective at disaggregating preformed Aβ fibrils. These properties of TF may explain its cytoprotective effect of Aβ-induced cell cytotoxicity (Figure 2). However, because the cells were incubated with monomeric Aβ42 at a lower concentration, 0.5 μM, rather than the value of 20 μM used in fibrillogenesis experiments, we further examined the effect of the flavonoids on Aβ fibrillogenesis under the same conditions (0.5 μM and incubation for 12 h) that were used in cell culture experiments. In addition, it was necessary to characterize Aβ intermediates formed in the absence or presence of flavonoids during the cell culture time frame because these intermediates are more toxic than fresh Aβ or fibrils[33,34] and thioflavin T cannot detect them.[35] Moreover, the thioflavin T fluorescence assay for Aβ fibril detection can be biased by the presence of flavonoids.[36] For this purpose, Aβ species formed in the incubated samples were imaged with TEM. Aβ incubated alone was predominantly composed of typical fibrils (Figure 6A). In the Aβ samples containing TF, however, fibrillar structures were not observed. Instead, small spherical and branched structures were detected (Figure 6A). The morphology and size of these structures are consistent with those of oligomer and protofibrils previously reported.[30,37,38] In the Aβ samples containing AP, both beaded protofibrillar structures and amorphous deposits were present (Figure 6A).

To confirm whether Aβ structures formed in the absence or presence of TF or AP were oligomers, we performed an immunoblotting assay using Aβ-specific antibody 6E10. In this experiment, we used a chemical cross-linker to cross-link the Aβ species prior to SDS−PAGE analysis to avoid SDS-induced dissociation of oligomers. To validate the method before probing the effect of the flavonoids on the formation of Aβ oligomers, we cross-linked preformed oligomers prepared as described in Materials and Methods and visualized them with the immunoblotting assay (Figure 6B). Compared to the incubated sample that was not cross-linked (Figure 6B, lanes 2 and 3), significantly more Aβ oligomers spanning a wide size range were detected in the cross-linked sample (Figure 6B, lane 4). On the other hand, cross-linking did not result in the formation of oligomers in freshly dissolved Aβ (data not shown). Thus, the immunoblotting assay using cross-linked samples is a reliable method for detecting oligomers.

Without added flavonoids, 0.5 μM Aβ incubated for 3 h (Figure 6C, lane 2) showed a range of higher-molecular mass oligomers and monomeric Aβ (band at the bottom of the gel). However, after incubation for 12 h, the monomeric Aβ band completely disappeared (Figure 6D, lane 2), indicating that all the Aβ had aggregated into higher-molecular mass species. When TF was added to the Aβ samples, increasing amounts of small Aβ oligomers (dimers, trimers, and tetramers) were detected with increasing TF concentrations after incubation for 3 h (Figure 6C,

Table 1. IC_{50} Values of Biflavonoids and AP[a]

flavonoid	polymerization (μM)	extension (μM)	destabilization (μM)
TF	2.41 ± 0.37	3.22 ± 0.39	13.0 ± 2.1
AP	23.3 ± 4.7	39.1 ± 5.9	>40
2′,8″-biapigenin	2.90 ± 0.35	4.19 ± 0.54	15.8 ± 2.2
amentoflavone	3.37 ± 0.40	6.91 ± 0.43	20.8 ± 1.4
sumaflavone	4.48 ± 0.96	11.2 ± 1.8	18.0 ± 1.8

[a] IC_{50} is defined as the concentration of the compound required to reduce the rate of polymerization, fibril extension of fresh Aβ42, or destabilization of preformed Aβ42 fibrils by 50%. The values were calculated by plotting fluorescence (percent) on a logarithmic scale vs concentrations of compounds as described previously.[20] Values are means ± the standard deviation drived from triplicate independent experiments.

