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Effects of Naturally Occurring Compounds on Fibril Formation and Oxidative Stress of β-Amyloid

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β-Amyloid (βA)-induced oxidative toxicity on neuronal cells is a principal route in Alzheimer's disease (AD), and its toxicity occurs after fibril formation. Inhibitory or promoting effects of naturally occurring compounds on βA fibril formation were evaluated. Among 214 tested compounds, curcuminoids, flavone type flavonoids, and naphthoquinones were shown to be potent inhibitors of βA fibrilization. The addition of the curcuminoids, curcumin, demethoxycurcumin, and bisdemethoxycurcumin strongly inhibited βA fibril formation. Flavonoids such as quercetin, rhamnetin, and fisetin strongly inhibited βA fibril formation. Limonoids, cinnamic acids, and catechins enhanced fibril formation in vitro. Anthothecol possessed the most enhancing activity on fibril formation of the compounds tested. On the other hand, it was found that curcuminoids showed cytotoxicity with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay and did not protect HT22 murine neuroblastoma cells from βA(25−35) insult. Two flavone type flavonoids, morin and quercetin, exhibited no cytotoxicity and strongly protected HT22 murine neuroblastoma cells from βA(25−35) oxidative attack. Conclusively, morin or quercetin could be a key molecule for the development of therapeutics for AD.

KEYWORDS: Curcumioids; flavonoids; Alzheimer's disease; β-amyloid; HT22 murine neuroblastoma cell; bA burden assay

INTRODUCTION

In aging, Alzheimer's disease (AD) is a common cause of progressive cognitive dysfunction as well as Parkinson's disease (*1*). In contrast, Huntington's disease can occur in young adults between 30 and 50 years old, with juveniles being especially affected by the disease (*2*). One statistical report demonstrates that AD affects about four million people in the United States and that AD is the cause of more than 100000 deaths each year (*3*). In addition, the number of AD patients is increasing in many Western countries and also in some Asian countries such as Korea (*4*).

Numerous epidemiological studies have shown that one of the principal pathological toxicities of AD is caused by extracellular β-amyloid (βA) deposition, leading to insoluble aggregation of βA (*5*). As βA deposition as senile plaques has been known to be one of the potential mechanisms of AD, a possible attenuation and modulation of βA toxicity has been highlighted as an important therapeutic approach to control the onset of AD. Interestingly, dietary components present in plants may protect against certain types of AD toxicities (*6*−*8*). Recently, curcuminoids such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin have shown potent inhibitory effects on βA-induced oxidative stress (*9*, *10*), and their functional β-diketone groups linked with two benzyl moieties seem to be essential for the inhibitory process. Even though some plant extracts and constituents are prepared as AD therapeutic drugs, other natural constituents have attracted relatively little attention as potentially valuable resources for drug discovery on AD treatments.

The objective of this study was therefore to investigate the effects of several classes of naturally occurring compounds, common in fruits, vegetables, or spices, on βA-induced insult and to elucidate structural features governing interaction between such natural products and βA aggregation. The information obtained was expected to provide a fundamental basis for in vivo investigation of effects of specific dietary compounds in

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mitigating βA-induced oxidative stress using HT22 murine neuroblastoma cells.

MATERIALS AND METHODS

General Chemicals. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and *N*,*N*-dimethyl formamide (DMF) were purchased from Sigma (St. Louis, MO). βA(1−42) were purchased from BaChem California Inc. (Torrance, CA). Culture media and supplements were obtained from Life Technologies (Grand Island, NY). Other general supplies needed for bioassay were purchased from Fisher Scientific (Itaca, IL). MTT was dissolved in sterile phosphate-buffered saline (PBS) (5 mg/mL) and further sterilized by filtration through a 0.2 μm filter. The sterilized MTT solution was stored at 4 °C. Lysis buffer was prepared by dissolving 10% sodium dodecyl sulfate (w/v) in 50% DMF in distilled water, and the pH was adjusted to 4.7 using HCl.

