Bi-flavonoids are Superior to Mono-flavonoid in Inhibiting Amyloid- β Toxicity and Fibrillogenesis through Accumulating Nontoxic Oligomer-like Structures

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Abstract

Polymerization of monomeric amyloid- β peptides (A β) 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 A β peptides can, therefore, be an effective therapeutic strategy for AD. In this study, we investigated the effects of mono- and bi-flavonoids on A β 42 toxicity and fibrillogenesis and found that the bi-flavonoid, taiwaniaflavone (TF) effectively and specifically inhibits A β toxicity and fibrillogenesis. Compared to TF, the mono-flavonoid apigenin (AP) is less effective and less specific. Our data showed that differential effects of the mono- and bi-flavonoids on A β fibrillogenesis correlate with their varying cytoprotective efficacies. We also found that other bi-flavonoids, namely 2',8''-biapigenin, amentoflavone, and sumafavone, can also effectively inhibit A β toxicity and fibrillogenesis, implying that the participation of two mono-flavonoids in a single bi-flavonoid molecule enhanced their activity. Bi-flavonoids, while strongly inhibited A β fibrillogenesis, accumulated nontoxic A β oligomeric structures, suggesting that these are off-pathway-oligomers. Moreover, TF abrogated the toxicity of preformed A β oligomers and fibrils, indicating that TF and other bi-flavonoids may also reduce the toxicity of toxic A β species. Altogether, our data clearly show that bi-flavonoids, possibly due to the possession of two A β binders separated by an appropriate size linker, are likely to be promising therapeutics to suppress A β toxicity.

Keywords: Amyloid- β , Taiwaniaflavone, Apigenin, Flavonoid, Fibrillogenesis, Toxicity

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive deposition of aggregated amyloid- β (A β) peptides in brain parenchyma and cerebral blood vessels [1]. Several isoforms of A β 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 A β are found in AD brains, A β 40 and A β 42. Native A β 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 A β oligomers and protofibrils formed during A β fibrillogenesis are the most toxic species and that their toxicity increases with the progression of AD^[2-4]. Although the A β 40 peptide is the most abundant isoform in the brain, A β 42 is enriched in AD brains as it progressively accumulates into extracellular senile plaques in AD brains^[5,6].

Flavonoids are naturally occurring polyphenolic compounds that have been shown to exhibit anti-fibrillogenic and cytoprotective properties^[19,21,23]. There are at least two types of flavonoids. Mono-flavonoids are planar with two terminal phenyl rings, which include myricetin, morin, quercetin, kaempferol, epicatechin and apigenin (AP), while bi-flavonoids consist of two interconnected mono-flavonoids (Fig. 1). Among bi-flavonoids, cytoprotective effects of taiwaniaflavone (TF) and its related flavones have been previously reported^[13], but their anti-fibrillogenic activity remains unclear. In the current study, we investigated the bi-flavonoids on the toxicity and fibrillogenesis of A β 42 and compared their

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Fig. 1. Chemical structures of TF, 2',8''-biapigenin, amentoflavone, sumaflavone, and epigenin

efficacies to that of a monomeric flavonoid, AP. We demonstrate that the bi-flavonoids exert cytoprotective effect against A β toxicity more efficiently and specifically than AP by potently inhibiting the fibrillogenic process.

2. Materials and Methods

2.1. Materials

TF, 2',8''-biapigenin, amentoflavone, and sumaflavone were isolated as previously described^[14]. AP, Thioflavin-T, staurosporine (STS), brefeldin A (BFA), etoposide (ETO) and dimethylsulphoxide (DMSO) were purchased from Sigma (St. Louis, MO). Phosphate buffered saline (PBS, 10 mM phosphate buffer, pH 7.4, 137 mM NaCl, and 2 mM KCl) was purchased from Amresco (Solon, Ohio). Dulbecco's modified eagle 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). ECL plus western blotting detection kit was obtained from Amersham biosciences (Piscataway, NJ). Monoclonal antibody, 6E10, was acquired from Signet laboratories (Dedham, MA). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Preparation of A β 42 Monomer, Oligomers, and Fibrils

Recombinant A β 42 was prepared as described earlier^[15]. Purified A β 42 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 use. For toxicity and fibrillogenesis experiments, the peptide was dissolved in 0.1% NH₄OH 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 A β 42 oligomers were prepared as previously described with slight modifications^[4]. Briefly, fresh A β 42 was dissolved in serum-free DMEM (without phenol red), vortexed for 30 seconds and incubated at 4°C for 24 h without agitation. Then, the sample was centrifuged for 15 min at 16,000 g and the supernatant, containing soluble A β 40 oligomers, was removed. Fresh A β 42 or A β 42 oligomers were further diluted in serum-free DMEM prior to cellular treatment (without phenol red). To prepare A β 42 fibrils, A β was incubated in PBS at 37°C for 3 days and centrifuged at 16,000 g for 15 min.

