3,3',4',5'-Tetrahydroxyflavone Induces Formation of Large Aggregates of Amyloid β Protein

Hiroko Ushikubo, a Yui Tanimoto, b Kazuho Abe, a Tomohiro Asakawa, a Toshiyuki Kan, b and Tatsushi Akashi * a

*Laboratory of Pharmacology, Faculty of Pharmacy and Research Institute of Pharmaceutical Sciences, Musashino University; Tokyo 202–8585, Japan; and b Synthetic Organic & Medicinal Chemistry, School of Pharmaceutical Sciences, University of Shizuoka; Shizuoka 422–8526, Japan.

Received September 9, 2013; accepted February 26, 2014

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by selective neuronal loss, senile plaques and neurofibrillary tangles in the brain. 1–3 Amyloid β protein (Aβ), the major component of senile plaques, is a 40-42 amino acid peptide that self-assembles into insoluble fibrillar aggregates and promotes neurotoxicity. 2,3 Based on the hypothesis that abnormal aggregation of Aβ into insoluble fibrillar deposits causes neurodegeneration in AD, 4 extensive efforts have been made to find drugs that can inhibit formation of Aβ fibrils. Aβ vaccine therapy has also been developed as an attractive therapeutic strategy to suppress AD. 5 However, the original amyloid cascade hypothesis for AD has been questioned in recent years and is currently quite controversial. Aβ aggregation is a complicated process and appears to involve more than a simple conversion of soluble monomers to insoluble fibrils. Assemblies ranging from dimers to 24-mers, or those of even higher molecular weight, have been reported as Aβ oligomers. 5–8 Soluble oligomers or prefibrillar aggregation intermediates of Aβ can be formed at time points preceding fibril formation. 9 Therefore, inhibitors of Aβ fibril formation could possibly cause accumulation of toxic Aβ oligomers, which may result in increased neurotoxicity.

Fisetin (3,3',4',5'-tetrahydroxyflavone, Fig. 1) is a flavonoid present in a number of commonly eaten foods, such as strawberries, 10 and has a variety of biological effects that may be beneficial for the treatment of AD. For example, fisetin protects nerve cells from oxidative stress-induced death 11 and promotes the differentiation of nerve cells, 12 indicating that it is neurotrophic. Furthermore, fisetin has been reported to inhibit Aβ fibril formation in vitro. 13,14 To find more effective drugs than fisetin, we have studied the structure–activity relationship by comparing the anti-amyloidogenic effects of several naturally occurring flavonoids and synthetic fisetin analogues, and found that the 7-hydroxy group is not necessary for the anti-amyloidogenic activity and increasing the number of hydroxyl groups on the B ring potentiates the activity. 15,16 The compound 3,3',4',5'-tetrahydroxyflavone (Fig. 1) was selected as a synthetic fisetin analogue that inhibits Aβ fibril formation more potently than fisetin. 17

To explore the potentiality of the fisetin analogue 3,3',4',5'-tetrahydroxyflavone as a therapeutic drug candidate for AD, the present study addressed the following two important issues. First, what kind of Aβ conformer(s) is (are) present in Aβ solutions when fibril formation is inhibited by 3,3',4',5'-tetrahydroxyflavone? Second, does inhibition of fibril formation by 3,3',4',5'-tetrahydroxyflavone result in a decrease or increase in Aβ neurotoxicity? To answer these questions, Aβ samples incubated in the absence and presence of the newly synthesized fisetin analogue 3,3',4',5'-tetrahydroxyflavone directly produces atypical, large Aβ aggregates and reduces Aβ toxicity.

Key words fisetin; amyloid β protein; 3,3',4',5'-tetrahydroxyflavone; neurotoxicity