Tested Chemicals. The chemicals used were acacetin, acetaldehyde, acetoanisol, acetophenone, 7-acetoxy-4-methylcoumarin, 3-acetylcoumarin, 8-acetyl-6,7-dimethoxycoumarin, 8-acetyl-7-hydroxycoumarin, alginic acid, allyl caproate, allyl cyclohexane propionate, allyl heptanoate, allyl isothio cyanate, allyl phenoxy acetate, angolensin, anisaldehyde, anthothecol, artemisinin, asarone, benzaldehyde, benzyl acetate, benzyl benzoate, benzyl formate, benzyl propionate, berberine, bergaptol, bicuculine, butanal, butyl acetate, butyl butylate, butyl formate, capsaicin, capsanthin, capyophyllene, (+)-catechin, (−)-catechin, chrysin, cinchonidine, cinchonine, chalcone, cinalool oxide, *cis*-3-hexenal, *cis*-3-hexenyl acetate, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl formate, cineol, citral, citronellal, citronellol, citronellyl acetate, criocitrin, curcumin, cyanidin, daidzein, daidzin, γ-decalactone, δ-decalactone, decanal, demethoxycurcumin, diethyl sebacate, (+)-dihydroquercetin, 3,4-dihydroxybenzoic acid, 3,4-dihydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, dimethyl anthranilate, diosmin, (+)-epicatechin, (−)-epicatechin, epigallocatechin, epigallocatechin 3-gallate, ethyl acetoacetate, ethyl benzoate, ethyl caproate, ethyl caprylate, ethyl decanoate, ethyl formate, ethyl heptanoate, ethyl isobutylate, ethyl isovalenate, ethyl lactate, ethyl octanoate, ethyl phenyl acetate, ethyl phenyl glycidate, ethyl-2-methyl-butynate, ethyl-β-methylthiopropionate, eugenol, ferulic acid, fisetin, flavone, furfural, furfuryl alcohol, gallic acid, gedunin, genistin, geraniol, geranyl acetate, gerayl formate, guineesine, hesperidin, hesperetin, hexanal, γ-hexalactone, hexanoic acid, hexanol, hexyl acetate, hexyl lactate, hinokitiol, *p*-hydroxybenzoic acid, 2-hydroxycinnamic acid, 3-hydroxycinnamic acid, 4-hydroxycinnamic acid, hydroxy citronellal, 4-hydroxycoumarin, 7-hydroxyflavone, 4-hydroxy-3-methoxycinnamic acid, 7-hydroxy-4-methoxymethylcoumarin, 4-hydroxy-6-methylcoumarin, 4-hydroxy-7-methylcoumarin, 6-hydroxy-4-methylcoumarin, 7-hydroxy-4-methylcoumarin, 5-hydroxypsoralen, 8-hydroxypsoralen (xanthotoxol), ichangin, imperatorin, α-ionone, β-ionone, isoamyl cinnamate, isoamyl formate, isoamyl propionate, isoamyl isobutyrate, isobutyl isobutyrate, isobutyl isovalerate, isobutyl valerate, isoeugenol, isosakuranetin, juglone, kaempferol, lapachol, lapachone, limonin, limonin glucoside, linalool, 8-methoxypsoralen (xanthotoxin), methyl anthranilate, 6-methyl-5-hepten-2-one, methyl heptine carbonate, methyl hexanoate, methyl isovalerate, 3,3-methylene-bis(4-hydroxy coumarin), 2-methyl butyl acetate, 2-methyl butyl butyrate, methyl β-methylthio-propionate, methyl phenyl carbinyl acetate, methyl phenyl glycidate, methyl salicylate, 8-methyl-*N*-vanillyl-6-nonenamide, morin, myricetin, naringenin, naringin, narirutin, neoeriocitrin, neohesperidin, neoponcitrin, nomilin, γ-nonalactone, obacunone, γ-octalactone, 1-octanol, 3-octanol, palmitine, 2-phenyl ethyl alcohol, phenyl ethyl butylate, pentan-2,3-dione, perilladehyde, α-pinene, β-pinene, pipereicosalidine, piperine, piperlongumine, piperettine, pipernonaline, piperoctadecalidine, pratol, propionic acid, propyl acetate, propyl disulfide, prunin, psoralen, pyrogallol, quercetin, quinidine, quinine, rhamnetin, rutin, scopoletin, sesamin, sesaminol, styrallyl acetate, sulfurol, tangeretin, taxifolin, techtochrysin, α-terpineol, terpinen-4-ol, α-terpinene, tetrahydrocurcumin, thymol, *trans*-cinnamic acid, *trans*-2-hexanal, *trans*-2-hexanol, triethyl citrate, tropolone, umbelliferone, valeric acid. All chemicals used were of the highest grade commercially available or the highest purified grade in my laboratory.