2.3. Cell Culture and Toxicity Assay

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's Modified Eagles Medium and Ham's F12

(1:1) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics in 5% CO₂ at 37°C. For cytotoxicity assay, cells were seeded at a density of 15,000 cells/well in 96-well plates. After incubation overnight, 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 μ M of A β 42 or other cytotoxic chemicals (25 nM STS, 1 μ M BFA or 1 μ M 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 assay. Briefly, 20 μ l of MTT solution (5 mg/ml) was added to each well. After 2 h, 100 μ l 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 to 16 h using a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA).

2.4. Caspase Activity Assay

The cells were then washed twice with ice-cold PBS before the addition of 40 μ l of cell lysis buffer (pH 7.0, 20 mM HEPES-NaOH, 1 mM EDTA, 1 mM EGTA, 20 mM NaCl, 10 mM DTT, 1 mM phenylmethanesulphonylfluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 2 μ g/ml aprotinin, and 25 μ g/ml ALLN). After incubating the sample on ice for 20 min, 60 μ l (50 μ l 2X) caspase assay buffer (20 mM HEPES-NaOH pH 7.0, 20 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA and 10 mM DTT) was added. DEVDase (Caspase-3/7-like) activity was initiated by adding 10 μ M 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, USA). Caspase activity was calculated as the initial slope of the emission intensity vs. time curves.

2.5. Circular Dichroism (CD) Spectroscopy

CD spectra of A β 42 in solution were recorded in a 1-mm path length cuvette at 0.5 nm interval and 1 nm resolution between 190 nm and 250 nm at 25°C using a Jasco Spectropolarimeter (Jasco Co., Tokyo, Japan). Scan rate was 50 nm/min. Five scans were recorded and averaged for each sample. A β spectra were determined by subtracting that of buffer (PBS containing 0.2% DMSO and 0.004% NH₄OH). The spectra of A β incu-

bated with TF or AP were obtained by subtracting background spectra given by TF or AP in buffer^[15]. The background subtracted and average spectra were smoothed using the means-movement algorithm using the Jasco spectra analysis program.

2.6. Transmission Electron Microscopy (TEM)

A 5 μ l aliquot of sample was adsorbed on Formvar-coated 200-mesh nickel grid for 15 min and air-dried overnight. 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, Japan) at an accelerating voltage of 80 kV at 40,000-fold magnification^[14].

2.7. Detection of A β 42 Oligomers by Immunoblotting Assay

To detect the oligomeric forms of A β 42, the peptide was incubated in PBS at 37°C in the absence or presence of flavonoids. Peptide species in the reaction mixtures were crosslinked before analyzed using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) except for freshly dissolved A β 42 (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 minutes. The crosslinking reaction was then terminated by 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 blocking 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 antibody 6E10 and the anti-mouse horseradish peroxidase-conjugated secondary antibodies^[14]. The blots were visualized using ECL plus reagent kit (Amersham Biosciences, Piscataway, NJ).

3. Results

3.1. TF Specifically and Potently Inhibited A β Toxicity

The chemical structures of TF and other bi-flavonoids are shown in Fig. 1. Note that TF, isolated from *Selag-*

inella tamariscina, is composed of two AP's connected by a 3,5'' linkage. First, we compared the cytoprotective effects of TF and AP on A β 42-induced toxicity in cultured SH-SY5Y cells using the MTT assay, which assesses cell viability by measuring mitochondrial reductase activity^[17]. Only ~1 μ M TF was necessary to reduce the cell viability by 50% (EC₅₀) (closed circles in Fig. 2A), while more than 10-times more AP was required to obtain a similar level of cell death (open circles in Fig. 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 protein transport from ER to Golgi), and ETO (a topoisomerase II inhibitor). TF was less effective than AP at protecting cells from STS-induced cell death (closed circles in Fig. 2B). The observed EC₅₀ of AP on STS-exposed cells was more than 5 μ M and AP was not able to completely suppress toxicity at concentrations up to 40 μ M (open circles in Fig. 2B). Moreover, TF was barely effective at rescuing BFA- and ETO-treated cells (closed circles in Fig. 2C and 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 (Fig. 2F). Known as a protein kinase inhibitor, STS induces mitochondria-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 caspase-activating pathway, although the exact mechanism remains to be elucidated. Note that the treatment of A β 42 induced only a low level of caspase-3/7 activity and consequently, the effects of the flavonoids on the activity were difficult to detect (Fig. 2E). Taken together, it is clear that TF was highly effective at rescuing cells from A β 42-induced toxicity, whereas AP was only moderately effective. In contrast to TF, AP demonstrated a broad spectrum, non-specific cytoprotection against several toxic insults including A β .

Fig. 2. Effect of TF and AP on the viability and caspase activity of SH-SY5Y cells treated with A β 42, STS, BFA and ETO. (A-D) Cell viability was assessed by MTT reduction assay in SH-SY5Y cells treated with 0.5 μ M A β 42 (A), 25 nM STS (B), 1 μ M BFA (C), and 1 μ M ETO (D) in the presence of TF (●) or AP (○) for 12 h (A β 42 and STS) or 24 h (BFA and ETO). Error bars indicate $\pm$ standard deviation of triplicate independent experiments. **(E, F)** DEVDase (caspase-3/7-like) activity in cells treated with 0.5 μ M A β 42 (E) or 25 nM STS (F) in the presence of TF (●) or AP (○) was monitored using 10 μ M Ac-DEVD-AMC after 12 h incubation.