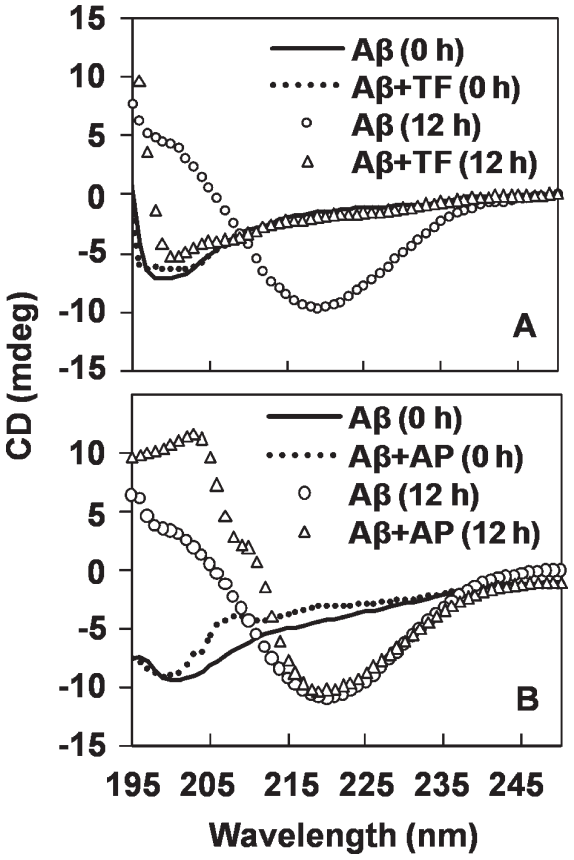

Figure 5. Effect of TF and AP on the structural transformation of Aβ42. CD spectra of 20 μM Aβ42 incubated alone or in the presence of 20 μM TF (A) or 20 μM AP (B) in PBS at 37 °C for 0 or 12 h. The spectra were obtained by subtracting the buffer background as described in Materials and Methods.

lanes 3−5). Significant amounts of monomeric Aβ remained in all of these samples. When incubated for longer times (12 h), all samples accumulated more small Aβ oligomers (Figure 6D, lanes 3−5) while still containing significant amounts of monomeric Aβ. With the addition of AP, larger oligomers (>30 kDa) were observed (Figure 6C,D, lanes 6−8), but not low-molecular mass Aβ oligomers. Taken together, our data show that TF, but not AP, favors the formation and accumulation of small Aβ oligomers. These data completely agree with TEM results (Figure 6A).