Preparation of βA Protein for Fibrillization Studies. Purified βA-(1−42) were purchased from BaChem California Inc. βA(1−42) stock solutions (250 μM) were prepared by completely dissolving the peptide in DMSO. Purified βA(25−35) were purchased from Sigma Co. βA-(25−35) were prepared as 10 mM stock solution in DMSO and diluted 10-fold with PBS to induce aggregation for 1 h incubation at room temperature.

Thioflavin T (ThT) Fluorescence Assay for Fibril Formation. Aggregation of βA peptide βA(1−42) was measured by the ThT binding assay, in which the fluorescence intensity reflects the degree of βA fibril formation. To each well of a 96 well microplate (blank) were added 5 μL of 250 μM βA42 and 45 μL of PBS buffer. The mixed solution was added to natural product samples and incubated for 1 h at room temperature. The ThT solution (150 μL of 5 μM ThT in 50 mM glycine, pH 8.5) was added to each well. The resulting ThT fluorescence was measured by using excitation and emission wavelengths of 440 and 485 nm, respectively (Safire, TECAN, Austria).

βA Burden and Cytotoxicity Assays. HT22 cells, a murine cell line of hippocampal origin, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 5% penicillin/streptomycin. At the outset, 90% confluent cells were dissociated and plated at 5×10^3 cells/well in a 96 well plate. When cells were attached to the plate, the medium was replaced with plain DMEM. Cells were treated with natural product samples. One hour after natural products treatment, 2.5 μL of prediluted 1 mM βA(25−35) was added to media and cells were further incubated for 18 h. Cell viability was checked by methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT was added to the culture medium (1:10 v:v; MTT solution/culture medium) and incubated for 4 h at 37 °C. Cells were then solubilized in 50% dimethylformamide and 10% sodium dodecyl sulfide (pH 4.7). The degree of cell survival was determined based on the absorbance measured at O.D. 570−630 nm using a plate reader (Sunrise, TECAN), and the percentage cytotoxicity was calculated. The survival rate from βA challenged cells with natural products was calculated and compared to βA-challenged cells. The innate cytotoxicity of natural products was measured from the MTT assay of non-βA-challenged cells with each natural product.

RESULTS

In vitro effects of flavonoids on βA fibril formation are shown in **Table 1**. Among the 39 flavonoids tested, the flavone type (**Figure 1**) generally exhibited potent inhibitory effects on βA fibril formation. Rhamnetin (IC_{50} = 2.3 μg/mL) and quercetin (IC_{50} = 2.4 μg/mL) were the most potent, followed by morin (IC_{50} = 7.4 μg/mL). The parent compound, flavone, showed moderate inhibition of fibril formation, whereas the flavanones tested did not show inhibitory activity. Cyanidin chloride (IC_{50} = 8.50 μg/mL) had a stronger inhibitory activity than flavone. Flavonoids with a C2−C3 double bond were more effective than the corresponding saturated homologues, while polyhydroxylation on flavonoids was effective as an increasing number of hydroxyl groups increased the effectiveness. Naphthoquinones such as juglone (IC_{50} = 2.50 μg/mL), 1,2-naphthoquinone (IC_{50} = 1.26 μg/mL), and 5,8-dihydroxy-1,4-naphthoquinone (IC_{50} = 3.56 μg/mL) (**Table 2** and **Figure 2**) showed a strong inhibitory effect in inhibiting fibril formation. The curcuminoids, curcumin (IC_{50} = 0.25 μg/mL), demethoxycurcumin (IC_{50} = 0.5 μg/mL), and bisdemethoxycurcumin (IC_{50} = 0.38 μg/mL) (**Table 2** and **Figure 2**), showed the strongest inhibition on fibril formation. However, tetrahydrocurcumin did not have any effect on fibril formation. Therefore, β-diketone groups linked with two double bonds were essential for inhibition of fibril formation.