3.2. Kinetic Studies on A β Fibrillogenesis/Fibril Destabilization in the Presence of TF or AP

Many polyphenolic compounds are known to influence A β fibrillogenesis by inhibiting or accelerating A β polymerization^[18]. We examined and compared the effect of TF or AP in the polymerization of A β 42 by Thioflavin-T fluorescence assay. In these experiments, 20 μ M of A β 42 was used because it is above the reported critical micelle concentration of the peptide^[19] and forms fibrils in a reasonable time frame such that its aggregation kinetics can be easily captured. Fig. 3A shows the fluorescence profiles of A β 42 aggregation with increasing TF concentration. The profiles exhibit the characteristic sigmoidal shape, expected for a nucleation-dependent polymerization mechanism^[19,28], where fluorescence plateaued after 6 h. The final fluorescence level, indicative of the amount of mature fibrils formed, decreased with increasing TF concentration (Fig. 3A).

Fig. 3. Effect of TF and AP on the fibrillogenesis of $A\beta_{42}$. Formation of fibrils was measured by Thioflavin-T binding assay. (A, B) Fluorescence intensity of 20 μM $A\beta_{42}$ 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, D) Logarithmic plot of $\{F(t)/[F(\infty)-F(t)]\}$ calculated from data in (A) and (B) (see materials and methods). A linear relationship was observed at the indicated time and concentrations (correlation coefficient, $R^2 = 0.99, 0.98, 0.99$, 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, F) Results from fibril extension assay. $A\beta_{42}$ fibrils (nuclei) (1.1 mM) were mixed with fresh $A\beta_{42}$ (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 rate of $A\beta_{42}$ nucleation-dependent polymerization in the presence of TF (●) or AP (○). The rates were determined by calculating the slope of early linear portions in (E) and (F). (H, I) Differential effects of TF (H) or AP (I) on $A\beta_{42}$ polymerization were shown according to the incubation times, 1 h (●, ○), 3 h (▲, △) and 12 h (△, △). These were re-drawn using data from (A) and (B). $F(t)$, the fluorescence unit at time t , was normalized by subtracting background.

The addition of AP also decreased Thioflavin-T fluorescence levels, but less effectively compared to TF (Fig. 3B). Semi-logarithmic plots of Thioflavin-T fluorescence vs. time gave a linear relationship for the indi-

cated time (Fig. 3C and 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^[16]. It is expected

that the $t_{1/2}$ value would increase if the flavonoids inhibited the nucleation process, the rate-determining step of the polymerization. For A β incubated with TF, $t_{1/2}$ values were 55 ± 2 , 62 ± 1 , 64 ± 1 and 70 ± 1 (average $\pm$ standard deviation) min, for 0, 0.5, 1 and 2 μ M of TF, respectively. For A β incubated with AP, $t_{1/2}$ values were 58 ± 2 , 65 ± 2 and 73 ± 2 min for at 1.25, 2.5, and 5 μ M of AP. The small changes of $t_{1/2}$ values with either TF or AP indicated that flavonoids marginally act on the nucleation step [16]. We also noticed that the disappearance of the linearity shown in Fig. 3C and D at the higher concentrations flavonoids or upon longer incubation times, implying the involvement of unaccounted processes and thus, rendering the logistic equation invalid.

Next, we compared the effect of TF or AP on A β fibril extension by measuring Thioflavin-T fluorescence of fresh A β samples seeded with preformed fibrils (nuclei). As shown in Fig. 3E and F, the fluorescence increased without a lag phase to give hyperbolic curves and reached plateau in about 2 h^[19,28]. 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 compared to AP (Fig. 3E and F). To further elucidate the difference between TF and AP in fibril extension, the initial rates of A β fibril extension, the slopes of the linear portion of the fluorescence profiles, were extrapolated and compared. TF and AP both decreased the rate of A β fibril extension with TF being the more effective inhibitor (Fig. 3G). TF and AP did

not significantly impact fibril nucleation during the lag phase (Fig. 3C and D), but once the rapid A β elongation phase is initiated, AP inhibited A β fibrillogenesis less efficiently (Fig. 3G), which resulted in a large difference in the final level of fibrils formed (Fig. 3H and I). Our data clearly demonstrated that the effects of the two flavonoids on the fibril formation may stem from the different effects they exert on A β fibril extension.

The initial rates of fibril extension in the absence or presence of TF were linearly dependent to A β 42 concentration (Fig. 4A). The linear relationship indicates that fibril extension is a first-order reaction with respect to A β concentration. The net rate of fibril extension has contributions from both polymerization and depolymerization^[20]. To further elucidate whether the observed reduced fibril growth rate by flavonoids is due to decreased polymerization or increased depolymerization, effect of the flavonoids on the disaggregation of preformed A β 42 fibrils was examined. As shown in Fig. 4C and 4B, 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 disaggregating fibrils. The IC₅₀ values of TF, AP and other bi-flavonoids for A β 42 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 to the same degrees (Table 1). Taken together, our data clearly show that TF is more anti-fibrillogenic than AP.

