BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Black Tea Theaflavins Inhibit Formation of Toxic Amyloid-β and α-Synuclein Fibrils

Gerlinde Grelle,[†] Albrecht Otto,[†] Mario Lorenz,[‡] Ronald F. Frank,[§] Erich E. Wanker,[†] and Jan Bieschke*[,†,∥]

[†]Max Delbrück Centrum für Molekulare Medizin, Robert-Roessle-Strasse 10, 13125 Berlin-Buch, Germany

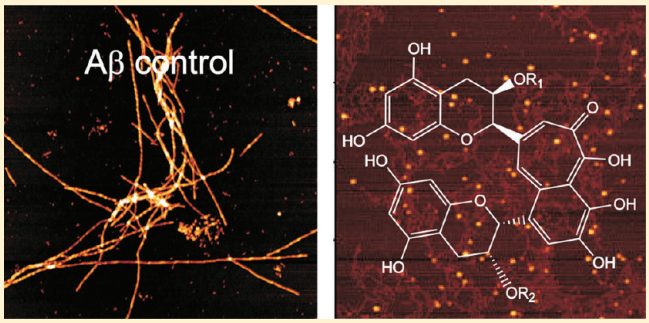
[‡]Medizinische Klinik für Kardiologie und Angiologie, Campus Mitte, Charité-Universitätsmedizin Berlin, Berlin, Germany

[§]Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany

[∥]Department of Biomedical Engineering, Washington University in St. Louis, One Brookings Drive, Campus Box 1097, St. Louis, Missouri 63130, United States

Supporting Information

ABSTRACT: Causal therapeutic approaches for amyloid diseases such as Alzheimer's and Parkinson's disease targeting toxic amyloid oligomers or fibrils are still emerging. Here, we show that theaflavins (TF1, TF2a, TF2b, and TF3), the main polyphenolic components found in fermented black tea, are potent inhibitors of amyloid-β (Aβ) and α-synuclein (αS) fibrillogenesis. Their mechanism of action was compared to that of two established inhibitors of amyloid formation, (−)-epigallocatechin gallate (EGCG) and congo red (CR). All three compounds reduce the fluorescence of the amyloid indicator dye thioflavin T. Mapping the binding regions of TF3, EGCG, and CR revealed that all three bind to two regions of the Aβ peptide, amino acids 12−23 and 24−36, albeit with different specificities. However, their mechanisms of amyloid inhibition differ. Like EGCG but unlike congo red, theaflavins stimulate the assembly of Aβ and αS into nontoxic, spherical aggregates that are incompetent in seeding amyloid formation and remodel Aβ fibrils into nontoxic aggregates. When compared to EGCG, TF3 was less susceptible to air oxidation and had an increased efficacy under oxidizing conditions. These findings suggest that theaflavins might be used to remove toxic amyloid deposits.



Protein misfolding diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), are among the most prevalent maladies of aging populations. Their underlying cause is the misfolding and subsequent aggregation into β-sheet rich assemblies of endogenous polypeptides, such as the β-amyloid peptides (Aβ) and tau protein in Alzheimer's disease and α-synuclein (αS) in Parkinson's disease.[1] While highly structured amyloid fibrils form the end point of most disease-associated aggregation processes, intermediate oligomeric assemblies have been indicated to be the main toxic agents in protein misfolding diseases,[2,3] while fully formed fibrils are less toxic and can even have physiological functions.[4,5] However, therapeutic approaches that address protein misfolding and aggregation are just beginning to emerge.[6,7]

Several potential treatment strategies exist to prevent or possibly reverse amyloid formation. The first strategy relies on limiting the supply of amyloidogenic monomers. β-Secretase inhibitors that prevent cleavage of the amyloid precursor protein, APP, inhibit the release of aggregation-prone Aβ peptides.[6,8,9] In transthyretin amyloidoses, formation of an aggregation-prone monomer can be prevented by small molecules that stabilize the native tetrameric structure of transthyretin.[10,11] A second strategy aims to identify compounds that bind to misfolded polypeptides and inhibit or reverse the formation of oligomeric and fibrillar aggregates (reviewed in ref 12). This strategy has been the focus of numerous drug discovery efforts; however, none of these molecules have been successfully evaluated in clinical studies.

Recently, a third therapeutic strategy has emerged. Natural compounds such as the polyphenol (−)-epigallocatechin gallate [EGCG (Figure 1)] that redirect amyloid formation pathways and efficiently promote the assembly of nontoxic aggregate structures have been identified.[13] EGCG affects the structure and stability of multiple target proteins, which are involved in neurodegeneration, oncogenesis, and even viral proliferation.[14−17] EGCG binds to unfolded proteins via hydrogen bonds and hydrophobic peptide backbone interactions.[14,18] In addition, conjugation of EGCG to lysines via Schiff base formation has recently been reported.[19] Interaction of EGCG with unfolded αS and Aβ polypeptides potently inhibits

Received: August 8, 2011
Revised: November 3, 2011
Published: November 4, 2011

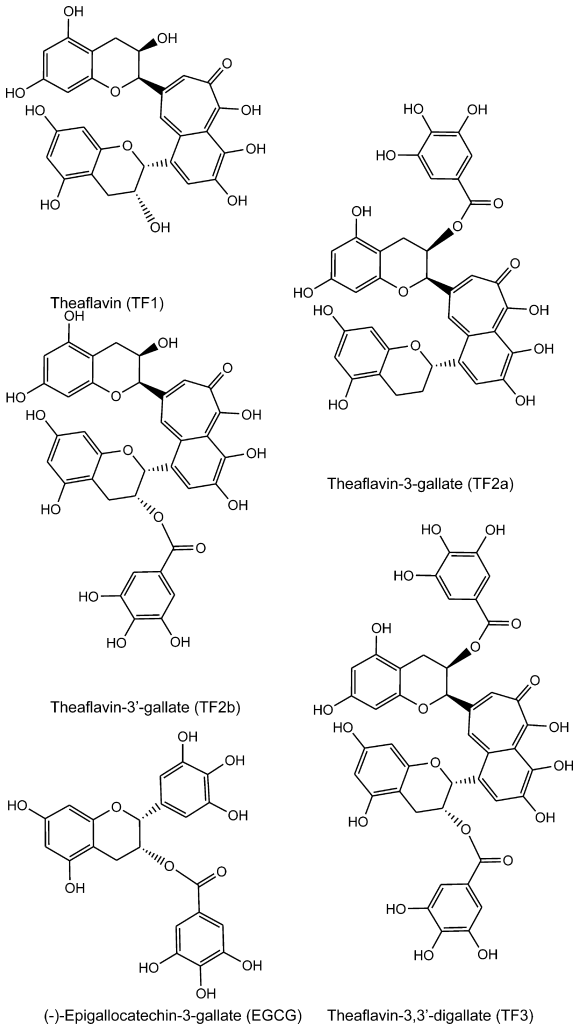


Figure 1. Structures of theaflavins.

amyloid formation and toxicity.[14,20−22] Similarly, EGCG was found to block the assembly of SDS-stable huntingtin protein aggregates.[13] Very recently, studies on a large number of amyloidogenic proteins have emerged: EGCG and other polyphenols prevent the conversion of prion protein PrP^{C} into its prion conformer PrP^{Sc} [23] as well as the propagation of the yeast prion Sup35.[24] EGCG inhibits fibril formation of lysozyme[25] and islet amyloid polypeptide IAPP[26] and destroys fibrils of transthyretin[27] and merozoite surface protein 2 (MSP2).[28]

Analysis of aggregation reactions with and without EGCG via electron microscopy (EM) or atomic force microscopy (AFM) revealed that compound treatment of amyloidogenic proteins results in the formation of small spherical or amorphous aggregates that are nontoxic for cells. These structures lack properties of amyloid, i.e., fibrillar morphology, cross-β structure, and the ability to bind amyloidophilic dyes such as CR and ThT.[14,17] Moreover, they have lost the ability to function as seeds and therefore do not catalyze the spontaneous formation of amyloid fibrils.[14]

Recently, we have also observed that EGCG can directly bind to preformed amyloid fibrils and remodels their characteristic cross-β-sheet structure.[29] Strikingly, this effect correlates with reduced amyloid toxicity in various cell models of AD and PD, suggesting that EGCG is a promising model substance for further therapy development that can detoxify preformed amyloid fibrils and oligomers. Studies with transgenic mouse models support these results. Oral administration of EGCG significantly reduced learning deficits and amyloid deposition in brains of AD model mice.[30] Similar effects also have been observed for a grape seed-derived polyphenol.[31] Moreover, it was shown that upregulation of endogenous catechols, such as dopamine, reduced the level of αS amyloid formation in cell model systems.[32]

EGCG is the main phenolic component of green tea; however, fermentation of tea leaves to produce different types of black tea causes oxidation of the compound.[33] This results in the formation of theaflavins,[34] a group of polycyclic polyphenols that are formed by quinonic condensation of the catechin structure.[33,34] Theaflavins that can be conjugated to either one (TF2a or TF2b) or two (TF3) gallic acid moieties are potent antioxidants (Figure 1) that are structurally similar to EGCG.[35−37] This led us to explore the potential antiamyloidogenic properties of different theaflavin compounds in the context of Aβ and αS amyloidogenesis.

EXPERIMENTAL PROCEDURES

Materials. $A\beta_{40}$ and $A\beta_{42}$ peptides as well as KLVFFA and EFAVFLK peptides were purchased from the laboratory of R. Volkmar-Engert (Institute for Medical Immunology, Charité, Berlin, Germany). Monomeric $A\beta_{40}$ and $A\beta_{42}$ peptide solutions were prepared as described previously.[38] Peptides were dissolved in hexafluoro-2-propanol (HFIP) overnight, sonicated for 30 min, lyophilized, and redissolved in a 10 mM NH_4OH solution. Aggregated forms of $A\beta_{40}$ were removed by filtering the solution first through a membrane filter with a pore size of 0.2 μm and then though a low-molecular weight membrane filter [$A\beta_{40}$, Microcon YM-10; $A\beta_{42}$, Microcon YM-30 (Millipore)]. Peptide concentrations were determined by OD_{280} (E_{280} = 1280 M^{-1}) in a Nanodrop 8000 photometer (Thermo Fisher). Filtrates were diluted to 30 μM in a 10 mM NH_4OH solution and mixed in a 1:1 ratio with 2× phosphate-buffered saline (PBS, pH 7.4) to final Aβ concentrations of 15 μM.

Recombinant αS protein was expressed in *Escherichia coli* and purified via ion exchange and size exclusion chromatography as described previously.[39] *E. coli* cells were grown at 37 °C in LB medium containing ampicillin to an OD_{600} of ∼0.5, induced with IPTG (1 mM), and grown overnight at 30 °C. Cells were pelleted for 30 min at 3000*g*, frozen at −80 °C, and then resuspended in Tris buffer (10 mM, pH 8.0) containing EDTA (1 mM) and PMSF (1 mM). Benzonase (1250 units) was added, and cells were lysed by sonication on ice (3 × 1 min, Branson Sonifier 450, Branson, Dunbury, CA), boiled for 20 min, and then centrifuged for 30 min at 20000*g*. αS was precipitated from the supernatant by an ammonium sulfate (360 mg/mL) treatment for 15 min followed by centrifugation (20000*g* for 30 min). The pellet was resuspended in Tris-HCl buffer (25 mM, pH 7.7) and purified on a Resource-Q anion exchange column using a NaCl gradient from 0 to 500 mM. αS fractions were pooled and run on a PD-10 gel chromatography column in 10 mM ammonium carbonate buffer, and eluted fractions were lyophilized.