On the other hand, morin and quercetin showed no cytotoxicity and strong protection of HT22 murine neuroblastoma cells from the βA oxidative attack (**Table 3**). However, rhamnetin showed lower protecting activity than quercetin, even they had

Table 1. In Vitro Inhibitory Effects of Flavonoids toward βA Fibrilogenesis Using βA1–42 in the ThT Fluorescence Assay[a]

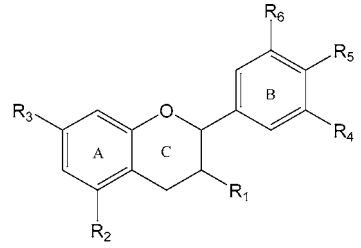
compd[b]	structure type	% of control at the concn of 10 ppm	IC_{50} (μg/mL)
acacetin	flavone	*c*	
angolensin	isoflavone		
(+)-catechin (**1**)	catechin	228.6	
(−)-catechin	catechin	226.7	
chalcone	flavanone		
chrysin	flavone		
criocitrin	flavanone		
cyanidin	anthocyanin	9.6	8.5
daidzein	isoflavone		
dihydroquercetin	flavone		
diosmin	flavone		
(+)-epicatechin (**2**)	catechin	183.8	
(−)-epicatechin	catechin	178.5	
fisetin (**3**)	flavone	27.3	12.5
flavone (**4**)	flavone	56.9	>20.0
genistin	isoflavone		
hesperidin	isoflavone		
hesperetin	isoflavone		
7-hydroxyflavone	flavone	48.5	18.7
isosakuranetin	flavanone		
kaempferol (**5**)	flavone	34.1	7.0
morin (**6**)	flavone	20.1	7.4
myricetin	flavone		
naringenin	flavanone		
naringin	flavanone		
narirutin	flavanone		
neoericitrin	flavanone		
neohesperidin	flavanone		
neoponcitrin	flavanone		
pratol	flavone		
prunin	flavanone		
quercetin (**7**)	flavone	7.4	2.4
rhamnetin (**8**)	flavone	7.3	2.3
rutin	flavone		
tangeretin	flavone		
taxifolin	flavanone		
tectochrysin	flavone		

[a] Values represent means of three independent experiments. [b] Boldface numbers in parentheses correspond to numbered structures in **Figure 1**. [c] Nondetectable.

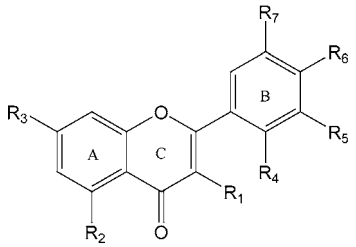
similar inhibitory effects on the βA aggregation (**Table 3**). However, naphthoquines did not protect the HT22 cells from the βA oxidative attack (**Table 3**) after the βA burden assay. In addition, curcuminoids exhibited about 50% of cytotoxicity at the concentration of 10 ppm against HT22 murine neuroblastoma cells (**Table 3**). By the βA burden assay, curcuminoids did not protect the HT22 cells from the βA oxidative attack (**Table 3**).