Fig. 4. Effect of TF on the disaggregation of A β 42 fibrils. (A) Initial rate of A β 42 fibril extension was determined as in Fig. 3G with varying concentrations of A β 42 in the absence (●) or the presence of 8 μ M TF (○). Linear relationship with the positive slope was found ($R^2 = 0.99$ for both data sets). (B) Destabilization of preformed A β 42 fibril. Fibrils (20 μ M) were incubated in the presence of 0 (●), 1 (○), 2 (■), 4 (□), 8 (◆), 16 (◇) and 20 (▲) μ M TF, or 0 (●), 1.25 (○), 2.5 (■), 5 (□), 10 (◆), 20 (◇) and 40 (▲) μ M AP for 0-12 h. Then, amounts of fibrils were measured as in Fig. 3A. $F(t)$, the fluorescence unit at time t , was normalized by subtracting background.

Table 1. IC₅₀ values of bi-flavonoids and AP

Flavonoid	Polymerization (μ M)	Extension (μ M)	Destabilization (μ M)
TF	2.41 $\pm$ 0.37	3.22 $\pm$ 0.39	13.0 $\pm$ 2.1
AP	23.3 $\pm$ 4.7	39.1 $\pm$ 5.9	>40
2',8''-biapigenin	2.90 $\pm$ 0.35	4.19 $\pm$ 0.54	15.8 $\pm$ 2.2
amentoflavone	3.37 $\pm$ 0.40	6.91 $\pm$ 0.43	20.8 $\pm$ 1.4
sumafavone	4.48 $\pm$ 0.96	11.2 $\pm$ 1.8	18.0 $\pm$ 1.8

IC₅₀ is defined as concentration of the compound required to reduce the polymerization, fibril extension rate of fresh A β 42 or destabilization of preformed A β 42 fibrils by 50%. The values were calculated by plotting fluorescence (%) in logarithmic scale versus concentrations of compounds described as before [25]. Means $\pm$ standard deviation are shown.

3.3. TF Inhibits A β 42 Structural Transformation

To further understand the inhibitory role of TF or AP in A β fibrillogenesis, we measured the secondary structures of A β 42 in the absence or presence of TF or AP by CD spectroscopy. 20 μ M A β 42 was used in these experiments. Freshly prepared A β 42 in PBS (pH 7.4) showed strong negative ellipticity at $\sim$ 195 nm (Fig. 5A and B), indicative of a largely unfolded, presumably

monomeric state with random-coil properties^[31,32]. Upon incubation at 37°C, the peptide showed changes in the far-UV CD spectrum, and reached a maximum negative ellipticity at 217 nm after 12 h (Fig. 5A and B). These changes arise from the formation of β -sheet structures in the peptides^[15]. The random coil to β -sheet transition was not observed in the presence of TF at 20 μ M A β where the peptide remained as a random coils for incubation times up to 12 h (Fig. 5A). TF thus effectively suppressed the formation of β -sheet structures of A β peptides. In contrast, AP was largely ineffective at inhibiting the formation of β -sheets (Fig. 5B), which is consistent with its poor anti-fibrillogenic activity compared to TF.

3.4. TF Accumulated Oligomeric A β Structures

Our fibrillogenesis and structural data (Fig. 3, 4 and 5) clearly showed that TF potently inhibits A β 42 fibrillogenesis and the formation of β -sheet structures. In addition, TF is also effective at disaggregating pre-formed amyloid fibrils. These properties of TF may explain its cytoprotective effect of A β -induced cell cytotoxicity (Fig. 2). However, because the cells were incubated with monomeric A β 42 at a lower concentration, 0.5 μ M, rather than the 20 μ M used in fibrillogenesis experiments, we further examined the effect of the flavonoids on A β fibrillogenesis in the same conditions (0.5 μ M and 12 h incubation) as those used in cell culture experiments. Additionally, the effect of the flavonoids on the formation of A β oligomeric structures also need to be characterized since the oligomers are more cytotoxic than the fibrils^[21,22] and Thioflavin-T could not detect the oligomeric species^[23]. For this purpose, A β species formed in the incubated samples were imaged with TEM. A β incubated alone was predomi-

Fig. 5. Effect of TF and AP on the structural transformation of A β 42. CD spectra of 20 μ M A β 42 incubated alone or in the presence of 20 μ M TF (A) or 20 μ M AP (B) in PBS at 37°C for 0 or 12 h. The spectra were obtained by subtracting buffer background as described in materials and methods.