Amyloid β protein (Aβ) self-assembles into insoluble fibrils, and forms the senile plaques associated with Alzheimer’s disease. 3,3',4',5'-Tetrahydroxyflavone, a synthetic analogue of the natural flavonoid fisetin, has been found to potently inhibit Aβ fibril formation. In the present study, it was investigated how inhibition of Aβ fibril formation by this flavonoid affects Aβ conformation and neurotoxicity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of Aβ1-42 (20 µM) incubated with or without 3,3',4',5'-tetrahydroxyflavone demonstrated that 3,3',4',5'-tetrahydroxyflavone (100 µM) rapidly caused formation of atypical Aβ conformers, which appeared as a very broad, smear-like band in the high molecular weight region and were distinguishable from soluble Aβ oligomers or mature Aβ fibrils. Transmission electron microscopy (TEM) revealed that large spherical Aβ aggregates were preferentially formed in the presence of 3,3',4',5'-tetrahydroxyflavone. The SDS-resistant, smear-like band on SDS-PAGE and the large spherical aggregates in TEM both disappeared after heat treatment (100°C, 10 min). Furthermore, a neurotoxicity assay with cultured rat hippocampal neurons demonstrated that Aβ incubated with 3,3',4',5'-tetrahydroxyflavone was significantly less toxic than Aβ incubated without the flavonoid. These results suggest that the newly synthesized fisetin analogue 3,3',4',5'-tetrahydroxyflavone directly produces atypical, large Aβ aggregates and reduces Aβ toxicity.

Fig. 1. Chemical Structures of Fisetin and 3,3',4',5'-Tetrahydroxyflavone

Fig. 1. Chemical Structures of Fisetin and 3,3',4',5'-Tetrahydroxyflavone

* To whom correspondence should be addressed. e-mail: tatuaka@musashino-u.ac.jp © 2014 The Pharmaceutical Society of Japan
of 3,3',4',5'-tetrahydroxyflavone were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or transmission electron microscopy (TEM), and neurotoxicity was investigated using primary cultured rat hippocampal neurons.

MATERIALS AND METHODS

Chemicals Aβ1-42 was purchased from Peptide Institute (Lot No. 600911; Osaka, Japan). An Aβ1-42 stock solution (250 μM) was prepared by dissolving the peptide in 0.1% amonia solution, and stored in small aliquots at −85°C until use. Fisetin was purchased from Sigma (St. Louis, MO, U.S.A.). 3,3',4',5'-Tetrahydroxyflavone was synthesized using our recently developed synthetic method for flavones.18,19 Fisetin and 3,3',4',5'-tetrahydroxyflavone were dissolved at 50 mM in 100% dimethyl sulfoxide and stored at −30°C until use. Immediately before use, flavonoids were diluted with 50 mM phosphate buffer (pH 7.4) to give the desired concentrations.

Thioflavin T Fluorescence Assay Fibril formation of Aβ1-42 was quantitatively measured by the Thioflavin T (ThT) fluorescence assay, in which ThT exhibits enhanced fluorescence upon binding to Aβ fibrils.20 Aβ1-42 was diluted to 20 μM in 50 mM phosphate buffer (pH 7.4) and incubated for 0, 2, 6 or 24 h at 37°C. Ten microliters of the Aβ solution was mixed well with 990 μM ThT in 50 mM glycine–NaOH buffer (pH 8.4), and the fluorescence intensity was measured at 450 nm excitation and 480 nm emission with a fluorescence spectrometer (RF-5300PC, Shimadzu Corp., Kyoto, Japan).

SDS-PAGE and Immunoblotting SDS-PAGE was performed as described by Okajima et al.21 Briefly, Aβ samples were mixed in a 1:2 ratio (vol/vol) with 1.5% Triton X-100 sample buffer (Bio-Rad Laboratories, Hercules, CA, U.S.A.) supplemented with 0.15 M dithiothreitol. In experiments testing the influence of heat on Aβ conformers, the samples were boiled (100°C) for 10 min. The samples were loaded onto SDS-polyacrylamide gels containing 3 M Tris–HCl (pH 8.85), and resolved in a running buffer containing 0.05 M Tris–HCl, 0.38 M glycine and 0.1% SDS. After transfer to polyvinylidene fluoride (PVDF) membrane, the membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.01% Tween 20 (TBS-T) for 1 h at room temperature, and incubated with monoclonal anti-Aβ antibody (1:1000; Covance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) diluted 1:5000 in TBS-T; Convance, Emeryville, CA, U.S.A.) overnight at 4°C.

Transmission Electron Microscopy (TEM) TEM was used to morphologically confirm Aβ fibril formation. After incubation, 10 μL of Aβ1-42 solution was placed on 400-mesh copper grids coated with collodion (Nissin EM, Tokyo, Japan). The grids were negatively stained with 2% phosphotungstic acid for 15 s, and observed under an electron microscope (H-7650, Hitachi High-Technologies Corp., Tokyo, Japan) operated at an accelerating voltage of 100 kV.