Three citrus limonoids, limonin, nomilin, and obacunone, and the structurally related limonoids, anthothecol and gedunin, increased βA fibril formation in vitro (**Table 2** and **Figure 3**). Anthothecol had the most potent activity on enhancing fibril formation, followed by limonin and nomilin. Interestingly, phenolic acids containing cinnamic acid moiety (**Figure 3**) showed about 2-fold greater fibril formation activity than control in vitro. Catechins also enhanced βA fibril formation.

Most isoflavonoids, phenolic acids, monoterpenoids, and coumarins tested did not have any inhibitory effect on fibril formation. A hydroxylated tropolone, hinokitiol, did not have an inhibitory effect on fibril formation, similar to tropolone, which had no effect. The alkaloids such as berberine, bicuculine, cinchonidine, cinchoinine, palmitine, piperine, pipereicosalidine, piperlongumine, piperoctadecalidine, piperettine, quinidine, and quinine, belonging to several different structural classes, did not inhibit fibril formation. Capsanthin, a capsaicinoid tested, also did not show any inhibition.



R_1=R_2=R_3=R_4=R_5=OH; R_6=H (catechin) **(1)**
R_1=R_2=R_3=R_5=R_6=OH; R_4=H (epicatechin) **(2)**



R_1=R_3=R_5=R_6=OH; R_2=R_4=R_7=H (fisetin) **(3)**
R_1=R_2=R_3=R_4=R_5=R_6=R_7=H (flavone) **(4)**
R_1=R_2=R_3=R_6=OH; R_4=R_5=R_7=H (kaempferol) **(5)**
R_1=R_2=R_3=R_4=R_6=OH; R_5=R_7=H (morin) **(6)**
R_1=R_2=R_3=R_5=R_6=OH; R_4=R_7=H (quercetin) **(7)**
R_1=R_2=R_5=R_6=OH; R_3=OCH3; R_4=R_7=H (rhamnetin) **(8)**

Figure 1. Structures of flavonoids and catechins possessing inhibitory or enhancing activities on βA fibril formation in vitro.

Table 2. In Vitro Inhibitory or Enhancing Effects of Naturally Occurring Compounds toward βA Fibrilogenesis Using βA1–42 in the ThT Fluorescence Assay[a]

compd[b]	structure type	% of control at the concn of 10 ppm	IC_{50} (μg/mL)
anthothecol (**9**)	limonoid	302.5	*c*
bisdemethoxycurcumin (**10**)	curcuminoid	13.3	0.38
curcucmin (**11**)	curcuminoid	2.3	0.25
demethoxycurcumin (**12**)	curcuminoid	4.2	0.50
5,8-dihydroxy-1,4-naphthoquinone (**13**)	naphthoquinone	38.7	3.56
gedunin (**14**)	limonoid	169.0	
2-hydroxy cinnamic acid (**15**)	phenolic acid	190.6	
3-hydroxy cinnamic acid (**16**)	phenolic acid	203.9	
juglone (**17**)	naphthoquinone	10.1	2.50
lapachol	naphthoquinone		
limonin (**18**)	limonoid	219.0	
1,2-naphthoquinone (**19**)	naphthoquinone	8.7	1.26
tetrahydrocurcumin	curcuminoid		

[a] Values represent means of three independent experiments. [b] Boldface numbers in parentheses correspond to numbered structures in **Figures 2** and **3**. [c] Nondetectable.

DISCUSSION

Flavonoids and cinnamic acids have been considered as primary antioxidants and play an important role as free radical acceptors and chain breakers in living organisms. Many reports demonstrate that inhibitory effects on βA fibril formation of natural products are significantly related to their antioxidative activities (*9*−*11*). However, antioxidant activities of polyphenols are associated with parameters such as their reduction potential, their abilities to stabilize and delocalize the unpaired electron, their reactions with other antioxidants, and their transition metal