Fig. 6. Effect of TF and AP on the morphology and size of A β 42 aggregates. (A) TEM images of A β 42 (0.5 μ M) incubated either alone or in the presence of TF or AP in PBS at 37°C for 12 h. Scale bar is shown at the bottom. (B) Immunoblot analysis of A β 42 oligomers prepared as described in Materials and methods using 6E10 monoclonal antibody. Lane 1, fresh A β 42 as a control; 2, A β 42 subjected to the oligomerization process; 3, A β 42 recovered in soluble fraction after centrifugation of the mixture from lane 2; 4, A β 42 of lane 3 crosslinked before immunoblot assay. (C, D) Immunoblot analysis of A β 42 aggregates in the presence of TF and AP. A β 42 (0.5 μ M) was incubated for 3 h (C) or 12 h (D) either alone or in the presence of TF or AP and then, crosslinked except lane 1 (fresh A β 42) before the immunoblot analysis. Lane 1, fresh A β 42; 2, A β 42 incubated alone; 3-8, A β 42 incubated in the presence of 2.5, 5 and 10 μ M TF (lanes 3-5) or AP (lanes 6-8), respectively. M, D and T indicate monomeric, dimeric, and trimeric A β 42, respectively. Molecular weights of protein standards are indicated on the left of panel B, C and D.

nantly composed of beaded structures 6-20 nm in width and ~200 nm in length (Fig. 6A). The morphology and size of these structures are consistent with those of protofibrils previously reported^[4,30,33]. Mature fibrils were not observed in this sample, implying that cellular toxicity (Fig. 2A) was likely to be due to A β oligomers and protofibrils. In the A β samples containing TF, however, protofibrillar structures were not observed. Instead, small spherical and branched structures were observed. In the A β samples containing AP, both beaded protofibrillar structures and amorphous deposits were present (Fig. 6A).

To confirm whether A β structures formed in the absence or presence of TF or AP were oligomers, immunoblotting assay was performed using A β -specific antibody 6E10. In this experiment, we used a chemical crosslinker to crosslink the A β species prior to SDS-PAGE analysis in order to avoid SDS-induced dissoci-

ation of oligomers. To validate the method before probing the effect of the flavonoids on the formation of A β oligomers, preformed oligomers prepared as described in Materials and methods were crosslinked and visualized by the immunoblotting assay (Fig. 6B). Compared to the incubated sample that was not crosslinked (Fig. 6B, lanes 2 and 3), significantly more A β oligomers spanning a wide size range was detected in the crosslinked sample (Fig. 6B, lane 4). Additionally, crosslinking did not result in the formation of oligomers in freshly dissolved A β (data not shown). Thus immunoblotting assay using crosslinked samples is a reliable method for detecting oligomers.

Without added flavonoids, 0.5 μ M A β incubated for 3 h (Fig. 6C, lane 2) showed a range of higher molecular weight oligomers and the amount of monomeric A β (band at the bottom of the gel) was not significantly different compared to those in a freshly prepared A β

sample (Fig. 6C, lane 1). However, after 12 h of incubation, the monomeric A β band completely disappeared (Fig. 6D, lane 2), indicating that all of A β have aggregated into higher molecular weight species. When TF was added to the A β samples, increasing amounts of small A β oligomers (dimers, trimers, and tetramers) were detected with increasing TF concentration after 3 h of incubation (Fig. 6C, lanes 3-5). Significant amounts of monomeric A β remained in all of these samples. When incubated for longer times (12 h), all samples accumulated more small A β oligomers (Fig. 6D, lanes 3-5) while still containing significant amounts of monomeric A β . With the addition of AP, larger oligomers (MW >30 kDa) were observed (Fig. 6C and D, lanes 6-8), but not small molecular weight A β oligomers. Taken together, our data show that TF, but not AP, favored the formation and accumulation of small A β oligomers. These results are consistent with our TEM results (Fig. 6A).

3.5. TF is Cytoprotective Against Preformed A β 42 Oligomers and Fibrils

Our results so far show that TF is cytoprotective to cells exposed to monomeric A β 42. The monomers, however, can be transformed into non-toxic oligomers in the presence of TF during the incubation time (12 h) (Fig. 6). It has been widely reported that soluble A β oligomers are the primary cytotoxic species^[21,22]. We

therefore tested whether TF or AP can counteract the toxicity of preformed oligomers and fibrils on cultured cells. Compared to freshly prepared A β 42, preformed A β oligomers induced ~16% more cell death after a 12 h incubation period (Fig. 2A and 7A). The addition of TF was effective at rescuing the cells from oligomer- and fibril-induced toxicity where 10 μ M of TF decreased cell toxicity induced by oligomers by ~40% and nearly ameliorated the toxicity induced by A β fibrils (Fig. 7). In contrast, AP did not rescue cells exposed to A β oligomers or fibrils (Fig. 7B). These data suggests that TF was effective at blocking the toxicity induced by A β oligomers and fibrils while AP was completely ineffective, implying that AP is not cytoprotective against the toxic A β oligomers and fibrils formed before its treatment. However, our earlier data indicated that AP did exert some rescuing effect towards toxicity induced by A β monomers (Fig. 2A). This is possibly due to weak, but significant anti-fibrillogenic property of AP at the early stages of fibrillogenesis as shown in Fig. 3 and 4.