Neurotoxicity Assay All animal experiments in this study were performed in accordance with the “Guiding Principles for the Care and Use of Laboratory Animals” approved by the Japanese Pharmacological Society. Primary neuronal cultures were prepared from the hippocampal of 18- to 20-d-old embryos of Wistar rats as described in our previous paper22 with some modifications. Briefly, dissociated hippocampal cells were suspended in Neurobasal medium (Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 2% B27 (Life Technologies), 25 μM l-glutamate and 10% fetal bovine serum, and seeded on polylysine-coated 96-well culture plates at a density of 1×10^4 cells/cm². The culture medium was changed to serum-free, B27-supplemented Neurobasal medium 1 d after plating and again to B27-supplemented Neurobasal medium containing cytosine arabinoside (5 μM) 3 d after plating. The cells were then cultured for a further 4 d. The culture medium was switched to unsupplemented Neurobasal medium 24 h prior to the toxicity assay. Aβ1-42 (20 μM) was incubated for 24 h in the presence of vehicle or 3,3',4',5'-tetrahydroxyflavone, and the solution was added to the culture at a dilution of 1:40. Since Aβ fibrils rapidly induced neuronal cell death, the cells were fixed with 4% paraformaldehyde 6 h after addition of Aβ. Since colorimetric methods are sometimes interfered by factors unrelated to cell viability,23 we chose the visual cell counting for quantitating Aβ toxicity as described previously.24 The cells were stained with 0.1% cresyl violet, and the number of neurons bearing processes at least twice as long as the cell body diameter, as an index of neuronal survival, was counted under a microscope.

RESULTS

To examine the time-course of changes in Aβ conformation, Aβ1-42 (20 μM) was incubated for 0, 2, 6 or 24 h at 37°C in 50 mM phosphate buffer (pH 7.4) and subjected to ThT fluorescence assay. As shown in Fig. 2A, ThT fluorescence intensities rapidly increased between 2–6 h of incubation and remained unchanged during 6–24 h after incubation, which is consistent with our previous study.16 Next, changes in soluble Aβ monomers and oligomers were analyzed by SDS-PAGE and immunoblotting with the anti-Aβ antibody 6E10. Immediately after preparation of Aβ1-42 solution (0 h), abundant Aβ monomers and substantial Aβ trimers and tetramers were observed on SDS-PAGE (Fig. 2B). Aβ dimers were rarely found at 0 to 2 h, but increased considerably 6 h after incubation (Fig. 2B). Six to 24 h after incubation, Aβ monomer, trimer and tetramer bands disappeared, and only a very low level of Aβ dimers remained (Fig. 2B). It is likely that most Aβ monomers and oligomers are converted into Aβ fibrils between 2–6 h, and that mature Aβ fibrils do not appear on SDS-PAGE gels in our experimental conditions, as they are highly insoluble and of very high molecular weight.

To investigate the effects of fisetin and 3,3',4',5'-tetrahydroxyflavone on conversion of soluble Aβ into insoluble fibrils, Aβ1-42 (20 μM) was incubated for 0, 2, 6 or 24 h in the presence of vehicle (0.2% dimethyl sulfoxide) or each flavonoid, and analyzed by SDS-PAGE and immunoblotting with 6E10. Time-dependent changes in the amounts of Aβ monomers and oligomers were virtually the same in the absence (Fig. 2B) and presence of vehicle (Figs. 3A, B), indicating that the vehicle has no effect on Aβ aggregation. Aβ monomers,
trimers and tetramers disappeared 6 h after incubation in the presence of vehicle or 10 \( \mu M \) fisetin, while they remained 6 h after incubation in the presence of 100 \( \mu M \) fisetin (Figs. 3A, C), indicating that fisetin delayed the conversion of \( \alpha \)-monomers and oligomers into fibrils. \( \alpha \)-monomers, trimers and tetramers disappeared 24 h after incubation in the presence of 100 \( \mu M \) fisetin, while they remained 6 h after incubation in the presence of 100 \( \mu M \) fisetin (Figs. 3A, C), indicating that fisetin delayed the conversion of \( \alpha \)-monomers and oligomers into fibrils. \( \alpha \)-monomers, trimers and tetramers disappeared 24 h after incubation in the presence of 100 \( \mu M \) fisetin, while they remained 24 h after incubation in the presence of 100 \( \mu M \) fisetin, indicating that the inhibitory effect of fisetin is not persistent. 3,3',4',5'-Tetrahydroxyflavone at 10 \( \mu M \) had the same effect as 100 \( \mu M \) fisetin, but at 100 \( \mu M \) showed very different effects (Figs. 3B, D). Two hours after incubation in the presence of 100 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone, a prominent smear-like band appeared in the high molecular weight region, and it remained unchanged 6–24 h later (denoted by an asterisk in Fig. 3B). The smear-like band was also observed 1 h after incubation in the presence of 100 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone (data not shown). The smear-like band is likely to represent SDS-resistant, high molecular weight \( \alpha \)-aggregates that are distinguishable from soluble \( \alpha \)-oligomers and mature \( \alpha \)-fibrils. \( \alpha \)-monomers, trimers and tetramers remained 24 h after incubation in the presence of 100 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone (Figs. 3B, D).