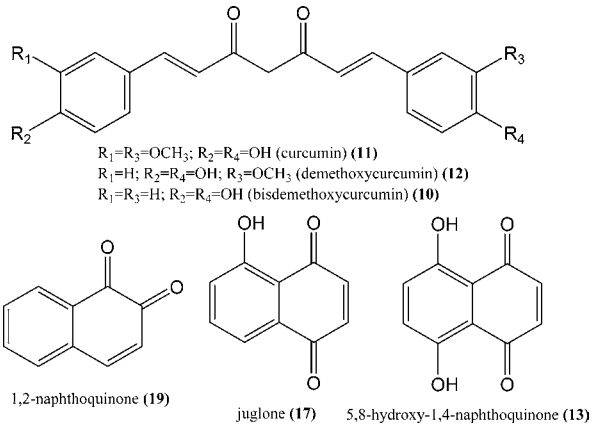


Figure 2. Structures of curcuminoids and naphthoquinones showing their inhibitory effects on βA fibril formation in vitro.

Table 3. In Vivo Inhibitory Effects[a] of Naturally Occurring Compounds toward βA Toxicity and Tests for Innate Cytotoxicity

		MTT assay with HT22 cells	
compd	chemical type	cytotoxicity (% of control) at the concn of 10 ppm	βA burden assay (% of control at 25 μM βA25–35)
bisdemethoxycurcumin	curcuminoid	55.8	66.1
curcumin	curcuminoid	55.5	73.9
cyanidin	flavone	115.5	165.7
demethoxycurcumin	curcuminoid	52.4	80.0
5,8-dihydroxy-1,4-naphthoquinone	nathphoquinone	65.3	94.6
fisetin	flavone	117.8	180.6
flavone	flavone	76.8	71.4
juglone (5-hydroxy-1,4-naphthoquinone)	naphthoquinone	52.7	101.8
kaempferol	flavone	107.5	171.7
morin	flavone	149.5	245.2
1,2-naphthoquinone	naphthoquinone	91.5	127.9
quercetin	flavone	126.1	198.9
rhamnetin	flavone	116.6	154.4

[a] Values represent means of three independent experiments.

chelating potential (*12*). Recently, one model system that used Fenton's reagent to generate highly reactive hydroxy radicals was reported (*13*). In this report, luteolin showed the strongest antioxidant activity, inhibiting malonaldehyde formation by 94 and 97% at the levels of 0.5 and 1.0 mM, respectively. A slightly lower antioxidant activity than luteolin was observed with rhamnetin, fisetin, kaempferol, morin, and quercetin, a group of flavonols with a free hydroxyl group at the C-3 position. Our studies showed similar results suggesting that the flavonol C-3 hydroxyl group was responsible for the high inhibitory activity to β fibril formation (**Table 1**). Loss of a hydroxyl group of quercetin at C-5 (fisetin) or C-3′ (kaempferol) had a significant effect on the inhibitory activity. Similarly, the ortho positioning of hydroxyl groups on the B ring (quercetin) did not influence the antioxidant activity, while a meta positioning of hydroxyl groups on the B ring (morin) also did not influence the antioxidant activity. In addition, morin and quercetin showed no cytotoxcity and protected HT22 murin neuroblastoma cells from the βA oxidative stress. These results were not reported previously.

Moreover, flavonoids are known to chelate metal ions at the 3′,4′-dihydroxy positions in the B ring, at the 3-hydroxy, 4-keto group, and at the 5-hydroxy (A ring), 4-keto group (*12*). It has been demonstrated that flavonoids can either enhance or inhibit the formation of •OH by Fenton type reaction (*14*). Similarly, a new class of inhibitors of βA fibril formation has been reported and their 10,11-dihydroxy groups of the D ring of apomorphine were required for the inhibitory effect (*15*). Methylation of these hydroxyl groups reduced their inhibitory potency (*15*). Our results showed that the 3-hydroxy, 4-keto groups of flavonols were essential for the inhibitory effect on βA fibril formation.