3.6. Effects of Other Related Bi-flavonoids in A β Toxicity and Fibrillogenesis

To determine whether the inhibition of A β 42 toxicity and fibrillogenesis is a unique property to TF or a general property of bi-flavonoids, we tested other related bi-flavonoids^[25,34], including 2',8''-biapigenin, ameto-flavone and sumafavone (Fig. 1). All bi-flavonoids tested increased the viability of the cells treated with 0.5 μ M fresh A β 42, albeit with slightly higher EC₅₀ (~2.5 μ M) compared to that of TF (Fig. 8A). These bi-flavonoids also suppressed A β fibrillogenesis at conditions similar to those in cell culture (Fig. 8B) or at 20 μ M A β 42 (Table 1). IC₅₀ for A β polymerization, extension and decomposition of the fibrils varied slightly among the bi-flavonoids (Table 1). TF was the most anti-fibrillogenic compared to other bi-flavonoids. However, all of the bi-flavonoids examined so far were more effective than AP, implicating the importance of the dimeric flavonoid structure for their anti-fibrillogenic and cytoprotective effects. TEM and immunoblotting assays also revealed that the bi-flavonoids, similar to TF, favored the formation and accumulated oligomer-like structures (Fig. 8C and D). Our data suggest that the accumulation of non-toxic A β oligomers as one of the mechanism by which bi-flavonoids exert their cytoprotective effect.

Fig. 7. Effect of TF and AP on the viability of SH-SY5Y cells treated with A β 42 oligomers and fibrils. Cells were treated with 0.5 μ M A β 42 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 MTT reduction assay. Error bars indicate $\pm$ standard deviation of triplicate independent experiments.

Fig. 8. The effect of other related bi-flavonoids 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 sumafavone (△) at the indicated concentrations for 12 h. (B) Immunoblot analysis of Aβ42 fibrillogenesis in the presence of different bi-flavonoids. Aβ42 (0.5 μM) was incubated for 12 h either alone or in the presence of bi-flavonoids, crosslinked except lane 1 and then, analyzed by immunoblot assay using 6E10 monoclonal antibody. Lane 1, fresh Aβ42; 2-8, Aβ42 incubated without bi-flavonoids (lane 2), or in the presence of 2',8''-biapigenin at 5 (lane 3) and 10 (lane 4) μM, amentoflavone at 5 (lane 5) and 10 (lane 6) μM, or sumafavone at 5 (lane 7) and 10 (lane 8) μM. (C) TEM images of Aβ42 incubated alone or in the presence of 10 μM bi-flavonoids 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 bi-flavonoids at the indicated doses. The fluorescence were measured at 0 h (white bar), 3 h (black bar), 6 h (dotted bar) and 12 h (hatched bar). Error bars indicate ± standard deviation of triplicate independent experiments (A and B).

4. Discussion

Our results show that the bi-flavonoid TF potently and specifically inhibits toxicity induced by monomeric, oligomeric, or fibrillar A β (Fig. 2A and 8A). In contrast, the rescuing effect of AP on Ab-induced toxicity was less potent and nonspecific, as AP reduced cytotoxicity induced by a number of compounds, including A β , STS, BFO, and ETO by a similar degree (Fig. 2A). Caspase-3 activation in STS-induced cell death was suppressed by AP, consistent with other reports^[24], but less efficiently by TF (Fig. 2B). Taken together, it is apparent that TF either interacts directly with A β or disrupts the specific toxicity pathway induced by A β . In contrast, AP exerts a general cytoprotective effect that is non-specific to A β toxicity.

TF appears to possess the three basic structural features that have been attributed to its antifibrillogenic and cytoprotective effects: high binding affinity to A β , two binding groups, and an optimal length between the two binding groups. TF is composed of two mono-flavonoids, AP-3' and 5''-AP. AP has been shown to efficiently block A β fibrillogenesis (Fig. 3), consistent with previous study showing that the IC₅₀ of mono-flavonoids such as myricetin, quercetin, and morin for the formation, extension and destabilization of fibrillar A β were found to be in the range of 0.1-1 μ M^[25]. TF and other bi-flavonoids may share a common mechanism for attenuating A β toxicity and fibrillogenesis, through the accumulation of non-toxic oligomeric forms of A β (Fig. 6 and 8). All bi-flavonoids tested were more effective than the mono-flavonoid AP (Table 1), suggesting that the possession of two mono-flavonoids in bi-flavonoids is a critical structural feature for inhibiting A β fibrillogenesis. In addition to possessing two flavonoids, TF also meets the third criteria of an optimal linker size between the two flavonoids (see Fig. 1). For the case of curcumin, the optimal length of the linker has been found to be between 8-16 Å^[8]. Furthermore, rigidity of the TF backbone may give it the additional advantage of being an efficient A β aggregation inhibitor^[8]. AP contains two phenyl groups connected by a heterocyclic ring. While these structural features may satisfy the first and second criteria, the length of the linker between the two rings may be shorter than the optimal length. Thus, the mono-flavonoid AP might act as a single binder.

TF appears to be more effective at inhibiting A β fibril

extension than nucleation (Fig. 3C). Characteristic A β secondary structures, namely β -sheets, were not found in the presence of TF (Fig. 5A). Furthermore, the linear relationship between the initial rate of fibril extension and the concentrations of A β implies that polymerization and depolymerization occur simultaneously (Fig. 4A). Our results suggest that TF fundamentally alters the aggregation pathway of A β , possibly by interacting with free A β and preventing it from misfolding into the β -sheet conformation found in fibrils. The stoichiometry of TF binding to A β , however, is unknown. The requirement of the two phenyl groups in curcumin for its ability to inhibit A β aggregation implies the existence of at least two binding sites on A β ^[8]. Insertion of long flexible linker (>16 Å) between them prevented from inhibiting the aggregation^[8], which probably exclude an interaction of curcumin with multiple A β 's. The similar speculation may be applicable to bi-flavonoids.