Lyophilized αS was dissolved in water, sonicated for 10 min in a water bath sonifier (TK-52, Bandelin), and centrifuged for 20 min at 200000*g*. The protein concentration was determined by OD_{280} (E_{280} = 6410 M^{-1}). Heptamer peptides were dissolved in DMSO at 10 mM, diluted to 200 μM in water, and then mixed in a 1:1 ratio with 2× PBS for compound binding.

dx.doi.org/10.1021/bi2012383|*Biochemistry* 2011, 50, 10624−10636

Theaflavin (TF1), theaflavin 3-monogallate (TF2a), theaflavin 3′-monogallate (TF2b), and theaflavin 3,3′-digallate (TF3) were kindly provided by Mitsui Norin Food Research Laboratories (Shizuoka, Japan). Theaflavins were dissolved in water at a concentration of 5 mM to produce stock solutions. EGCG was obtained from Sigma-Aldrich (Hamburg, Germany), and stock solutions (10 mM) were freshly prepared in water. TF3 stock solutions were filtered through a 3 kDa membrane filter (YM-3, Millipore) where indicated.

Atomic Force Microscopy (AFM). Sheet mica (Nanoworld) was glued to a microscope slide, and samples (20 μL) were adsorbed for 10 min onto the freshly cleaved mica, washed with freshly filtered deionized water (4 × 30 μL), and dried overnight. AFM images were recorded on a Nanowizard II/Zeiss Axiovert setup (JPK, Berlin, Germany) using intermittent contact mode and FEBS cantilevers (Veeco, Santa Barbara, CA).

Protein Aggregates and Seeding Experiments. Fibrillar αS aggregates were formed by incubation of the αS protein (50 μM) on a rotary shaker (180 rpm) at 37 °C in SP buffer [100 mM sodium phosphate (pH 7.4) and 10 mM NaCl]. Fibrillar Aβ aggregates were grown for 24 or 36 h at a peptide concentration of 15 μM in PBS at 37 °C in the presence or absence of the indicated amounts of theaflavins, EGCG, or CR. For seeding experiments, $A\beta_{40}$ aggregates were sonicated for 15 min in a cold water bath and added to fresh monomeric $A\beta_{40}$ peptide solutions (15 μM).

ThT Aggregation Kinetics. Aliquots (10 μL) of an αS solution were mixed with 90 μL of ThT (20 μM) in SP buffer. The resulting fluorescence was measured immediately after mixing using a fluorescence plate reader (InfinitE M200, Tecan) at excitation and emission wavelengths of 440 and 485 nm, respectively. The ThT kinetics for $A\beta_{40}$ and $A\beta_{42}$ were recorded as described in ref 38 at 37 °C [shaking for 5 s every 10 min using a fluorescence plate reader (InfinitE M200)].

Analysis of TF3 and EGCG Oxidation. Stock solutions of TF3 or EGCG were diluted into PBS to a final concentration of 25 μM. Then, samples were incubated in a 1.5 mL reaction tube on a rotary tumbler at 37 °C for 30 min or 1 h for subsequent HPLC analysis. EGCG and TF3 concentrations were determined by reverse phase HPLC (μRPC C2/C18SC 2.1/10, GE Healthcare) in a water (0.1% TFA)/acetonitrile (0.1% TFA) gradient and quantified by UV absorption at 215 nm. EGCG and TF3 solutions used in kinetic fibril formation assays were preincubated for 1 or 24 h in PBS at a concentration of 1 mM while being shaken at 37 °C.

Filter Retardation Assays. Equal volumes of denaturation buffer (100 mM DTT and 4% SDS) were added to αS or Aβ solutions (50 μL). Then, samples were boiled for 5 min and filtered through a cellulose acetate membrane filter (Bio-Rad) using a 96-well vacuum apparatus. For detection of Aβ or αS, membranes were blocked in milk and stained using an anti-Aβ (1:2000, mAb 6E10, Signet) or anti-αS antibody (1:2000, mAB 211, Santa Cruz Biotechnology), respectively.

Cell Toxicity Assays. PC12 cells (rat pheochromocytoma, American Type Culture Collection) were cultured in DMEM (Gibco BRL) supplemented with 5% fetal bovine serum, 10% horse serum, and 3.0 mM glutamine in a 5% CO_2 humidified environment at 37 °C.[40] Cells were plated at a density of 10000 cells/well on 96-well plates in 90 μL of fresh medium. After 24 h, the αS or Aβ protein aggregates were added and cells were further incubated for 3 days at 37 °C. Cellular metabolic activity was measured utilizing a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Promega). Formazan formation was quantified by absorption at 590 nm in a plate reader (Infinite M200, Tecan).

Peptide Array Binding Assays. An array of 33 peptides representing the complete $A\beta_{42}$ sequence was synthesized on a membrane support to define potential drug binding regions in Aβ peptides.[41] Each synthetic peptide was composed of 10 amino acids, which overlapped in sequence with the next peptide by nine residues (Figure S1A of the Supporting Information). Membranes were incubated in a compound solution (300 μM in PBS) for 15 min and then washed overnight in PBSTT (PBS with 0.05% Tween 20 and 0.2% Triton X-100). Heptamer peptides (100 μM) were mixed with either CR, EGCG, or TF3 (300 μM) for 15 min and then dot blotted on a nitrocellulose membrane using a vacuum apparatus. Binding of compounds was visualized either directly (CR and TF3) or after NBT staining (EGCG and TF3). NBT staining of proteins electroblotted onto nitrocellulose membranes has been described by Paz et al.[42] Binding was quantified via spot intensity with AIDA (Raytest). Binding data were normalized by setting the background from unspecific membrane binding outside the peptide spots to 0% and the maximal spot signal to 100%. Peptides that inhibited unspecific drug interaction [as for CR (Figure S1B of the Supporting Information)] resulted in negative normalized signals.

Statistical Analysis. *P* values for data from MTT and seeding assays were calculated from three independent samples using the unpaired two-tailed *t* test assuming equal variances.

RESULTS

Theaflavins Inhibit Aβ Fibril Formation and Induce the Formation of Spherical Aggregates. First, the effect of theaflavins on $A\beta_{40}$ amyloid fibril formation was analyzed. Solutions of $A\beta_{40}$ monomers (15 μM) were incubated for 48 h at 37 °C with theaflavins TF1, TF2a, TF2b, and TF3. Formation of aggregates was systematically imaged by AFM (Figure 2). We found that the formation of $A\beta_{40}$ fibrils was inhibited when compared to that in an untreated control sample (Figure 2A–F). Instead, spherical aggregates were predominantly observed in the presence of theaflavins, suggesting that the compounds redirect $A\beta_{40}$ momomers into aggregates of a new type. This effect was most pronounced in the presence of TF3, which induced formation of spherical aggregates at a molar ratio of 1:0.2 between $A\beta_{40}$ and TF3 (Figure 2D). Therefore, further more detailed experiments on the dosage dependence, compound stability, and mechanism of action were conducted with TF3.

TF3 was added to $A\beta_{42}$ monomers under the same conditions. Like the $A\beta_{40}$ peptide, $A\beta_{42}$ formed spherical aggregates in the presence of TF3 (Figure 2G) but formed fibrils in its absence (Figure 2J). Thus, the effect of TF3 on $A\beta_{42}$ was similar to that previously observed for EGCG.[14]

$A\beta_{40}$ was incubated with equimolar CR under the same conditions to compare its effect on fibril formation to that of the theaflavins (Figure 2H). CR treatment did not prevent fibrillization but reduced the amount of mature twisted fibrils, so that more protofibrils and single-strand fibrils were present compared to the number for the untreated $A\beta_{40}$ control (Figure 2I). CR did not induce the formation of spherical oligomers, suggesting that its mechanism of amyloid inhibition differs from that of the theaflavins.

Theaflavins Inhibit ThT Fluorescence. ThT fluorescence is a widely used tool for monitoring how small molecules or proteins modulate aggregation and amyloid formation. However, a number of antiamyloidogenic compounds have

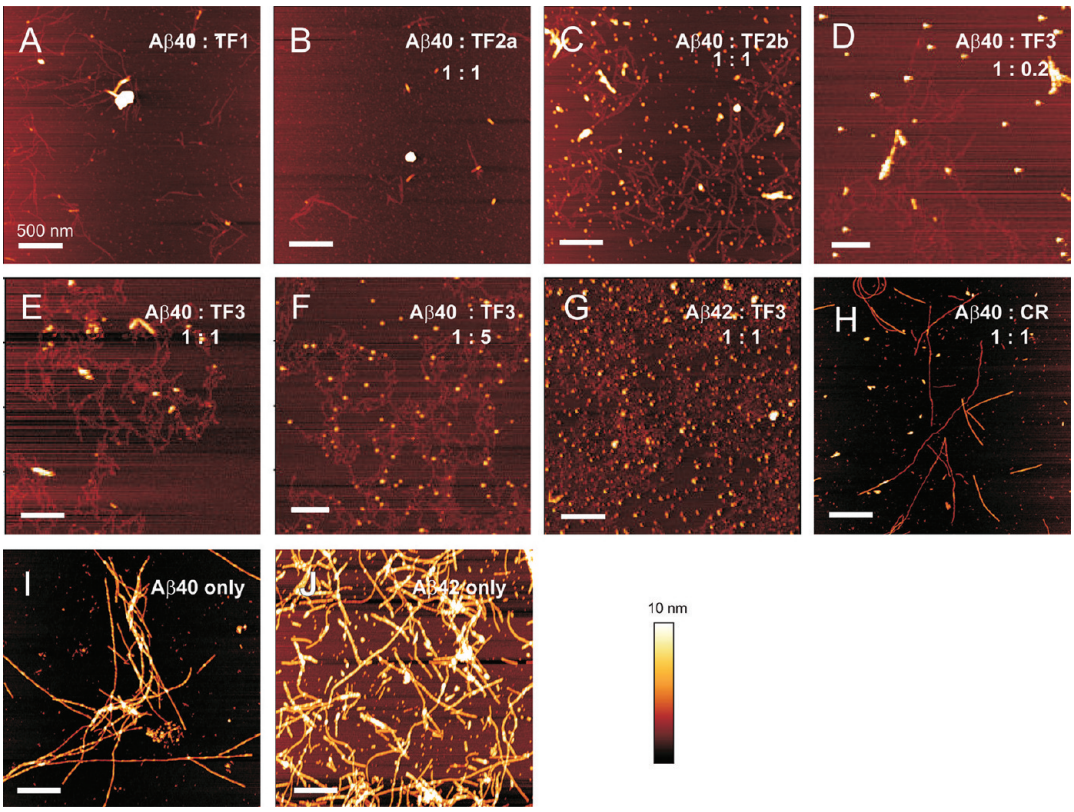


Figure 2. Theaflavins induce the assembly of spherical aggregates. $A\beta_{40}$ and $A\beta_{42}$ peptides (15 μM) were incubated in the presence or absence of the indicated ratios of theaflavins TF1, TF2a, TF2b, TF3, and CR in a rotary shaker in PBS at 37 °C for 48 h. Spherical structures 4−10 nm in height were predominantly observed in theaflavin-treated samples, whereas fibrils and protofibrils were observed in CR samples and untreated controls.

been observed to interfere with ThT fluorescence either by spectral interference or by direct binding to micellar ThT.[43] We analyzed the effect of theaflavins TF1, TF2a, TF2b, and TF3 on $A\beta_{40}$ aggregation kinetics using the previously described thioflavin T (ThT) dye binding assay.[44] All theaflavins efficiently inhibited ThT fluorescence of $A\beta_{40}$ aggregates by ~90% at equimolar concentrations (Figure 3A), supporting the results by AFM (Figure 2). Moreover, compound treatment prolonged the lag phase of $A\beta_{40}$ aggregation, indicating that theaflavins influence nucleation, an early step in the amyloid formation cascade, during which the seeds for fibril formation are formed.[45] Similar results were observed upon incubation of $A\beta_{42}$ with theaflavins at equimolar concentrations. All theaflavins reduced ThT fluorescence, and all but TF1 prolonged the lag phase of aggregation (Figure 3B). To test whether binding to a nonamyloidogenic protein would prevent the effect on aggregation kinetics, we added BSA to $A\beta_{40}$ and $A\beta_{42}$ aggregation assays at twice the $A\beta$ concentration (Figure S2A,B of the Supporting Information). TF3 still inhibited ThT fluorescence and delayed the lag phase of $A\beta_{40}$ aggregation in the presence of BSA, although to a lesser degree than in the absence of BSA (Figure 3B). This may suggest that TF3 partitions between $A\beta$ and BSA binding, which would lower its effective concentration.