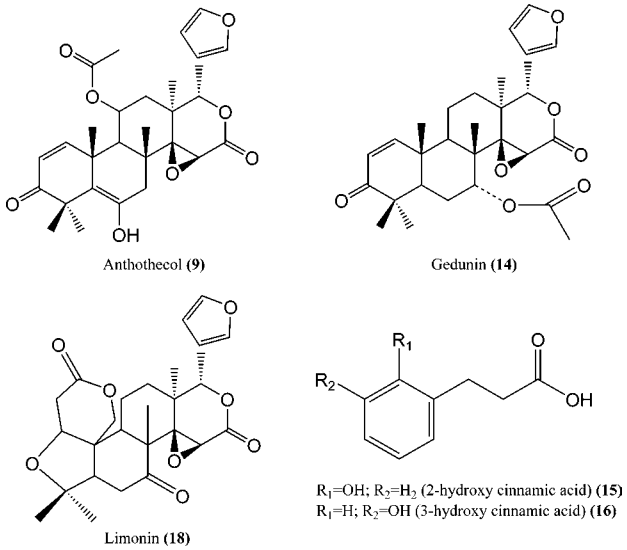


Figure 3. Structures of limonoids and phenolic acids showing their enhancing effects on βA fibril formation in vitro.

However, there are significantly different results for cinnamic acid derivatives, which are known as strong antioxidants. In **Table 2**, 2-hydroxy cinnamic acid and 3-hydroxy cinnamic acid showed increase βA fibril formation. From our results, they stimulated βA fibril formation. Hydroxylation on the benzene ring increased the fibril formation, while methylation of the hydroxyl groups suppressed the enhancing effect. In addition, saturation of the 2,3-double bond and removal of the 4-oxo group in the C ring of quercetin formed catechins, which caused similar increasing activity on βA fibril formation as compared to cinnamic acids. It may be caused by the presence of the carboxyl moiety of the cinnamic acids and the absence of the keto group on the C ring of flavonoids. In contrast, gallic acid derivatives did not increase βA fibril formation. However, we need further studies to explain these different actions of antioxidants on βA fibril formation. Limonoids are one group of natural products that increase fibril formation. Among them, anthothecol has a carboxyl ester group in its structure. However, we cannot explain properly why they possess such character.

Curcuminoids have been known to possess potent inhibition on fibril formation or βA toxicity to neuron cells in vitro and in vivo (*7*, *9*, *10*). Curcumin is a β-diketone constituent of the spice turmeric, *Curcuma longa* L., and has been found to possess anticarcinogenic properties in several model systems (*16*). Recently, curcumin was considered for further evaluation as a candidate chemopreventive agent (*17*). When we tested a series of analogues of curcumin (**Figure 3**) to determine the structural features necessary for their inhibitory effects on βA fibril formation, tetrahydrocurcumin dramatically lost the inhibitory effect. Demethoxycurcumin lacking a methoxy group on one of the aromatic rings possessed a slightly lower inhibitory effect than curcumin. Similarly, bisdemethoxycurcumin, which lacks two methoxy groups on both of the aromatic rings, showed less inhibitory activity than curcumin. Therefore, among the curcumin analogues, a β-diketone unit linking two phenyl groups

and the double bonds are essential structural features for inhibition of βA fibril formation.

However, the inhibitory activity of curcuminoids decreased after we did check their in vivo activity using HT22 murine neuroblastoma cells. Curcuminoids showed cytotoxicity and no inhibitory effect of βA against the cells. Therefore, in vivo results using HT22cells suggest that all candidate drugs may be checked when they are in human or animals. This result differs from those of previous results that curcuminoids had a protection from βA (*9*). We did not explain why they were different.

Conclusively, the results of this study showed that curcumins, flavone type flavonoids, and naphthoquinone had a significant influence on inhibition of βA fibril formation in vitro. However, curcuminoids did not protect HT22 murine neuroblastoma cells from βA oxidative attack. These findings provide a basis for further study on relationships between naturally occurring compounds in the diet and reduced risk of βA-induced AD patients.

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