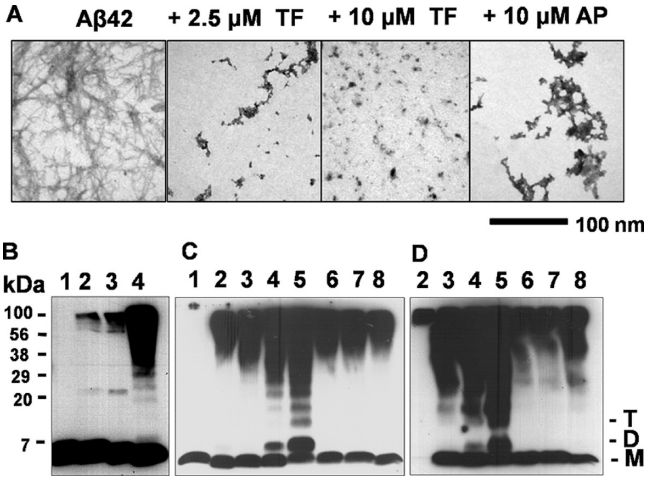

Figure 6. Effect of TF and AP on the morphology and fibrillogenesis of Aβ42. (A) TEM images of Aβ42 (0.5 μM) incubated either alone or in the presence of TF or AP in PBS at 37 °C for 12 h. The scale bar is shown at the bottom. (B) Immunoblot analysis of Aβ42 oligomers probed with the 6E10 monoclonal antibody: lane 1, fresh Aβ42 as a control; lane 2, Aβ42 subjected to the oligomerization process; lane 3, Aβ42 recovered in the soluble fraction after centrifugation of the mixture from lane 2; lane 4, Aβ42 of lane 3 cross-linked before the immunoblot assay. (C and D) Immunoblot analysis of Aβ42 aggregates in the presence of TF and AP. Aβ42 (0.5 μM) was incubated for 3 h (C) or 12 h (D) either alone or in the presence of TF or AP and then cross-linked, except lane 1 (fresh Aβ42), before the immunoblot analysis: lane 1, fresh Aβ42; lane 2, Aβ42 incubated alone; lanes 3−8, Aβ42 incubated in the presence of 2.5, 5, and 10 μM TF (lanes 3−5, respectively) or AP (lanes 6−8, respectively). M, D, and T indicate monomeric, dimeric, and trimeric Aβ42, respectively. Molecular masses of protein standards are given to the left of panels B−D.

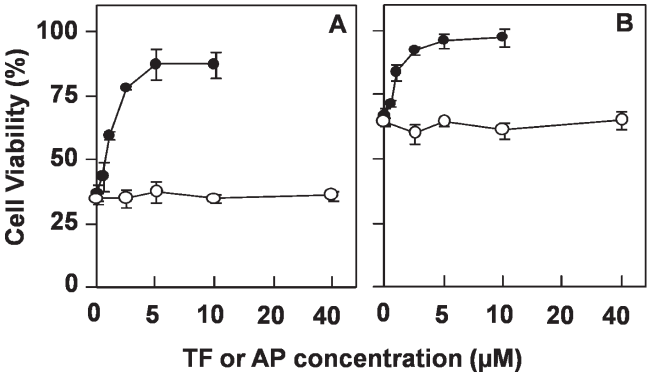

Figure 7. Effect of TF and AP on the viability of SH-SY5Y cells treated with Aβ42 oligomers and fibrils. Cells were treated with 0.5 μM Aβ42 oligomers (A) or fibrils (B) prepared as described in Materials and Methods in the presence of TF (●) or AP (○) at the indicated doses for 12 h. Cell viability was assessed by the MTT reduction assay. Error bars indicate the standard deviation of triplicate independent experiments.

TF Is Cytoprotective against Preformed Aβ42 Oligomers and Fibrils. Our results so far show that TF is cytoprotective to cells exposed to monomeric Aβ42. The monomers, however, can be transformed into nontoxic oligomers in the presence of TF during the incubation time (12 h) (Figure 6). It has been widely reported that soluble Aβ oligomers are the primary cytotoxic species.[34,35] We therefore tested whether TF or AP can counteract the toxicity of preformed oligomers and fibrils on cultured cells.