In general, heat can break the bonds that maintain the three-dimensional structure of proteins. To characterize the smear-like \( \alpha \)-band produced in the presence of 3,3',4',5'-trihydroxyflavone at 10 \( \mu M \) had the same effect as 100 \( \mu M \) fisetin, but at 100 \( \mu M \) showed very different effects (Figs. 3B, D). Two hours after incubation in the presence of 100 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone, a prominent smear-like band appeared in the high molecular weight region, and it remained unchanged 6–24 h later (denoted by an asterisk in Fig. 3B). The smear-like band was also observed 1 h after incubation in the presence of 100 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone (data not shown). The smear-like band is likely to represent SDS-resistant, high molecular weight \( \alpha \)-aggregates that are distinguishable from soluble \( \alpha \)-oligomers and mature \( \alpha \)-fibrils. \( \alpha \)-monomers, trimers and tetramers remained 24 h after incubation in the presence of 100 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone (Figs. 3B, D).

Fig. 2. Time–Course of Conversion of \( \alpha \)-Monomers and Oligomers into \( \alpha \)-Fibrils in Vitro
\( \alpha \)-f(42) (20 \( \mu M \)) was incubated for 0, 2, 6 or 24 h at 37°C in 50 mM phosphate buffer (pH 7.4). (A) \( \alpha \)-Fibril formation as determined by the ThT fluorescence assay. The ordinate indicates the fluorescence intensities of ThT. Data are the mean±S.E.M. of five experiments. (B) \( \alpha \)-monomers and oligomers analyzed by SDS-PAGE and immunoblotting with 6E10. Bands corresponding to monomers to tetramers are indicated by arrowheads. Representative data from one experiment are shown here. Similar results were obtained in three independent experiments.

Fig. 3. Effects of Fisetin and 3,3',4',5'-Tetrahydroxyflavone on Time-Dependent Changes in the Amounts of \( \alpha \)-Monomers and Oligomers in Vitro
\( \alpha \)-f(42) (20 \( \mu M \)) was incubated for 0, 2, 6 or 24 h at 37°C in the presence of vehicle (0.2% dimethyl sulfoxide) or flavonoids. The samples were analyzed by SDS-PAGE and immunoblotting with 6E10. (A, B) Representative results showing the effects of fisetin (A) and 3,3',4',5'-tetrahydroxyflavone (B). The asterisk denotes smear-like bands observed in the high molecular weight region. (C, D) Quantitative data. The intensities of the bands corresponding to \( \alpha \)-monomers (approximately 4 kDa) were quantitated with Multi Gauge V3.0 software (FUJIFILM), and expressed as the percentage of the value at 0 h in each group. White columns: vehicle; gray columns: 10 \( \mu M \) fisetin (C) or 10 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone (D); black columns: 100 \( \mu M \) fisetin (C) or 100 \( \mu M \) 3,3',4',5'-tetrahydroxyflavone (D). The data are the mean±S.E.M. of 5–9 experiments. *p<0.05 vs. vehicle, Dunn’s test.
of Aβ neurotoxicity, cytotoxicity was investigated using primary cultures of hippocampal neurons. Rat hippocampal neurons extended long processes and survived stably in our culture conditions (Fig. 5A, none). When Aβ1-42 (20µM) was incubated for 24h in the presence of vehicle and added at a dilution of 1:40 to hippocampal neuron cultures, almost all cells lost their processes and died within 6h (Fig. 5A, Aβ+ vehicle). When Aβ1-42 (20µM) was incubated for 24h in the presence of 100µM 3,3',4',5'-tetrahydroxyflavone and added at a dilution of 1:40, the neurons retained normal morphology and were alive at 6h after addition (Fig. 5A, Aβ+3,3',4',5'-tetrahydroxyflavone). Quantitative analysis by counting of viable neurons bearing processes demonstrated that Aβ incubated in the presence of 100µM 3,3',4',5'-tetrahydroxyflavone is significantly less toxic than Aβ incubated in the presence of vehicle (Fig. 5B). For comparison, 3,3',4',5'-tetrahydroxyflavone (100µM) alone was added at a dilution of 1:40 to hippocampal neuron cultures, but it had no effect on cell viability in this experimental condition (data not shown, n=5).