Recently, it has been observed that aggregates rather than monomers of some anti-amyloid compounds were the active species in inhibiting fibril formation.[46] While TF3, like EGCG, is highly soluble in water, we wanted to test whether this hypothesis applies to TF3. Therefore, we filtered a TF3 stock solution through a low-molecular mass (3 kDa) cutoff membrane filter and compared the efficacy of the filtered and unfiltered solution in ThT aggregation assays (Figure S2C of the Supporting Information). Both TF3 solutions were equally effective in preventing the formation of ThT binding aggregates, indicating that TF3 can inhibit $A\beta$ fibrillization without preaggregation.

The effects of TF3 on ThT aggregation kinetics were then compared to those of EGCG[14] and CR (reviewed in ref 47), two previously described inhibitors of amyloid fibril formation. $A\beta_{40}$ was incubated with TF3, EGCG, or CR at molar ratios of 1:0.1 to 1:1 (Figure 3C−E). Like TF3, both EGCG and CR strongly decreased the magnitudes of ThT signals of $A\beta_{40}$ at equimolar ratios (Figure 3D,E and ref 14). In contrast to polyphenols EGCG and TF3, however, CR did not prolong the lag phase of $A\beta_{40}$ polymerization. Both polyphenols inhibited nucleation of $A\beta_{40}$ fibril formation, resulting in a prolonged lag phase, whereas CR reduced the magnitude of only the ThT signal of the mature fibrils. The observation that fibrillar aggregates were present in $A\beta_{40}$ aggregation samples after incubation with CR (Figure 2H) suggests that CR interferes with ThT binding or fluorescence rather than with aggregation under these aggregation conditions. Thus, the mechanism of action of theaflavin TF3 is different from that of the previously described amyloid inhibitor CR. Rather, its effect is similar to that of polyphenol EGCG.[14]

TF3 Binds to Hydrophobic Regions in $A\beta$ Peptides. Next, we asked whether the different effects of TF3, EGCG, and CR on $A\beta_{40}$ aggregation kinetics may be due to different interactions with the $A\beta$ peptide. We probed the interaction of TF3, EGCG, and CR with $A\beta$ to define potential binding regions using an array of 33 overlapping peptides that represent the complete $A\beta_{42}$ sequence (Figure S1A of the Supporting

dx.doi.org/10.1021/bi2012383|*Biochemistry* 2011, 50, 10624−10636

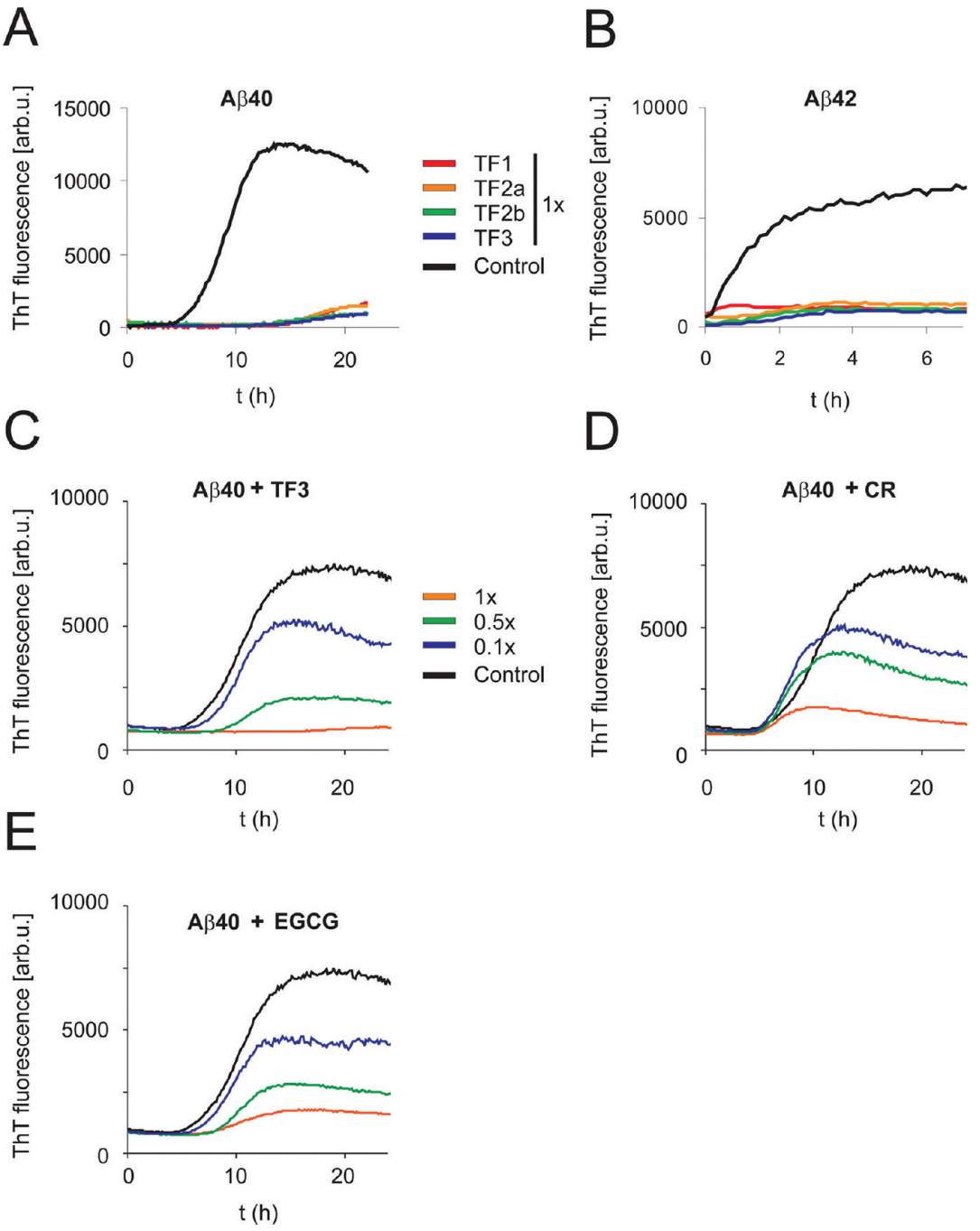


Figure 3. Theaflavins inhibit the formation of thioflavin T binding aggregates. Monomeric solutions of $A\beta_{40}$ (15 μM) or $A\beta_{42}$ (15 μM) were incubated at 37 °C in PBS in the presence of theaflavins under agitation (5 s every 10 min) in a fluorescence plate reader, and aggregation kinetics were recorded by ThT (20 μM) fluorescence. Theaflavins TF1, TF2a, TF2b, and TF3 strongly inhibited $A\beta_{40}$ (A) and $A\beta_{42}$ (B) fluorescence. (C–E) Concentration-dependent inhibition of ThT fluorescence in $A\beta_{40}$ (15 μM) aggregation reactions by TF3 (C), CR (D), or EGCG (E).

Information). Analysis of array binding data revealed that TF3 most strongly interacts with those peptides that contain nonpolar, hydrophobic amino acids (e.g., peptide 12, VHHQKLVFFA, amino acids 12–21; or peptide 24, VGSNKGAIIG, amino acids 24–33), while peptide sequences with polar amino acids (e.g., peptide 1, DAEFRHDSGY, amino acids 1–10) were not recognized (Figure 4A and Figure S1B of the Supporting Information).

Like TF3, EGCG and CR also both bound to the regions around amino acids 12–23 and 24–36 (Figure 4A). However, closer analysis revealed differences among the three compounds. Binding regions of both TF3 and EGCG were broad, spanning peptides 7–17 (amino acids 7–26) and 20–28 (amino acids 20–37). The relative affinities of both compounds for the first and second binding region are reversed. While EGCG interacts more strongly with the first region, TF3 binds stronger to the second region. In contrast to both polyphenols, CR bound with similar affinity to both regions but exhibited very narrow specific interactions with peptides 12 and 13 (amino acids 12–22) and 24–27 (amino acids 24–36). This suggests that CR interacts with $A\beta$ at two specific interaction sites, whereas the polyphenols bind more broadly to multiple peptides spanning