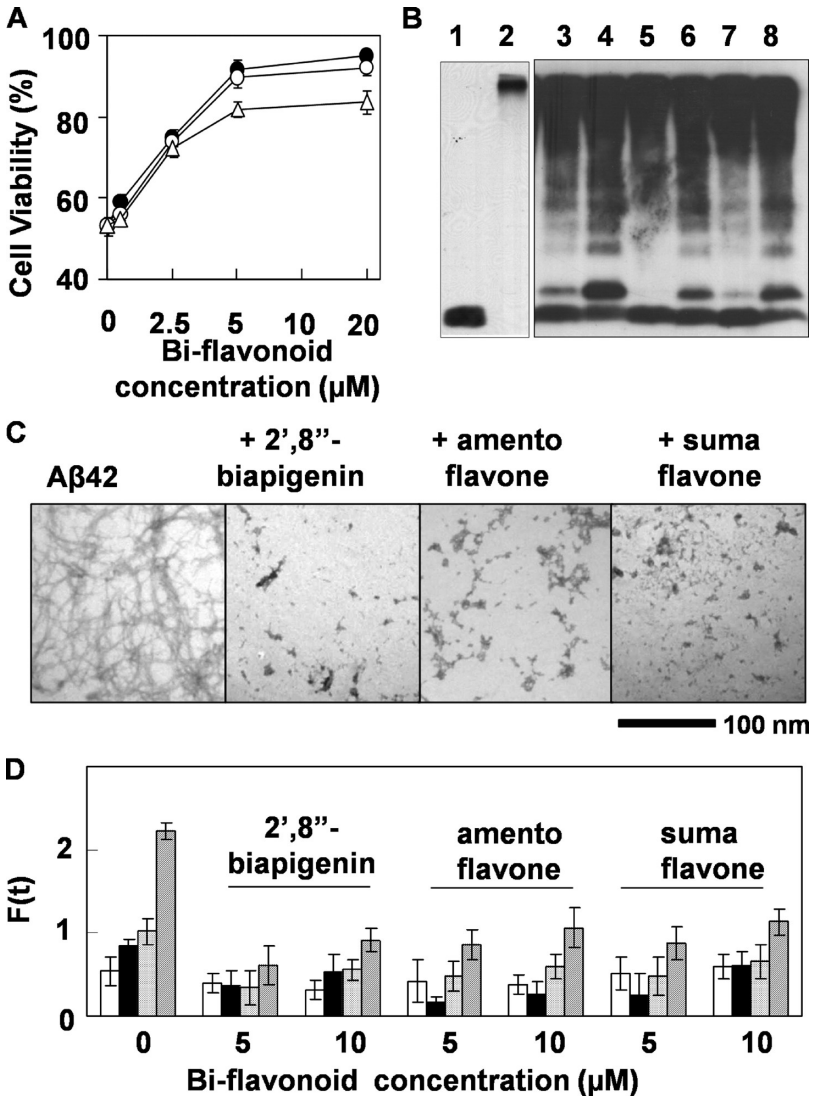

Figure 8. Effect of other related biflavonoids on Aβ42 toxicity and fibrillogenesis. (A) Viability of cells treated with Aβ (0.5 μM) in the absence or presence of 2′,8′′-biapigenin (●), amentoflavone (○), or sumaflavone (△) at the indicated concentrations for 12 h. (B) Immunoblot analysis of Aβ42 fibrillogenesis in the presence of other different biflavonoids. Aβ42 (0.5 μM) was incubated for 12 h either alone or in the presence of biflavonoids, cross-linked (except lane 1), and then analyzed by an immunoblot assay using monoclonal antibody 6E10: lane 1, fresh Aβ42; lanes 2−8, Aβ42 incubated without biflavonoids (lane 2) or with 2′,8′′-biapigenin at 5 μM (lane 3) and 10 μM (lane 4), amentoflavone at 5 μM (lane 5) and 10 μM (lane 6), or sumaflavone at 5 μM (lane 7) and 10 μM (lane 8). (C) TEM images of Aβ42 incubated alone or in the presence of 10 μM biflavonoids for 12 h. Scale bars are shown at the bottom. (D) Thioflavin T assay of Aβ42 (0.5 μM) incubated in the absence or presence of biflavonoids at the indicated doses. The fluorescence was measured at 0 h (white bar), 3 h (black bar), 6 h (dotted bar), and 12 h (hatched bar). Error bars indicate the standard deviation of triplicate independent experiments (A and B).

Compared to freshly prepared Aβ42, preformed Aβ oligomers induced ∼16% more cell death after a 12 h incubation period (Figures 2A and 7A). The addition of TF was effective at rescuing the cells from oligomer- and fibril-induced toxicity where 10 μM TF decreased cell toxicity induced by oligomers by ∼40% and nearly ameliorated the toxicity induced by Aβ fibrils (Figure 7). In contrast, AP did not rescue cells exposed to Aβ oligomers or fibrils (Figure 7B). These data suggest that TF is effective at blocking the toxicity induced by Aβ oligomers and fibrils whereas AP is completely ineffective, implying that AP is not cytoprotective against the toxic Aβ oligomers and fibrils formed before its treatment. However, our earlier data indicated that AP did exert some rescuing effect toward toxicity induced by Aβ monomers (Figure 2A). This is possibly due to the weak but significant antifibrillogenic property of AP at the early stages of fibrillogenesis as shown in Figures 3 and 4.