As shown in Fig. 6, the presence of TF suppressed the formation of mature A β fibrils, but led to the formation and accumulation of A β oligomer-like structures. These results are in agreement with TF's anti-fibrillogenic property. However, since oligomeric A β species have been found to be more toxic than monomeric or fibrillar A β species^[21,22], the presence of TF might increase A β cytotoxicity if the accumulated A β oligomers are the same as the "on-pathway" or toxic A β oligomers. This is clearly not that case, because cell viability increased significantly in the presence of TF (Fig. 2A). Thus, the oligomeric aggregates accumulated in the presence of TF are not only structurally different than on-pathway A β aggregates (Fig. 5), they are also functionally different than oligomers obtained by A β incubation without TF. Consistent with earlier studies^[26], TF appears to redirect A β fibril formation to form unstructured and nontoxic "off-pathway oligomers"^[26]. Moreover, TF abrogated the toxicity of pre-formed A β oligomers and fibrils (Fig. 7), probably by disaggregating the toxic aggregates.

Development of small molecule inhibitors to inhibit toxicity has emerged as an encouraging approach to treat AD. Hence, several flavonoids have been currently being examined for their therapeutic potentials against amyloid-mediated diseases. Compared to mono-flavonoids, dimeric bi-flavonoids may be promising therapeutics because its monomeric moieties are good A β

binders and that these moieties are separated by an appropriate length linker that satisfies structural requirements for the inhibition of A β fibrillogenesis. In conclusion, our results show that bi-flavonoids more specifically inhibit A β 42-induced cytotoxicity than mono-flavonoid by redirecting and/or reverting toxic A β aggregates to form off-pathway, nontoxic, and unstructured A β oligomers and intermediate. This comparative study of mono- and bi-flavonoids suggests that dimeric flavonoids may be useful in AD therapy.

Abbreviations: A β , 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; DMSO, dimethylsulphoxide; EC₅₀, concentration to reduce the cell viability by 50%; ETO, etoposide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IC₅₀, concentration of the compound required to reduce the polymerization, fibril extension rate of fresh A β 42 or destabilization of preformed A β 42 fibrils by 50%; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffer saline; SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis; STS, staurosporine; TEM, transmission electron microscopy; TF, taiwanflavone

References

- [1] C. Haass, A. Y. Hung, M. G. Schlossmacher, T. Ottersdorf, D. B. Teplow and D. J. Selkoe, "Normal cellular processing of the beta-amyloid precursor protein results in the secretion of the amyloid beta peptide and related molecules", *Ann N Y Acad Sci*, Vol. 695, pp. 109-116, 1993.
- [2] J. Hardy and D. J. Selkoe, "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics", *Science*, Vol. 297, pp. 353-356, 2002.
- [3] R. Kaye and C. G. Glabe, "Conformation-dependent anti-amyloid oligomer antibodies", *Methods Enzymol*, Vol. 413, pp. 326-344, 2006.
- [4] A. Petit, F. Bihel, C. Alves da Costa, O. Pourquie, F. Checler and J. L. Kraus, "New protease inhibitors prevent gamma-secretase-mediated production of Abeta40/42 without affecting Notch cleavage", *Nat Cell Biol*, Vol. 3, pp. 507-511, 2001.
- [5] D. Commenges, V. Scotet, S. Renaud, H. Jacqmin-Gadda, P. Barberger-Gateau and J. F. Dartigues, "Intake of flavonoids and risk of dementia", *Eur J Epidemiol*, Vol. 16, pp. 357-363, 2000.
- [6] L. Pugliese, R. E. Tanzi and D. M. Kovacs, "Alzheimer's disease: the cholesterol connection", *Nat Neurosci*, Vol. 6, pp. 345-351, 2003.
- [7] T. D. Rea, J. C. Breitner, B. M. Psaty, A. L. Fitzpatrick, O. L. Lopez, A. B. Newman, W. R. Hazard, P. P. Zandi, G. L. Burke, C. G. Lyketsos, C. Bernick and L. H. Kuller, "Statin use and the risk of incident dementia: the Cardiovascular Health Study", *Arch Neurol*, Vol. 62, pp.1047-1051, 2005.
- [8] A. A. Reinke and J. E. Gestwicki, "Structure-activity relationships of amyloid beta-aggregation inhibitors based on curcumin: influence of linker length and flexibility", *Chem. Biol. Drug. Des.*, Vol. 70, pp. 206-215, 2007.
- [9] K. H. Lee, B. H. Shin, K. J. Shin, D. J. Kim and J. Yu, "A hybrid molecule that prohibits amyloid fibrils and alleviates neuronal toxicity induced by beta-amyloid (1-42)", *Biochem Biophys Res Commun*, Vol. 328, pp. 816-823, 2005.
- [10] D. E. Ehrnhoefer, J. Bieschke, A. Boeddrich, M. Herbst, L. Masino, R. Lurz, S. Engemann, A. Pastore and E. E. Wanker, "EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers", *Nat. Struct. Mol. Biol.*, Vol. 15, pp. 558-566, 2008.
- [11] Y. S. Han, W. H. Zheng, S. Bastianetto, J. G. Chabot and R. Quirion, "Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C", *Br J. Pharmacol*, Vol. 141, pp. 997-1005, 2004.
- [12] J. J. Yerbury, S. Poon, S. Meehan, B. Thompson, J. R. Kumita, C. M. Dobson and M. R. Wilson, "The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures", *FASEB J.*, Vol. 21, pp. 2312-2322, 2007.
- [13] S. S. Kang, J. Y. Lee, Y. K. Choi, S. S. Song, J. S. Kim, S. J. Jeon, Y. N. Han, K. H. Son and B. H. Han, "Neuroprotective effects of naturally occurring bioflavonoids", *Bioorg. Med. Chem. Lett.*, Vol. 15, pp. 3588-3591, 2005.
- [14] Y. R. Pokharel, J. W. Yang, J. Y. Kim, H. W. Oh, H. G. Jeong, E. R. Woo and K. W. Kang, "Potent inhibition of the inductions of inducible nitric oxide synthase and cyclooxygenase-2 by taiwanflavone", *Nitric Oxide*, Vol. 15, pp. 217-225, 2006.
- [15] M. Shahnawaz, A. Thapa and I. S. Park, "Stable activity of a deubiquitylating enzyme (Usp2-cc) in