dx.doi.org/10.1021/bi2012383|*Biochemistry* 2011, 50, 10624–10636

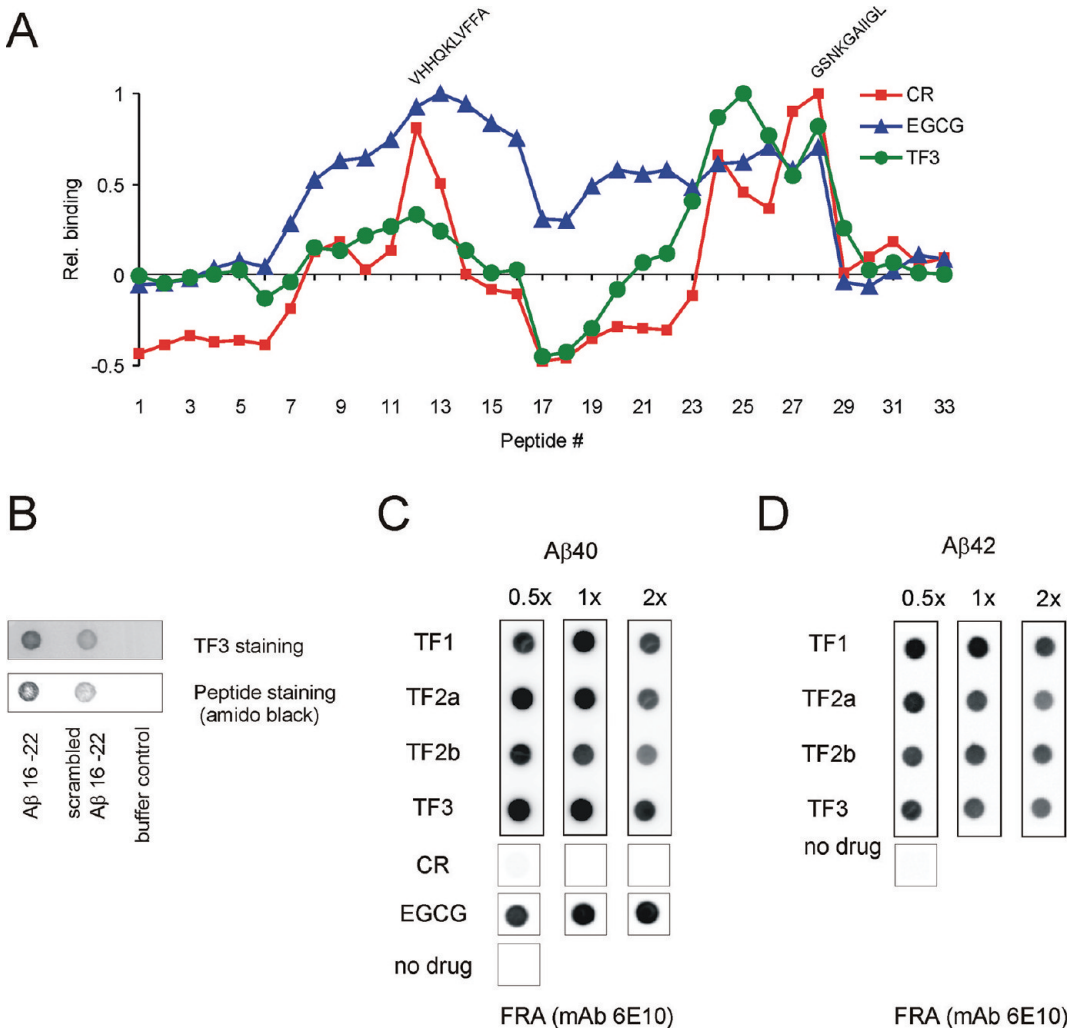


Figure 4. Theaflavins bind Aβ and induce the formation of SDS-stable aggregates. (A) Relative binding of TF3, EGCG, and CR to a 10-mer peptide array covering the Aβ_{42} sequence. Peptide numbers represent the starting amino acids of 10-mer peptides. All compounds bind in two regions to Aβ peptides detected (peptides 8−16 and 23−28) but with different relative affinities. (B) Binding of TF3 to Aβ 16−22 and scrambled Aβ 16−22 peptide. TF3 binding was quantified colorimetrically. Peptides were quantified by amido black staining. (C) Filter retardation assay of SDS-resistant Aβ_{40} aggregates. The Aβ_{40} peptide was incubated at 37 °C in the presence of TF1, TF2a, TF2b, and TF3 at molar ratios of 0.5−2 for 24 h in PBS under agitation (shaking for 5 s every 10 min). Samples were boiled in 2% SDS, and SDS-insoluble aggregates were analyzed by a filter retardation assay (FRA). Aggregates were stained for Aβ (mAb and 6E10). (D) FRA of the Aβ_{42} peptide incubated at 37 °C in the presence of TF1, TF2a, TF2b, and TF3 as for panel C. All theaflavins induced the formation of SDS-stable aggregates.

the hydrophobic regions of the Aβ peptide. It should be noted that peptides that interacted with EGCG or TF3 all contained lysine residues (K16 and K28). Our results are therefore compatible with the emerging view that formation of Schiff bases to lysines contributes to polyphenol binding.

TF3 Binding Is Not Sequence-Specific. We proceeded to test whether the binding of TF3 to the hydrophobic regions of the Aβ peptide is sequence-specific or whether it depends solely on the amino acid composition. We incubated TF3 with two heptamer peptides, the peptide KLVFFAE that represents amino acids 16−22 of the Aβ sequence and a scrambled EFAVFLK peptide that is composed of the same amino acids in a different sequence. The peptides were then dot blotted to detect peptide-bound TF3 by its red color (Figure 4B). TF3 bound strongly to the KLVFFAE peptide, but it also stained, albeit to a lesser degree, the scrambled peptide. No staining of the membrane was observed in the absence of peptides (Figure 4B and Figure S1C of the Supporting Information). We counterstained peptides with amido black to quantify the amount of peptide that was retained on the membrane and found that relative intensities of TF3 staining matched those of amido black staining (Figure 4B).

Therefore, we conclude that binding of TF3 to the hydrophobic region at amino acids 16−22 is not sequence-specific, but rather that hydrophobic amino acid side chains, and possibly also Schiff base formation with lysine residue K16, mediate binding. When we incubated TF3 and EGCG with the two peptides, we observed similar binding patterns, i.e., strong binding to the native sequence peptide and weaker binding to the scrambled peptide (Figure S1C of the Supporting Information). In contrast, binding of CR depended on the proper Aβ sequence, and the scrambled peptide was not stained by the CR dye (Figure S1C of the Supporting Information).

Theaflavin-Induced Aβ Aggregates Are Stable to SDS Denaturation. Because previous studies with EGCG have demonstrated that compound treatment results in the

dx.doi.org/10.1021/bi2012383|*Biochemistry* 2011, 50, 10624−10636

formation of SDS-stable Aβ aggregates, we next investigated whether the aggregates produced by theaflavins had similar properties. $A\beta_{40}$ peptides were incubated for 24 h with compounds TF1, TF2a, TF2b, and TF3, boiled in 2% SDS, and then analyzed by a FRA (Figure 4C). We found that even substoichiometric amounts of the theaflavins induced the formation of SDS-resistant $A\beta_{40}$ aggregates, whereas no SDS-resistant aggregates were detected in untreated controls (Figure 4C).

The same results were obtained upon incubation of the theaflavins with the $A\beta_{42}$ peptide (Figure 4D). In contrast, no SDS-stable Aβ aggregates were observed in the presence of CR under these conditions (Figure 4C), corroborating the observation that theaflavins modulate Aβ amyloid formation by a mechanism that is similar to that of EGCG but distinct from that of CR.

Induction of Aβ Aggregates Does Not Depend on Antioxidant Properties. To further scrutinize the mechanism of TF3-induced formation of SDS-stable aggregates, we tested whether the antiamyloidogenic effect of theaflavins depends on their antioxidant properties. We investigated the stability of TF3 and EGCG against oxidation by air using HPLC and compared these results with data from ThT aggregation assays. Solutions (25 μM) of EGCG and TF3 in PBS were agitated in an overhead shaker at 37 °C in the dark. Concentrations of both compounds were analyzed by reverse phase HPLC after 30 and 60 min (Figure 5A). After 60 min, the amplitude of the EGCG elution peak was reduced to ∼20% of that of the initial signal, while ∼70% of TF3 was still present (Figure 5A), indicating that the theaflavin is less rapidly oxidized by air than EGCG. The oxidation products of EGCG and TF3 were not analyzed in detail. However, a large number of peaks were eluted from the HPLC column, indicating that a number of different oxidation products are formed (data not shown).

To test whether the oxidation products of EGCG and TF3 were still competent to inhibit amyloidogenesis, both compounds were shaken for 1 or 24 h at 37 °C and then added to $A\beta_{40}$ monomers at equimolar ratios. Formation of $A\beta_{40}$ aggregates was monitored over 18 h by ThT fluorescence.[44] Both compounds completely inhibited the formation of ThT positive aggregates when added freshly to $A\beta_{40}$ peptide (Figure 5B,C). However, prolonged preoxidation of EGCG reduced its efficacy. $A\beta_{40}$ peptide treated with EGCG that had been preincubated for 24 h showed aggregation kinetics with a 30% prolonged lag phase (12 h vs 9 h) and 10% of the ThT fluorescence when compared to those of the untreated $A\beta_{40}$ peptide (Figure 5B). In contrast, no ThT signal was recorded from $A\beta_{40}$ peptide treated with TF3, regardless of preincubation (Figure 5C). Our results indicate that TF3 is more resistant to oxidation by air than EGCG and retains its anti-amyloid activity under oxidizing conditions.

TF3 Reduces the Seeding Competence and Toxicity of Aβ Aggregates. Next, we assessed whether the ThT-negative spherical aggregates formed in the presence of TF3 are seeding competent structures that are able to accelerate the conversion of $A\beta_{40}$ monomers into amyloid fibrils.[45] $A\beta_{40}$ monomers were treated with TF3 at equimolar ratios, and spherical oligomers were produced after incubation for 24 h at 37 °C. $A\beta_{40}$ was incubated in the presence of EGCG and CR or without the addition of small molecules in control experiments under the same conditions. $A\beta_{40}$ oligomers and/or aggregates were then sonicated and added to fresh monomeric $A\beta_{40}$ peptide (2%, v/v) to seed aggregate formation (Figure 6A). The seeding competence of these seeds was quantified by measuring the lag phase ($t_{50\%}$) until the half-maximal ThT fluorescence was reached (Figure 6B). We found that addition of EGCG and TF3 generated $A\beta_{40}$ oligomers and/or aggregates that had significantly longer lag phases than seeds derived from untreated $A\beta_{40}$ fibrils (Figure 6B), supporting the results of previous studies that showed that polyphenol-generated aggregates have a diminished seeding competence.[14] Interestingly, CR did not reduce the seeding competence of $A\beta_{40}$ aggregates, supporting the hypothesis that this compound has a mechanism of action different from that of the theaflavins and EGCG (Figure 6A,B).

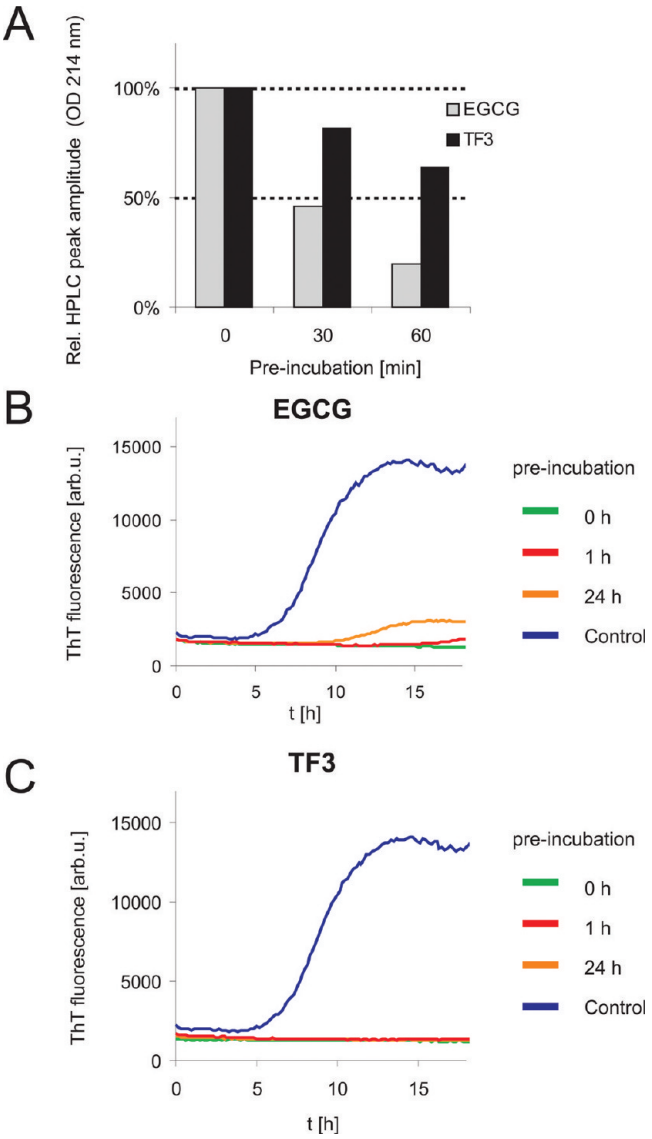


Figure 5. TF3 is less rapidly oxidized than EGCG. EGCG and TF3 (25 μM) solutions in PBS were shaken in an Eppendorf tube tumbler at 37 °C. (A) Relative concentrations of EGCG and TF3 were analyzed by reverse phase HPLC after 30 and 60 min. (B and C) EGCG and TF3 (1 mM) were shaken in PBS for 1 or 24 h and then added to fresh monomeric $A\beta_{40}$ peptide (15 μM) at equimolar ratios. Reaction mixtures were incubated at 37 °C for 18 h in PBS and ThT (20 μM) while being agitated in a fluorescence plate reader in which ThT fluorescence was recorded.