Effects of Other Related Biflavonoids on Aβ Toxicity and Fibrillogenesis. To determine whether the inhibition of Aβ42 toxicity and fibrillogenesis is a unique property to TF or a general property of biflavonoids, we tested other related biflavonoids,[24,39] including 2′,8′′-biapigenin, amentoflavone, and sumaflavone (Figure 1). All tested biflavonoids increased the viability of the cells treated with 0.5 μM fresh Aβ42, albeit with slightly higher EC_{50} values (∼2.5 μM) compared to that of TF (Figure 8A). These biflavonoids also suppressed Aβ fibrillogenesis under conditions similar to those in cell culture (Figure 8B) or at 20 μM Aβ42 (Table 1). IC_{50} values for Aβ polymerization, extension, and decomposition of the fibrils varied slightly among

the biflavonoids (Table 1). TF and all the other the biflavonoids examined so far are more effective than AP, implicating the importance of the dimeric flavonoid structure for their antifibrillogenic and cytoprotective effects. TEM and immunoblotting assays also revealed that the biflavonoids, similar to TF, favor the formation and accumulation of oligomer-like structures (Figure 8C,D). These data clearly show that biflavonoids exert their cytoprotective effect in Aβ-induced toxicity by accumulating nontoxic oligomers.

DISCUSSION

The monoflavonoid, AP, reduces Aβ toxicity by abrogating the activation of caspase-3 and the release of cytochrome *c*.[40] The cytoprotective effect of AP is also linked with its antifibrillogenic and antioxidative activity. On the other hand, biflavonoids have also been shown to reduce Aβ toxicity and oxidative stress,[24,39] but the underlying molecular mechanism(s) by which biflavonoids attenuate Aβ fibrillogenesis and toxicity remains unexplored. In this study, we investigated and compared the effects of mono- and biflavonoids to gain insights into the structure−function relationship of mono- and biflavonoids in Aβ-induced toxicity and fibrillogenesis.

Our results show that the TF and other related biflavonoids potently and specifically inhibit the toxicity induced by Aβ (Figures 2A and 8A). In contrast, the rescuing effect of AP in Aβ-induced toxicity was less potent and nonspecific, as AP reduced cytotoxicity induced by a number of compounds, including Aβ, STS, BFO, and ETO, by a similar degree (Figure 2). AP suppressed caspase-3 activation in STS-induced cell death as described previously[41] much more efficiently than TF (Figure 2B). Altogether, it is apparent that TF or other biflavonoids either interact directly with Aβ or disrupt the specific toxicity pathway induced by Aβ. In contrast, AP exerts a general cytoprotective effect that is not specific to Aβ toxicity, and it may be due to an anti-apoptotic effect of AP.[40,41]

TF is composed of two monoflavonoids, AP-3′ and 3‴-AP. TF efficiently blocked Aβ fibrillogenesis (Figure 3). All the other biflavonoids tested in this study are more effective than the monoflavonoid, AP (Table 1), suggesting that the possession of two monoflavonoids in biflavonoids is a critical structural feature for potently inhibiting Aβ fibrillogenesis. TF is more effective in inhibiting Aβ fibril extension than nucleation (Figure 3C). Characteristic Aβ secondary structures, namely β-sheets, were not found in the presence of TF (Figure 5A). Furthermore, the linear relationship between the initial rate of fibril extension and the concentrations of Aβ implies that polymerization and depolymerization occur simultaneously (Figure 4A). Our results suggest that TF fundamentally alters the aggregation pathway of Aβ, possibly by interacting with free Aβ and preventing it from misfolding into the β-sheet conformation found in fibrils. The stoichiometry of TF binding to Aβ, however, is unknown. The requirement that the two phenyl groups in curcumin be able to inhibit Aβ aggregation implies the existence of at least two binding sites on Aβ.[12] Insertion of a long flexible linker (>19 Å) between them prevented them from inhibiting the aggregation,[12] which probably excludes an interaction of curcumin with multiple Aβ proteins. Similar speculation may be applicable to other biflavonoids tested here.