Fig. 4. Large Aβ Aggregates Produced in the Presence of 3,3',4',5'-Tetrahydroxyflavone Are Distinguishable from Aβ Fibrils

Aβ1-42 (20µM) was incubated for 24h in the presence of vehicle or 100µM 3,3',4',5'-tetrahydroxyflavone, and subjected to heat treatment (100°C for 10min). (A) SDS-PAGE of Aβ incubated in the presence (a) or absence (b) of 3,3',4',5'-tetrahydroxyflavone before or after heat treatment. (B) ThT fluorescence of Aβ incubated in the presence of vehicle before or after heat treatment. (C) TEM images of Aβ incubated in the presence of vehicle (a,b) or 3,3',4',5'-tetrahydroxyflavone (c,d) before (a,c) or after (b,d) heat treatment. Scale bar, 200nm.
DISCUSSION

In our previous study, incubation of Aβ with fisetin at 100 µM, but not 10 µM, resulted in a significant decrease in the ThT fluorescence intensity of Aβ. SDS-PAGE analysis in the present study demonstrated that fisetin at 100 µM, but not 10 µM, delayed disappearance of Aβ monomers, trimers and tetramers on gels. Furthermore, 3,3',4',5'-tetrahydroxyflavone at 100 µM decreased the ThT fluorescence intensity of Aβ in our previous study and delayed disappearance of Aβ monomers, trimers and tetramers in the present study. These results support the notion that our previous observation is not an artifact of the ThT fluorescence analysis, but represents inhibition of Aβ fibril formation by these flavonoids. In addition, 100 µM fisetin and 10 µM 3,3',4',5'-tetrahydroxyflavone delayed conformational changes in Aβ, but their effects were not persistent and did not result in a significant increase in Aβ oligomers.

In addition to the anti-amyloidogenic effect, 3,3',4',5'-tetrahydroxyflavone had a unique effect that could not be seen in the ThT fluorescence assay. SDS-PAGE analysis in the present study revealed that 3,3',4',5'-tetrahydroxyflavone at 100 µM caused formation of atypical Aβ conformers, which appeared as a very broad, smear-like band in the high molecular weight region. The smear-like band is likely to represent SDS-resistant, smear-like aggregates whose molecular weight is higher than tetramers and lower than mature fibrils. Furthermore, TEM examination revealed that 3,3',4',5'-tetrahydroxyflavone at 100 µM caused formation of very large, spherical aggregates. Since the SDS-resistant, smear-like band in SDS-PAGE and the large spherical aggregates in TEM both disappeared after heat treatment (100°C for 10 min), they are likely to be identical.
The Aβ aggregates produced in the presence of 100 $\mu M$ 3,3',4',5'-tetrahydroxyflavone are clearly distinguishable from mature Aβ fibrils. The structure of mature Aβ fibrils was not changed by heat treatment (100°C for 10 min), as indicated by the ThT fluorescence analysis and TEM examination. SDS-PAGE analysis demonstrated that heat treatment of Aβ samples containing fibrils did not produce Aβ monomers or oligomers. These results suggest that mature Aβ fibrils are formed by stronger bonding and are relatively resistant to heat. On the other hand, the large spherical Aβ aggregates produced in the presence of 100 $\mu M$ 3,3',4',5'-tetrahydroxyflavone disappeared with heat treatment, as demonstrated by TEM examination. SDS-PAGE analysis revealed that heat treatment caused a decrease of the high molecular weight smear-like band, which was accompanied by a prominent increase of Aβ monomers.

These results suggest that the large spherical Aβ aggregates are formed by weak bonding and are disassembled into monomers upon heating.