dx.doi.org/10.1021/bi2012383|*Biochemistry* 2011, 50, 10624–10636

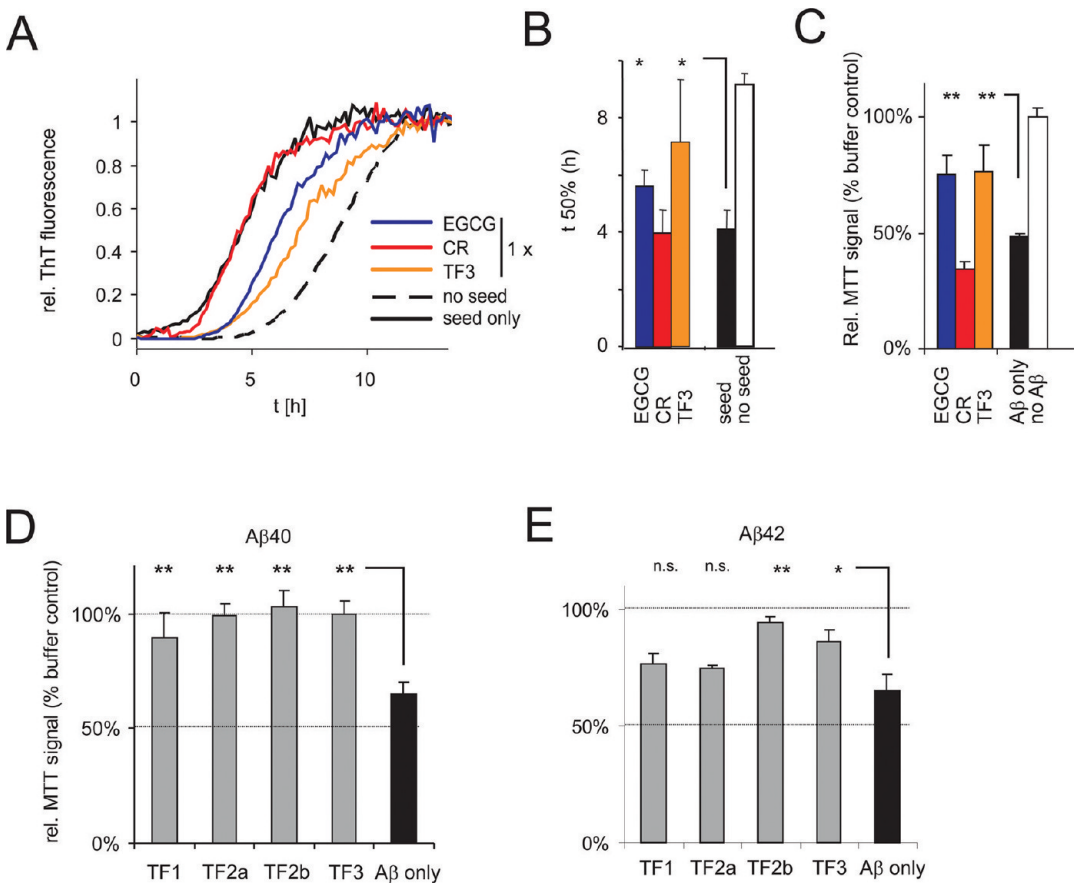


Figure 6. Theaflavins reduce amyloid seeding competence and toxicity. Monomeric $A\beta_{40}$ peptide (15 μM) was incubated under agitation with TF3, EGCG, or CR in PBS at the indicated molar ratios for 24 h at 37 °C and then sonicated for 15 min to produce seeds. These seeds (2%, v/v) were then added to monomeric $A\beta_{40}$ peptide (15 μM) to stimulate aggregation. (A) Seeded and unseeded aggregation kinetics were monitored by ThT fluorescence. (B) Times to reach half-maximal fluorescence ($t_{50\%}$) were plotted for quantitative analysis of lag phases in a seeded aggregation assay (means ± standard deviation). (C) Drug-treated and untreated seeds were added to the medium of PC12 cells at a final $A\beta_{40}$ concentration of 375 nM and incubated for 3 days. Metabolic activities of cells were measured by MTT reduction. Bar graphs show MTT signals relative to buffer controls (means ± standard deviation). (D) Seeds produced as described for panel A in the presence of TF1, TF2a, TF2b, and TF3 at 2:1 theaflavin:$A\beta_{40}$ molar ratios were assayed in the MTT assay as described for panel C. (E) $A\beta_{42}$ aggregates formed in the presence of equimolar concentrations of theaflavins were added to PC12 cells at 50 nM $A\beta$, and metabolic activities were assayed by MTT reduction after 3 days. All theaflavins significantly reduce the toxicity of $A\beta_{40}$ and $A\beta_{42}$ when compared to untreated controls. *P* values in panels B–E: $^*P < 0.05$; $^{**}P < 0.002$; n.s., not significant ($n = 3$).

In parallel with their seeding competence, we quantified the effects of $A\beta_{40}$ aggregates formed in the presence of TF3, EGCG, and CR on the metabolic activity of PC12 cells. Metabolic activities were quantified by the MTT reduction assay after cells had been incubated with compound-generated $A\beta_{40}$ aggregates for 3 days. Both EGCG and TF3 significantly inhibited the effect of $A\beta_{40}$ fibrils on the metabolic activity of PC12 cells when compared to untreated controls (Figure 6C). In contrast, CR-generated $A\beta_{40}$ aggregates slightly exacerbated the deleterious effect of amyloid structures on the metabolism of PC12 cells (Figure 6C). Thus, our data indicate that TF3 treatment reduces the seeding competence of $A\beta_{40}$ amyloid structures and that seeding competence correlates with cellular toxicity measured by the MTT assay.

We then tested the other theaflavins for their effect on $A\beta$ toxicity by incubating $A\beta_{40}$ for 24 h in the presence of a 2-fold excess of TF1, TF2a, TF2b, or TF3 and then performing an MTT assay in PC12 cells (Figure 6D). All theaflavins significantly improved MTT reduction. TF2a, TF2b, and TF3 treatment of $A\beta_{40}$ completely rescued the metabolic activity of PC12 cells. The same test was repeated for the $A\beta_{42}$ peptide (Figure 6E). Here, only TF2b and TF3 significantly rescued metabolic activity, whereas TF1 and TF2a had little effect on $A\beta_{42}$ toxicity. The $A\beta_{42}$ peptide has a higher aggregation propensity and therefore, supposedly, a higher toxicity than $A\beta_{40}$.[6] The different efficacies of TF1 and TF2a with $A\beta_{40}$ and $A\beta_{42}$ toxicity support this interpretation. The reduced efficacy of TF1 when compared with those of TF2b and TF3 also mirrors the previous observation in catechins that their anti-amyloid activity depends on the gallate moiety.[14,29]

Theaflavins Remodel Preformed Aβ Fibrils into Non-toxic Aggregates. Because EGCG was found to remodel and detoxify amyloid fibrils,[14,21] we tested whether theaflavins TF1, TF2a, TF2b, TF3, and CR can also remodel amyloid fibrils into nontoxic aggregate structures. $A\beta_{40}$ aggregates with fibrillar morphology were prepared in vitro (15 μM $A\beta$, shaking for 36 h, 37 °C, PBS). $A\beta_{40}$ fibrils were then incubated with TF1, TF2a, TF2b, TF3, or CR (each at 15 μM), and ThT

dx.doi.org/10.1021/bi2012383 | *Biochemistry* 2011, 50, 10624–10636

fluorescence was recorded over 12 h in PBS at 37 °C (shaking for 5 s every 10 min). The resulting aggregates were then analyzed by atomic force microscopy and MTT reduction assays (Figure 7).

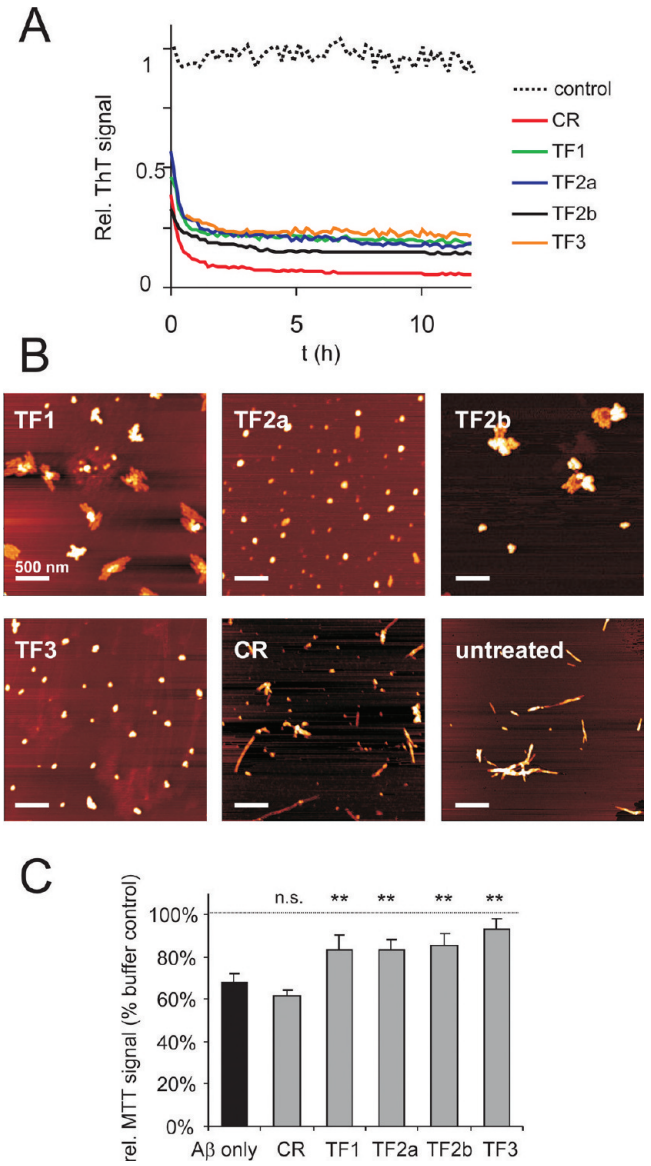


Figure 7. Theaflavins remodel preformed Aβ_{40} fibrils. Monomeric Aβ_{40} peptide (15 μM) was incubated under agitation at 37 °C in PBS and ThT (20 μM) for 36 h to form fibrillar aggregates. (A) Theaflavins TF1, TF2a, TF2b, and CR were added to these aggregates at a concentration of 15 μM, and fibrils were further incubated at 37 °C under agitation (shaking for 5 s every 10 min) and ThT signals recorded. (C) Resulting aggregates from panel A were imaged by AFM. (D) Resulting aggregates from panel A were added to the medium of PC12 cells at a final Aβ concentration of 375 nM and incubated for 3 days, and metabolic activities were assayed by MTT reduction. All compounds reduced the magnitudes of ThT signals, but only the theaflavins induced the formation of spherical aggregates and significantly rescued metabolic activities when compared to untreated controls; incubation with CR had no effect on toxicity. $^{**}P < 0.002$; n.s., not significant ($n = 3$).