As shown in Figure 6, the presence of TF suppressed the formation of mature Aβ fibrils but led to the formation and accumulation of Aβ oligomer-like structures. These results are in agreement with TF's antifibrillogenic property. However, because oligomeric Aβ species have been found to be more toxic than monomeric or fibrillar Aβ species,[33,34] the presence of TF might increase Aβ cytotoxicity if the accumulated Aβ oligomers are the same as the "on-pathway" or toxic Aβ oligomers. This is clearly not the case, because cell viability increased significantly in the presence of TF or other biflavonoids (Figures 2A and 8A). Thus, the oligomeric aggregates that accumulated in the presence of TF are not only structurally different from on-pathway Aβ aggregates (Figure 5) but also functionally different from oligomers obtained by Aβ incubation without TF. Moreover, TF abrogated the toxicity of preformed Aβ oligomers and fibrils (Figure 7), probably by disaggregating the toxic aggregates. Previously, it was demonstrated that polyphenol (−)-epigallocatechin gallate efficiently inhibited the fibrillogenesis of Aβ by directly binding to the unfolded polypeptides and promoted the formation of unstructured and nontoxic Aβ, resulting in off-pathway, highly stable oligomers.[17] Similarly, TF or other related biflavonoids appear to redirect Aβ fibril formation to form unstructured and nontoxic "off-pathway oligomers".[17,42]

Development of small molecule inhibitors to inhibit Aβ toxicity has emerged as an encouraging approach to treating AD. Hence, several flavonoids have been examined for their therapeutic potentials against amyloid-mediated diseases. Compared to monoflavonoids, dimeric biflavonoids may be promising therapeutics because their monomeric moieties are good Aβ binders and these moieties are separated by an appropriate length linker that satisfies structural requirements for the inhibition of Aβ fibrillogenesis. On the other hand, some monoflavonoids were shown to have IC_{50} values 1 order of magnitude lower than that of TF.[20] It will be interesting to examine whether the dimeric forms of those monoflavonoids would have also lower IC_{50} values as shown here.

In conclusion, our results show that biflavonoids more specifically inhibit Aβ42-induced toxicity than monoflavonoids by redirecting and/or reverting toxic Aβ aggregates to form off-pathway, nontoxic, and unstructured Aβ oligomers and intermediates. This comparative study of mono- and biflavonoids suggests that dimeric flavonoids may be useful AD therapeutics.

AUTHOR INFORMATION

Corresponding Author

*Department of Biomaterials Engineering, Chosun University, Gwanju 501-759, Republic of Korea. Phone: 8262-230-6753. Fax: 8262-227-834. E-mail: parkis@chosun.ac.kr.

Author Contributions

A.T. and E.-R.W. contributed equally to this work.

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ABBREVIATIONS

Aβ, amyloid-β peptide; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; AD, Alzheimer's disease; AP,

apigenin; BFA, brefeldin A; CD, circular dichroism spectroscopy; DMF, dimethylformamide; EC_{50}, concentration that reduces the cell viability by 50%; ETO, etoposide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IC_{50}, concentration of the compound required to reduce the rate of polymerization, fibril extension of fresh Aβ42, or destabilization of preformed Aβ42 fibrils by 50%; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; STS, staurosporine; TEM, transmission electron microscopy; TF, taiwaniaflavone.

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