- the presence of high concentrations of urea and its application to purify aggregation-prone peptides", *Biochem Biophys Res Commun*, Vol. 359, pp. 801-805, 2007.
- [16] H. Naiki, K. Hasegawa, I. Yamaguchi, H. Nakamura, F. Gejyo and K. "Nakakuki, Apolipoprotein E and antioxidants have different mechanisms of inhibiting Alzheimer's beta-amyloid fibril formation in vitro", *Biochemistry*, Vol. 37, pp. 17882-17889, 1998.
- [17] M. B. Hansen, S. E. Nielsen and K. Berg, "Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill", *J. Immunol Methods*, Vol. 119, pp. 203-210, 1989.
- [18] M. Necula, R. Kaye, S. Milton and C. G. Glabe, "Small molecule inhibitors of aggregation indicate that amyloid beta oligomerization and fibrillization pathways are independent and distinct", *J Biol Chem*, Vol. 282, pp. 10311-10324, 2007.
- [19] E. Y. Chi, S. L. Frey, A. Winans, K. L. Lam, K. Kjaer, J. Majewski and K. Y. Lee, "Amyloid-beta fibrillogenesis seeded by interface-induced peptide misfolding and self-assembly", *Biophys. J.*, Vol. 98, pp. 2299-2308, 2010.
- [20] H. Naiki and K. Nakakuki, "First-order kinetic model of Alzheimer's beta-amyloid fibril extension in vitro", *Lab Invest.*, Vol. 74, pp. 374-383, 1996.
- [21] J. L. Tomic, A. Pensalfini, E. Head and C. G. Glabe, "Soluble fibrillar oligomer levels are elevated in Alzheimer's disease brain and correlate with cognitive dysfunction", *Neurobiol Dis.*, Vol. 35, pp. 352-358, 2009.
- [22] C. A. McLean, R. A. Cherny, F. W. Fraser, S. J. Fuller, M. J. Smith, K. Beyreuther, A. I. Bush and C. L. Masters, "Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease", *Ann Neurol.*, Vol. 46, pp. 860-866, 1999.
- [23] A. Jan, O. Gokce, R. Luthi-Carter and H. A. Lashuel, "The ratio of monomeric to aggregated forms of Abeta40 and Abeta42 is an important determinant of amyloid-beta aggregation, fibrillogenesis, and toxicity", *J. Biol. Chem.*, Vol. 283, pp. 28176-28189, 2008.
- [24] M. A. Vargo, O. H. Voss, F. Poustka, A. J. Cardounel, E. Grotewold and A. I. Doseff, "Apigenin-induced-apoptosis is mediated by the activation of PKCdelta and caspases in leukemia cells", *Biochem Pharmacol*, Vol. 72, pp. 681-692, 2006.
- [25] K. Ono, Y. Yoshiike, A. Takashima, K. Hasegawa, H. Naiki and M. Yamada, "Potent anti-amyloidogenic and fibril-destabilizing effects of polyphenols in vitro: implications for the prevention and therapeutics of Alzheimer's disease", *J. Neurochem.*, Vol. 87, pp. 172-181, 2003.
- [26] M. Masuda, N. Suzuki, S. Taniguchi, T. Oikawa, T. Nonaka, T. Iwatsubo, S. Hisanaga, M. Goedert and M. Hasegawa, "Small molecule inhibitors of alpha-synuclein filament assembly", *Biochemistry*, Vol. 45, pp. 6085-6094, 2006.