When incubated with Aβ_{40} fibrils, all compounds decreased ThT fluorescence in a time-dependent manner (Figure 7A). Interestingly, the residual fluorescence was lowest after incubation with CR (~10% of the initial signal), whereas all theaflavins reduced ThT fluorescence to ~25% of the initial signal when compared to untreated controls (Figure 7A). The reduction in ThT fluorescence indicates that all compounds either remodeled the Aβ_{40} fibril structure or displaced ThT from the fibril.

The morphologies of the resulting aggregates were analyzed by AFM (Figure 7B). While incubation with CR left the fibrillar structure of Aβ_{40} aggregates intact, incubation with theaflavins resulted in a loss of fibrillar structures (Figure 7B). Instead, spherical and small amorphous aggregates were observed, indicating that the theaflavins remodel fibrillar Aβ_{40}. Incubation with theaflavins therefore remodeled Aβ fibrils in a manner similar to that of EGCG,[29] resulting in the formation of spherical, ThT negative aggregates.

Finally, the drug-treated Aβ_{40} aggregates were added to PC12 cells, and metabolic activities were assayed after incubation for 3 days using an MTT reduction assay. All theaflavins significantly rescued amyloid-mediated cellular toxicity (Figure 7C). However, such an effect was not observed when CR-treated aggregates were added to PC12 cells. Thus, our data indicate that remodeling of amyloid fibrils with theaflavins correlates with reduced cellular toxicity. In contrast, CR, although it reduces ThT fluorescence, altered neither the morphology nor the toxicity of preformed amyloid fibrils, suggesting that it may simply displace ThT from amyloid fibrils.

Theaflavins Modulate the Assembly of αS Fibrils. To assess whether the anti-amyloid activity of theaflavins is specific to Aβ or whether these compounds more generally affect amyloid structures, we incubated monomeric αS protein (50 μM) with compounds TF1, TF2a, TF2b, and TF3 at equimolar concentrations for 26 h. Predominantly spherical structures were observed by AFM after incubation of αS monomers with TF2b and TF3, whereas spherical and chainlike structures were also observed in the presence of TF1 and TF2a (Figure 8A). In comparison, αS incubated in the absence of chemical compounds formed fibrils (Figure 8A).

Similar to Aβ, αS fibrils bound the dye ThT and increased ThT fluorescence (Figure 8B), while incubation of αS in the presence of theaflavins TF1, TF2a, TF2b, and TF3 at equimolar concentrations reduced ThT fluorescence to less than 0.5% of the untreated control (Figure 8B). SDS-resistant αS aggregates were detected in FRA after incubation of αS with the theaflavins for 26 h, whereas no SDS resistance was observed in buffer-treated controls (Figure S3 of the Supporting Information). While treatment with TF3 and TF2b produced similar amounts of SDS-resistant αS aggregates as treatment with EGCG, smaller amounts were observed after treatment with TF1 and TF2a. It should be noted that reducing conditions did not significantly influence the assembly of SDS-resistant αS aggregates in the presence of TF3 or EGCG (Figure S3 of the Supporting Information), indicating that oxidation of the compound is not a prerequisite for the induction of SDS-resistant aggregates.

Finally, compound-treated αS aggregates were added to PC12 cells, and metabolic activities were monitored by MTT reduction. We found that all theaflavins significantly rescued the metabolic inhibition induced by αS aggregates (Figure 8C). Taken together, these data indicate that the effects of theaflavins on fibril formation and toxicity are not restricted to Aβ aggregation. A similar effect can also be observed with the aggregation-prone protein αS.

dx.doi.org/10.1021/bi2012383|*Biochemistry* 2011, 50, 10624–10636

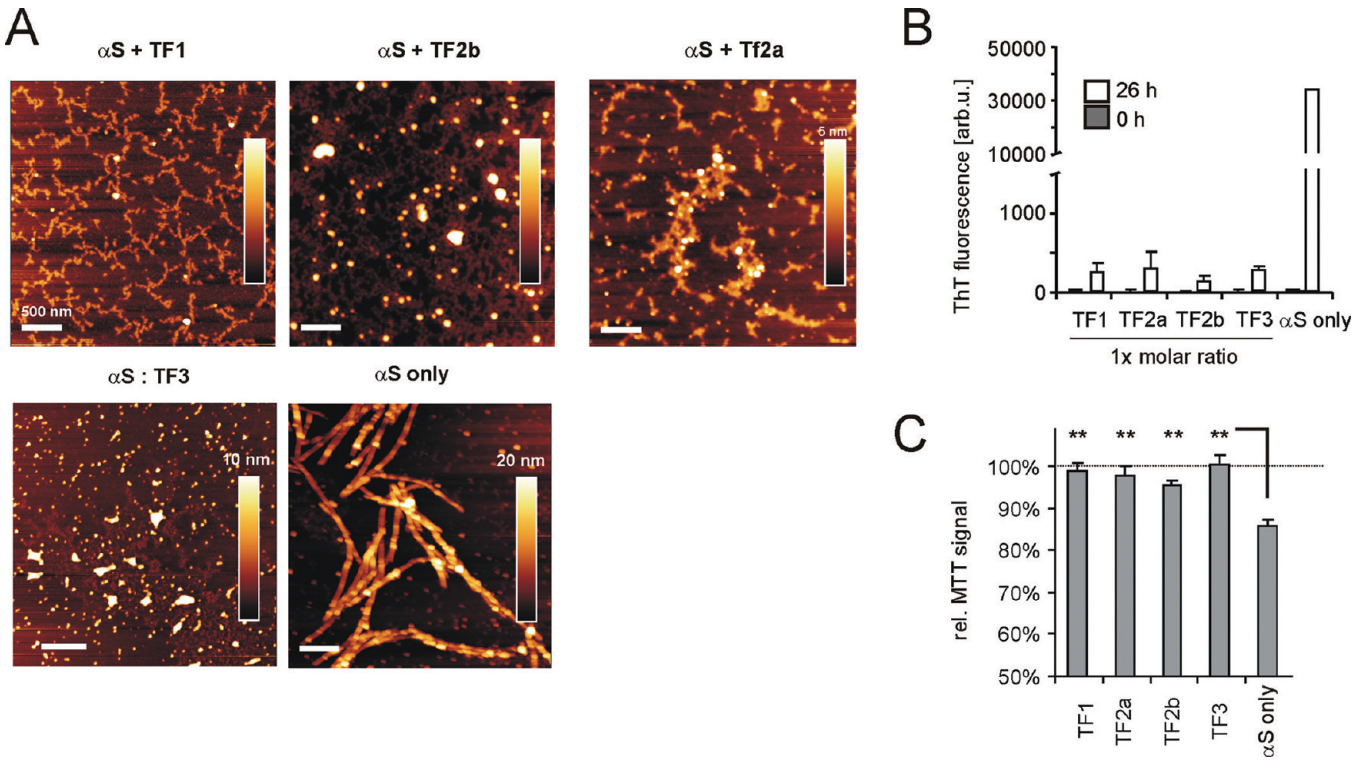


Figure 8. Theaflavins induce the formation of benign αS aggregates. αS (50 μM) were incubated with equimolar concentrations of TF1, TF2a, TF2b, and TF3 at 37 °C in SP buffer for 26 h under constant agitation in an overhead shaker; control samples were incubated without small molecules. (A) AFM images of TF3-treated and control samples after 26 h. (B) ThT signals of αS samples before and after incubation for 26 h (n = 3). (C) αS aggregates from panel B were added to the medium of PC12 cells at a final concentration of 75 nM and incubated for 3 days. MTT signals were determined as described above. All theaflavins significantly rescued the metabolic activities of PC12 cells when compared to untreated controls. **P < 0.002 (n = 3).

■ DISCUSSION

Epidemiological studies suggest that consumption of tea correlates with a reduced incidence of neurological diseases. A higher level of consumption of green tea was found to be inversely associated with the prevalence of cognitive impairment in a Japanese study.[48] Tea drinking also lowered the risk for Parkinson's disease.[49,50] Other studies found beneficial effects for Parkinson's disease when black tea was consumed.[51] However, the results are not uniform. Green tea consumption was not associated with a decreased risk of cognitive decline in another Japanese population.[52] On the other hand, a beneficial effect of green tea on systemic amyloid diseases has been observed.[53,54]

In this study, we have observed that theaflavins weaken the spontaneous formation of protofibrils and fibrils of Aβ_{40}, Aβ_{42}, and αS in vitro (Figure 2). Like classical anti-amyloid compounds, such as CR, theaflavins strongly reduced ThT signals in aggregation kinetics (Figure 3). TF3, EGCG, and CR all bind to two hydrophobic regions of the Aβ peptide centered on amino acids 16−22 and 27−35 (Figure 4A). Interactions of CR, on the other hand, were more narrowly defined, suggesting two specific interaction sites of the compound. Although the details of binding of CR to amyloid fibrils are still not quite resolved, structural models and fluorescence polarization data suggest that both CR and ThT bind at specific sites with their long axis parallel to the fibril axis and perpendicular to the peptide strands.[55−57] The amyloidophilic dye ThT is reported to bind to the same region and possibly the same site as CR.[57] Therefore, a loss of ThT signal may partially reflect the fact that drug candidates compete with the ThT reporter for binding sites. It has to be noted that CR did not prevent the formation of Aβ_{40} fibrils or the inhibition of MTT reduction by Aβ peptides under our assay conditions. This is in contrast to previous studies in which CR inhibited Aβ_{40} fibril formation and rescued Aβ toxicity at equimolar concentrations.[55] A possible explanation is that much higher concentrations of Aβ and CR were used in previous studies, which may increase drug affinity as well as change the Aβ assembly mechanism.

In contrast to CR, polyphenols TF3 and EGCG display a broad binding pattern in peptide arrays, suggesting that they may interact with multiple amino acids, rather than a single specific binding site (Figure 4A). The broad interaction pattern would correspond to the broad range of interactions between EGCG and αS that was observed previously in NMR experiments.[14] TF3 bound with similar strength to peptides spanning the hydrophobic region at amino acids 16−22 either as an intact or as a scrambled sequence (Figure 4B), which suggests that the interaction is governed by amino acid side chains, rather than the sequence. EGCG also weakly stained the scrambled sequence peptide (Figure S1C of the Supporting Information). Thermodynamic studies concluded that the binding of EGCG to Aβ depended on hydrophobic interactions and hydrogen bonding.[18] In addition, a conjugation of EGCG to lysines via Schiff base formation was reported recently for binding to albumin.[19] Our peptide binding data are compatible with such a model. However, TF3 and EGCG staining was not uniform for the different peptides that contained lysine residues

dx.doi.org/10.1021/bi2012383|*Biochemistry* 2011, 50, 10624−10636

(Figure 4A,B and Figure S1B,C of the Supporting Information). This would suggest that different types of interactions contribute to their binding.