Another notable characteristic of the Aβ aggregates produced in the presence of 100 $\mu M$ 3,3',4',5'-tetrahydroxyflavone is the rapidity with which they are formed. More than 2 h was required for the conversion of Aβ monomers or oligomers into Aβ fibrils in our experimental conditions, as indicated by the ThT fluorescence and SDS-PAGE analyses. On the other hand, the SDS-resistant, smear-like band appeared on SDS-PAGE within 1–2 h after incubation. This result suggests that, in the presence of 3,3',4',5'-tetrahydroxyflavone, atypical Aβ aggregates are formed faster than Aβ fibrils. It is likely that atypical Aβ aggregates are formed by a direct effect of 3,3',4',5'-tetrahydroxyflavone, but not as a result of inhibition of fibril formation.

It has recently been proposed that formation of amyloid fibers is a two-step process in which proteins first aggregate into colloidal spheres of approximately 20 nm diameter, and the spheres then join together to form linear chains. The spherical Aβ aggregates observed using TEM in our present study were very large, ranging from 500 nm to 1 µm in diameter, and are probably different from the colloidal spheres that can evolve into mature fibrils. Very recently, Ladhawa et al. have reported using atomic force microscopy that resveratrol, a stilbene found largely in the skins of red grapes, remodels soluble oligomers and fibrils of Aβ into large spherical aggregates. The shape and size of Aβ aggregates observed in those studies are very similar to those found in the present study. However, it remains unclear how these small molecules cause the unusual conformational changes in Aβ. In our preliminary study, 100 $\mu M$ 3,3',5'-triiodothyroxyfluavone produced large spherical Aβ aggregates, very similar to 3,3',4',5'-tetrahydroxyflavone, suggesting that the 3,3',4',5'-tetrahydroxy group of the B ring is crucial for the effect of flavonoids. Considering that resveratrol (3,4',5'-trihydroxystilbene) does not have the 3,3',4',5'-triiodothyroxyfluavone and resveratrol may bind to Aβ in different manners. To clarify molecular mechanisms by which these flavonoids produce large spherical Aβ aggregates, computational modeling and molecular simulation studies are underway in our laboratory.

Little is known about the physiological or pathological role of atypical Aβ aggregates produced by 3,3',4',5'-tetrahydroxyflavone. A neurotoxicity assay with cultured rat hippocampal neurons demonstrated that Aβ incubated in the presence of 100 $\mu M$ 3,3',4',5'-tetrahydroxyflavone was significantly less toxic than Aβ incubated in the presence of vehicle, suggesting that the large spherical aggregates produced by 3,3',4',5'-tetrahydroxyflavone are less toxic Aβ conformers. Further investigations are underway in our laboratory to clarify how 3,3',4',5'-tetrahydroxyflavone affects Aβ conformation and neurotoxicity in the brains of animals.

In conclusion, we have found for the first time that 3,3',4',5'-tetrahydroxyflavone rapidly produces large, spherical Aβ aggregates whose bonding is resistant to SDS, but sensitive to heat. Furthermore, we have confirmed that inhibition of Aβ fibril formation by 3,3',4',5'-tetrahydroxyflavone results in a decrease of Aβ neurotoxicity. Therefore, this newly synthesized fisetin analogue is a promising lead compound for the development of therapeutic drugs for AD. For clinical application, further medicinal chemical studies are underway in our laboratory to find fisetin analogues with increased bioavailability.