Unlike classical amyloid formation inhibitors, theaflavins prevent formation of fibrils and protofibrils by inducing the formation of spherical and amorphous structures. These structures are nontoxic and lack the competence to catalyze amyloid formation (Figure 6). Self-association of the hydrophobic core region around amino acids 16−22 is critical for spontaneous amyloid polymerization.[58] It is tempting to speculate that the broad interaction with this region prevents its β-sheet formation, thus derailing amyloid formation.

EGCG and black tea extract were recently both found to inhibit αS oligomer formation[59] and prevent membrane destabilization by Aβ_{42}.[60] Because the effects of theaflavins on αS and Aβ fibril formation closely mirror the effects of EGCG on amyloid polypeptides, it is reasonable to assume that theaflavins affect amyloid formation pathways by a molecular mechanism similar to that of EGCG. We found no evidence that an aggregated rather than the monomeric form of the compound was responsible for its effect on Aβ aggregation, as has been reported for other compounds.[46]

Comparison of catechins in their effects on amyloid formation revealed that the activity of the compounds correlates with the number of phenolic ring structures in catechin molecules. These rings are thought to form a "hydroxyl edge" on one side of polyphenolic compounds, which might improve binding to polypeptide chains.[13,29] It had been deduced very early from time-resolved spectroscopy data that catechins may adopt such a conformation.[61] NMR studies of EGCG suggest an interaction of the molecule with multiple sites in the peptide backbone of soluble αS polypeptides.[14] The fact that theaflavins present at least one face of multiple phenolic groups in a planar orientation suggests that these regions are critical for their effects on amyloid formation processes.[15]

Although theaflavin TF1 that lacked the gallate moiety was less effective than the digallate TF3 in rescuing metabolic inhibition by Aβ_{40} and Aβ_{42} (Figure 6D,E), we did not detect a striking difference in the effects on aggregate morphology, SDS resistance, or ThT fluorescence or MTT signals between the different theaflavins and EGCG. This observation is in contrast to the therapeutic efficacy of tea polyphenols for tumor or cardiovascular phenotypes.[37,62,63] Individual black tea theaflavins showed a higher potency than EGCG in vasorelaxation in aortic rings and in NO production and in bovine aortic endothelial cells.[63]

However, it should be noted that the observed mechanisms of action of theaflavins on amyloid formation strongly differ from those in cardiovascular models. While these studies were performed using cellular systems that involved receptor-mediated intracellular signaling pathways, our data strongly suggest that tea polyphenols such as theaflavins directly influence protein misfolding in vitro and in vivo by interacting with aggregation-prone proteins. Our results do not rule out the possibility that other activities of polyphenols in cells might affect amyloid diseases, and effects of tea polyphenols on information propagation in signaling pathways, redox potentials of mammalian cells, and the balance of divalent cations in cells have been described.[64]

Large variations in bioavailability have been reported for the ingestion of EGCG.[65] These may be due to either oxidation or metabolic modification of the compound.[66] Enhanced concentrations of black tea polyphenols when compared to EGCG have been reported in different organs.[67] Theaflavins may thus have better long-term bioavailability than EGCG. However, detailed pharmacokinetic data on theaflavins need to be collected. We observed that theaflavin TF3 is less readily oxidized by air than EGCG and that TF3 solutions retain their antiamyloidogenic properties after prolonged oxidation by air (Figure 5). This suggests that polymeric condensation products that are formed during oxidation[68] may contribute to the antiamyloidogenic activity.

Black tea is much more widely consumed in Western countries than green tea. Our data demonstrate that theaflavins are similar in mechanism and at least equal in efficacy to EGCG in preventing and reversing amyloid formation. This suggests that theaflavins, which are found in large amounts in black tea, are promising "nutraceuticals" that may delay the onset of age-related amyloid diseases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Effect of filtration and BSA addition on TF3, Aβ peptide array binding, and evidence that theaflavins induce SDS-resistant aggregates of αS. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jbiesch@mdc-berlin.de. Phone: +49-30-9406-2871.

Funding

J.B. acknowledges support from NGFN Grant 01GS08132, from DFG Grant BI1409/1, and from the Helmholtz Gemeinschaft.

■ ACKNOWLEDGMENTS

Recombinant αS was prepared by S. Kostka; clones were a gift from the lab of P. Selenko (Forschungsinstitut für Molekulare Pharmakologie, Berlin, Germany). Theaflavins were kindly provided by Mitsui Norin Food Research Laboratories.

■ REFERENCES

(1) Chiti, F., and Dobson, C. M. (2006) Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem. 75*, 333−366.

(2) Walsh, D. M., Klyubin, I., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., and Selkoe, D. J. (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature 416*, 535−539.

(3) Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Brett, F. M., Farrell, M. A., Rowan, M. J., Lemere, C. A., Regan, C. M., Walsh, D. M., Sabatini, B. L., and Selkoe, D. J. (2008) Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat. Med. 14*, 837−842.

(4) Rousseau, F., Schymkowitz, J., and Serrano, L. (2006) Protein aggregation and amyloidosis: Confusion of the kinds? *Curr. Opin. Struct. Biol. 16*, 118−126.

(5) Fowler, D. M., Koulov, A. V., Alory-Jost, C., Marks, M. S., Balch, W. E., and Kelly, J. W. (2006) Functional amyloid formation within mammalian tissue. *PLoS Biol. 4*, e6.

(6) Jakob-Roetne, R., and Jacobsen, H. (2009) Alzheimer's disease: From pathology to therapeutic approaches. *Angew. Chem., Int. Ed. 48*, 3030−3059.

(7) Dasilva, K. A., Shaw, J. E., and McLaurin, J. (2009) Amyloid-β fibrillogenesis: Structural insight and therapeutic intervention. *Exp. Neurol. 223*, 311−321.
(8) Haass, C., Koo, E. H., Mellon, A., Hung, A. Y., and Selkoe, D. J. (1992) Targeting of cell-surface β-amyloid precursor protein to lysosomes: Alternative processing into amyloid-bearing fragments. *Nature 357*, 500−503.
(9) Vassar, R. (2001) The β-secretase, BACE: A prime drug target for Alzheimer's disease. *J. Mol. Neurosci. 17*, 157−170.
(10) Hammarstrom, P., Wiseman, R. L., Powers, E. T., and Kelly, J. W. (2003) Prevention of transthyretin amyloid disease by changing protein misfolding energetics. *Science 299*, 713−716.
(11) Johnson, S. M., Wiseman, R. L., Sekijima, Y., Green, N. S., Adamski-Werner, S. L., and Kelly, J. W. (2005) Native state kinetic stabilization as a strategy to ameliorate protein misfolding diseases: A focus on the transthyretin amyloidoses. *Acc. Chem. Res. 38*, 911−921.
(12) Cohen, F. E., and Kelly, J. W. (2003) Therapeutic approaches to protein-misfolding diseases. *Nature 426*, 905−909.
(13) Ehrnhoefer, D. E., Duennwald, M., Markovic, P., Wacker, J. L., Engemann, S., Roark, M., Legleiter, J., Marsh, J. L., Thompson, L. M., Lindquist, S., Muchowski, P. J., and Wanker, E. E. (2006) Green tea (−)-epigallocatechin-gallate modulates early events in huntingtin misfolding and reduces toxicity in Huntington's disease models. *Hum. Mol. Genet. 15*, 2743−2751.
(14) Ehrnhoefer, D. E., Bieschke, J., Boeddrich, A., Herbst, M., Masino, L., Lurz, R., Engemann, S., Pastore, A., and Wanker, E. E. (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat. Struct. Mol. Biol. 15*, 558−566.
(15) Kuzuhara, T., Suganuma, M., and Fujiki, H. (2008) Green tea catechin as a chemical chaperone in cancer prevention. *Cancer Lett. 261*, 12−20.
(16) Hauber, I., Hohenberg, H., Holstermann, B., Hunstein, W., and Hauber, J. (2009) The main green tea polyphenol epigallocatechin-3-gallate counteracts semen-mediated enhancement of HIV infection. *Proc. Natl. Acad. Sci. U.S.A. 106*, 9033−9038.
(17) Hudson, S. A., Ecroyd, H., Dehle, F. C., Musgrave, I. F., and Carver, J. A. (2009) (−)-Epigallocatechin-3-gallate (EGCG) maintains kappa-casein in its pre-fibrillar state without redirecting its aggregation pathway. *J. Mol. Biol. 392*, 689−700.
(18) Wang, S. H., Liu, F. F., Dong, X. Y., and Sun, Y. (2010) Thermodynamic analysis of the molecular interactions between amyloid β-peptide 42 and (−)-epigallocatechin-3-gallate. *J. Phys. Chem. B 114*, 11576−11583.
(19) Ishii, T., Ichikawa, T., Minoda, K., Kusaka, K., Ito, S., Suzuki, Y., Akagawa, M., Mochizuki, K., Goda, T., and Nakayama, T. (2011) Human serum albumin as an antioxidant in the oxidation of (−)-epigallocatechin gallate: Participation of reversible covalent binding for interaction and stabilization. *Biosci., Biotechnol., Biochem. 75*, 100−106.
(20) Porat, Y., Abramowitz, A., and Gazit, E. (2006) Inhibition of amyloid fibril formation by polyphenols: Structural similarity and aromatic interactions as a common inhibition mechanism. *Chem. Biol. Drug Des. 67*, 27−37.
(21) Ono, K., Condron, M. M., Ho, L., Wang, J., Zhao, W., Pasinetti, G. M., and Teplow, D. B. (2008) Effects of grape seed-derived polyphenols on amyloid β-protein self-assembly and cytotoxicity. *J. Biol. Chem. 283*, 32176−32187.
(22) Bastianetto, S., Yao, Z. X., Papadopoulos, V., and Quirion, R. (2006) Neuroprotective effects of green and black teas and their catechin gallate esters against β-amyloid-induced toxicity. *Eur. J. Neurosci. 23*, 55−64.
(23) Rambold, A. S., Miesbauer, M., Olschewski, D., Seidel, R., Riemer, C., Smale, L., Brumm, L., Levy, M., Gazit, E., Oesterhelt, D., Baier, M., Becker, C. F., Engelhard, M., Winklhofer, K. F., and Tatzelt, J. (2008) Green tea extracts interfere with the stress-protective activity of PrP and the formation of PrP. *J. Neurochem. 107*, 218−229.
(24) Roberts, B. E., Duennwald, M. L., Wang, H., Chung, C., Lopreiato, N. P., Sweeny, E. A., Knight, M. N., and Shorter, J. (2009) A synergistic small-molecule combination directly eradicates diverse prion strain structures. *Nat. Chem. Biol. 5*, 936−946.
(25) He, J., Xing, Y. F., Huang, B., Zhang, Y. Z., and Zeng, C. M. (2009) Tea catechins induce the conversion of preformed lysozyme amyloid fibrils to amorphous aggregates. *J. Agric. Food Chem. 57*, 11391−11396.
(26) Meng, F., Abedini, A., Plesner, A., Verchere, C. B., and Raleigh, D. P. (2010) The flavanol (−)-epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils, and protects cultured cells against IAPP-induced toxicity. *Biochemistry 49*, 8127−8133.
(27) Ferreira, N., Saraiva, M. J., and Almeida, M. R. (2011) Natural polyphenols inhibit different steps of the process of transthyretin (TTR) amyloid fibril formation. *FEBS Lett. 585*, 2424−2430.
(28) Chandrashekaran, I. R., Adda, C. G., Macraild, C. A., Anders, R. F., and Norton, R. S. (2011) EGCG disaggregates amyloid-like fibrils formed by *Plasmodium falciparum* merozoite surface protein 2. *Arch. Biochem. Biophys. 513*, 153−157.
(29) Bieschke, J., Russ, J., Friedrich, R. P., Ehrnhoefer, D. E., Wobst, H., Neugebauer, K., and Wanker, E. E. (2010) EGCG remodels mature α-synuclein and amyloid-β fibrils and reduces cellular toxicity. *Proc. Natl. Acad. Sci. U.S.A. 107*, 7710−7715.
(30) Rezai-Zadeh, K., Arendash, G. W., Hou, H., Fernandez, F., Jensen, M., Runfeldt, M., Shytle, R. D., and Tan, J. (2008) Green tea epigallocatechin-3-gallate (EGCG) reduces β-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice. *Brain Res. 1214*, 177−187.
(31) Wang, J., Ho, L., Zhao, W., Ono, K., Rosensweig, C., Chen, L., Humala, N., Teplow, D. B., and Pasinetti, G. M. (2008) Grape-derived polyphenolics prevent Aβ oligomerization and attenuate cognitive deterioration in a mouse model of Alzheimer's disease. *J. Neurosci. 28*, 6388−6392.
(32) Mazzulli, J. R., Mishizen, A. J., Giasson, B. I., Lynch, D. R., Thomas, S. A., Nakashima, A., Nagatsu, T., Ota, A., and Ischiropoulos, H. (2006) Cytosolic catechols inhibit α-synuclein aggregation and facilitate the formation of intracellular soluble oligomeric intermediates. *J. Neurosci. 26*, 10068−10078.
(33) Yang, C., Li, D., and Wan, X. (2008) Combination of HSCCC and Sephadex LH-20 methods. An approach to isolation and purification of the main individual theaflavins from black tea. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 861*, 140−144.
(34) Lin, L. Z., Chen, P., and Harnly, J. M. (2008) New phenolic components and chromatographic profiles of green and fermented teas. *J. Agric. Food Chem. 56*, 8130−8140.
(35) Porter, L. J. (1993) Flavans and proanthocyanines. In *The Flavonoids: Advances in research since 1986* (Harborne, J. B., Ed.) pp 23−54, Chapman & Hall, London.
(36) Miller, N. J., Castelluccio, C., Tijburg, L., and Rice-Evans, C. (1996) The antioxidant properties of theaflavins and their gallate esters: Radical scavengers or metal chelators? *FEBS Lett. 392*, 40−44.
(37) Leung, L. K., Su, Y., Chen, R., Zhang, Z., Huang, Y., and Chen, Z. Y. (2001) Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J. Nutr. 131*, 2248−2251.
(38) Bieschke, J., Zhang, Q., Powers, E. T., Lerner, R. A., and Kelly, J. W. (2005) Oxidative metabolites accelerate Alzheimer's amyloidogenesis by a two-step mechanism, eliminating the requirement for nucleation. *Biochemistry 44*, 4977−4983.
(39) Hoyer, W., Cherny, D., Subramaniam, V., and Jovin, T. M. (2004) Rapid self-assembly of α-synuclein observed by in situ atomic force microscopy. *J. Mol. Biol. 340*, 127−139.
(40) Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature 416*, 507−511.
(41) Beutling, U., Stading, K., Stradal, T., and Frank, R. (2008) Large-scale analysis of protein-protein interactions using cellulose-bound peptide arrays. *Adv. Biochem. Eng. Biotechnol. 110*, 115−152.

(42) Paz, M. A., Fluckiger, R., Boak, A., Kagan, H. M., and Gallop, P. M. (1991) Specific detection of quinoproteins by redox-cycling staining. *J. Biol. Chem. 266*, 689−692.
(43) Hudson, S. A., Ecroyd, H., Kee, T. W., and Carver, J. A. (2009) The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds. *FEBS J. 276*, 5960−5972.
(44) LeVine, H. III (1999) Quantification of β-sheet amyloid fibril structures with thioflavin T. *Methods Enzymol. 309*, 274−284.
(45) Harper, J. D., and Lansbury, P. T. Jr. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: Mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. *Annu. Rev. Biochem. 66*, 385−407.
(46) Feng, B. Y., Toyama, B. H., Wille, H., Colby, D. W., Collins, S. R., May, B. C., Prusiner, S. B., Weissman, J., and Shoichet, B. K. (2008) Small-molecule aggregates inhibit amyloid polymerization. *Nat. Chem. Biol. 4*, 197−199.
(47) Frid, P., Anisimov, S. V., and Popovic, N. (2007) Congo red and protein aggregation in neurodegenerative diseases. *Brain Res. Rev. 53*, 135−160.
(48) Kuriyama, S., Hozawa, A., Ohmori, K., Shimazu, T., Matsui, T., Ebihara, S., Awata, S., Nagatomi, R., Arai, H., and Tsuji, I. (2006) Green tea consumption and cognitive function: A cross-sectional study from the Tsurugaya Project 1. *Am. J. Clin. Nutr. 83*, 355−361.
(49) Hu, G., Bidel, S., Jousilahti, P., Antikainen, R., and Tuomilehto, J. (2007) Coffee and tea consumption and the risk of Parkinson's disease. *Mov. Disord. 22*, 2242−2248.
(50) Tan, E. K., Tan, C., Fook-Chong, S. M., Lum, S. Y., Chai, A., Chung, H., Shen, H., Zhao, Y., Teoh, M. L., Yih, Y., Pavanni, R., Chandran, V. R., and Wong, M. C. (2003) Dose-dependent protective effect of coffee, tea, and smoking in Parkinson's disease: A study in ethnic Chinese. *J. Neurol. Sci. 216*, 163−167.
(51) Tan, L. C., Koh, W. P., Yuan, J. M., Wang, R., Au, W. L., Tan, J. H., Tan, E. K., and Yu, M. C. (2008) Differential effects of black versus green tea on risk of Parkinson's disease in the Singapore Chinese Health Study. *Am. J. Epidemiol. 167*, 553−560.
(52) Shimbo, M., Nakamura, K., Jing Shi, H., Kizuki, M., Seino, K., Inose, T., and Takano, T. (2005) Green tea consumption in everyday life and mental health. *Public Health Nutr. 8*, 1300−1306.
(53) Hunstein, W. (2007) Epigallocathechin-3-gallate in AL amyloidosis: A new therapeutic option? *Blood 110*, 2216.
(54) Shanafelt, T. D., Call, T. G., Zent, C. S., Laplant, B., Bowen, D. A., Roos, M., Secreto, C. R., Ghosh, A. K., Kabat, B. F., Lee, M. J., Yang, C. S., Jelinek, D. F., Erlichman, C., and Kay, N. E. (2009) Phase I Trial of Daily Oral Polyphenon E in Patients with Asymptomatic Rai Stage 0 to II Chronic Lymphocytic Leukemia. *J. Clin. Oncol. 27*, 3808−3814.
(55) Lorenzo, A., and Yankner, B. A. (1994) β-Amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc. Natl. Acad. Sci. U.S.A. 91*, 12243−12247.
(56) Krebs, M. R., Bromley, E. H., and Donald, A. M. (2005) The binding of thioflavin-T to amyloid fibrils: Localisation and implications. *J. Struct. Biol. 149*, 30−37.
(57) Biancalana, M., and Koide, S. (2010) Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochim. Biophys. Acta 1804*, 1405−1412.
(58) Kim, W., and Hecht, M. H. (2006) Generic hydrophobic residues are sufficient to promote aggregation of the Alzheimer's Aβ42 peptide. *Proc. Natl. Acad. Sci. U.S.A. 103*, 15824−15829.
(59) Caruana, M., Hogen, T., Levin, J., Hillmer, A., Giese, A., and Vassallo, N. (2011) Inhibition and disaggregation of α-synuclein oligomers by natural polyphenolic compounds. *FEBS Lett. 585*, 1113−1120.
(60) Gauci, A. J., Caruana, M., Giese, A., Scerri, C., and Vassallo, N. (2011) Identification of Polyphenolic Compounds and Black Tea Extract as Potent Inhibitors of Lipid Membrane Destabilization by Aβ42 Aggregates. *J. Alzheimer's Dis.*, DOI: doi: 10.3233/JAD-2011-111061.
(61) Bergmann, W. R., Viswanadhan, V. N., and Mattice, W. L. (1988) Conformations of polymeric proanthocyanidins composed of (+)-catechin or (−)-epicatechin joined by 4 → 6 interflavan bonds. *J. Chem. Soc., Perkin Trans. 2*, 45−47.
(62) Liang, Y. C., Chen, Y. C., Lin, Y. L., Lin-Shiau, S. Y., Ho, C. T., and Lin, J. K. (1999) Suppression of extracellular signals and cell proliferation by the black tea polyphenol, theaflavin-3,3′-digallate. *Carcinogenesis 20*, 733−736.
(63) Lorenz, M., Urban, J., Engelhardt, U., Baumann, G., Stangl, K., and Stangl, V. (2009) Green and black tea are equally potent stimuli of NO production and vasodilation: New insights into tea ingredients involved. *Basic Res. Cardiol. 104*, 100−110.
(64) Weinreb, O., Mandel, S., Amit, T., and Youdim, M. B. (2004) Neurological mechanisms of green tea polyphenols in Alzheimer's and Parkinson's diseases. *J. Nutr. Biochem. 15*, 506−516.
(65) Chow, H. H., Cai, Y., Hakim, I. A., Crowell, J. A., Shahi, F., Brooks, C. A., Dorr, R. T., Hara, Y., and Alberts, D. S. (2003) Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. *Clin. Cancer Res. 9*, 3312−3319.
(66) Singh, M., Arseneault, M., Sanderson, T., Murthy, V., and Ramassamy, C. (2008) Challenges for research on polyphenols from foods in Alzheimer's disease: Bioavailability, metabolism, and cellular and molecular mechanisms. *J. Agric. Food Chem. 56*, 4855−4873.
(67) Henning, S. M., Aronson, W., Niu, Y., Conde, F., Lee, N. H., Seeram, N. P., Lee, R. P., Lu, J., Harris, D. M., Moro, A., Hong, J., Pak-Shan, L., Barnard, R. J., Ziaee, H. G., Csathy, G., Go, V. L., Wang, H., and Heber, D. (2006) Tea polyphenols and theaflavins are present in prostate tissue of humans and mice after green and black tea consumption. *J. Nutr. 136*, 1839−1843.
(68) Kusano, R., Andou, H., Fujieda, M., Tanaka, T., Matsuo, Y., and Kouno, I. (2008) Polymer-like polyphenols of black tea and their lipase and amylase inhibitory activities. *Chem. Pharm. Bull. 56*, 266−272.