REFERENCES

1) Selkoe DJ. Alzheimer’s disease: genes, proteins, and therapy. Physiol. Rev., 81, 741–766 (2001).
2) Lorenzo A, Yankner BA. β-Amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. Proc. Natl. Acad. Sci. U.S.A., 91, 12243–12247 (1994).
3) Martin JB. Molecular basis of the neurodegenerative disorders. N. Engl. J. Med., 340, 1970–1980 (1999).
4) Hardy JA, Higgins GA. Alzheimer’s disease: the amyloid cascade hypothesis. Science, 256, 184–185 (1992).
5) Lambracht-Washington D, Rosenberg RN. Advances in the development of vaccines for Alzheimer’s disease. Discov. Med., 15, 319–326 (2013).
6) Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer’s amyloid β-peptide. Nat. Rev. Mol. Cell Biol., 8, 101–112 (2007).
7) Glabe CG. Structural classification of toxic amyloid oligomers. J. Biol. Chem., 283, 29639–29643 (2008).
8) Roychaudhuri R, Yang M, Hoshi MM, Teplow DB. Amyloid β-protein assembly and Alzheimer’s disease. J. Biol. Chem., 284, 4749–4753 (2009).
9) Harper JD, Wong SS, Lieber CM, Lansbury PT Jr. Observation of metastable Aβ amyloid protofibrils by atomic force microscopy. Chem. Biol., 4, 119–125 (1997).
10) Klein WL, Krafft GA, Finch CE. Targeting small Aβ oligomers: the solution to an Alzheimer’s disease conundrum? Trends Neurosci., 24, 219–224 (2001).
11) Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer’s disease: progress and problems on the road to therapeutics. Science, 297, 353–356 (2002).
12) Arai Y, Watanabe S, Kimira M, Shimosi K, Mochizuki R, Kinae N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J. Nutr., 130, 2243–2250 (2000).
13) Ishige K, Schubert D, Sagara Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Radic. Biol. Med., 30, 433–446 (2001).
14) Sagara Y, Vanhansy J, Maher P. Induction of PC12 cell differentiation by flavonoids is dependent upon extracellular signal-regulated kinase activation. J. Neurochem., 90, 1144–1155 (2004).
15) Kim H, Park BS, Lee KG, Choi CY, Jang SS, Kim YH, Lee SE. Effects of naturally occurring compounds on fibril formation and oxidative stress of β-amylloid. J. Agric. Food Chem., 53, 8537–8541 (2005).
16) Akaishi T, Morimoto T, Shibao M, Watanabe S, Sakai-Kato K, Utsunomiya-Tate N, Abe K. Structural requirements for the flavonoid fisetin in inhibiting fibril formation of amyloid β protein. *Neurosci. Lett.*, **444**, 280–285 (2008).
17) Abe K, Ushikubo H, Watanabe S, Hiza A, Ogawa T, Asakawa T, Kan T, Akaishi T. Inhibitory effects of synthetic fisetin analogs on fibril formation of amyloid β protein. *J. Pharmacol. Sci.*, **115** (suppl. 1), 246P (2011).
18) Furuta T, Nakayama M, Suzuki H, Tajimi H, Inai M, Nukaya H, Wakimoto T, Kan T. Concise synthesis of chafurosides A and B. *Org. Lett.*, **11**, 2233–2236 (2009).
19) Asakawa T, Hiza A, Nakayama M, Inai M, Oyama D, Koide H, Shimizu K, Wakimoto T, Harada N, Tsukada H, Oku N, Kan T. PET imaging of nobiletin based on a practical total synthesis. *Chem. Commun.*, **47**, 2868–2870 (2011).
20) Khurana R, Coleman C, Ionescu-Zanetti C, Carter SA, Krishna V, Grover RK, Roy R, Singh S. Mechanism of thioflavin T binding to amyloid fibrils. *J. Struct. Biol.*, **151**, 229–238 (2005).
21) Okajima T, Tanabe T, Yasuda T. Nonurea sodium dodecyl sulfate-polyacrylamide gel electrophoresis with high-molarity buffers for the separation of proteins and peptides. *Anal. Biochem.*, **211**, 293–300 (1993).
22) Abe K, Takayanagi M, Saito H. Effects of recombinant human basic fibroblast growth factor and its modified protein CS23 on survival of primary cultured neurons from various regions of fetal rat brain. *Jpn. J. Pharmacol.*, **53**, 221–227 (1990).
23) Abe K, Saito H. Both oxidative stress-dependent and independent effects of amyloid β protein are detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. *Brain Res.*, **830**, 146–154 (1999).
24) Xu S. Aggregation drives “misfolding” in protein amyloid fiber formation. *Amyloid*, **14**, 119–131 (2007).
25) Ladiwala ARA, Lin JC, Bale SS, Marcelino-Cruz AM, Bhattacharya M, Dordick JS, Tessier PM. Resveratrol selectively remolds soluble oligomers and fibrils of amyloid Aβ into off-pathway conformers. *J. Biol. Chem.*, **285**, 24228–24237 (2010).
26) Ladiwala ARA, Mora-Pale M, Lin JC, Bale SS, Fishman ZS, Dordick JS, Tessier PM. Polyphenolic glycosides and aglycones utilize opposing pathways to selectively remodel and inactivate toxic oligomers of amyloid β. *ChemBioChem*, **12**, 1749–1758 (2011).