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# Fluphenazine·HCl and Epigallocatechin Gallate Modulate the Rate of Formation and Structural Properties of Apolipoprotein C-II Amyloid Fibrils

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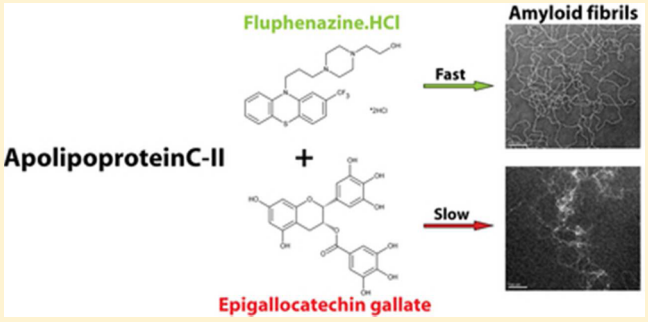
Supporting Information

**ABSTRACT:** Protein misfolding and aggregation, leading to amyloid fibril formation, are characteristic of many devastating and debilitating amyloid diseases. Accordingly, there is significant interest in the mechanisms underlying amyloid fibril formation and identification of possible intervention tools. Small molecule drug compounds approved for human use or for use in phase I−III clinical trials were investigated for their effects on amyloid formation by human apolipoprotein (apo) C-II. Several of these compounds modulated the rate of amyloid formation by apoC-II. Epigallocatechin gallate (EGCG), a green tea catechin, was an effective inhibitor of apoC-II fibril formation, and the antipsychotic drug, fluphenazine·HCl, was a potent activator. Both EGCG and fluphenazine·HCl exerted concentration-dependent effects on the rate of fibril formation, bound to apoC-II fibrils with high affinity, and competitively reduced thioflavin T binding. EGCG significantly altered the size distribution of fibrils, most likely by promoting the lateral association of fibrils and subsequent formation of large aggregates. Fluphenazine·HCl did not significantly alter the size distribution of fibrils, but it may induce the formation of a small population of rod-like fibrils that differ from the characteristic ribbon-like fibrils normally observed for apoC-II. The findings of this study emphasize the effects of small molecule drugs on the kinetics of amyloid fibril formation and their roles in determining fibril structure and aggregate size.




Amyloid deposits are characteristic of a large range of protein misfolding diseases, such as Alzheimer's disease, Creutzfeldt−Jakob disease, Type II diabetes, AL amyloidosis, and senile systemic amyloidosis.[1−3] Amyloid fibrils, the primary component of these deposits, have hallmark features such as fibrillar morphology, cross-$\beta$ structure, and the ability to bind the dyes Congo red and thioflavin T (ThT). While amyloid deposits and plaques are associated with amyloid disease, small oligomers formed during the aggregation process or by fragmentation of fibrils, are widely believed to contribute to amyloid toxicity.[4−9] Amyloid fibril formation has been proposed, therefore, as a potentially protective process for the sequestration of toxic, oligomeric misfolded proteins.[10,11] As the pathway for amyloid fibril formation is not fully understood, there is increasing interest in conditions that inhibit or enhance the mechanisms underlying amyloid formation.

Apolipoproteins are a group of serum proteins that is associated with amyloid fibril formation both *in vivo* and *in vitro*.[12] Human apolipoprotein (apo) C-II is a 79 amino acid protein that is normally bound to chylomicrons and very-low-density lipoproteins (VLDL). *In vitro*, apoC-II readily forms homogeneous amyloid fibrils under quiescent conditions and at physiological pH.[13,14] ApoC-II is one of several apolipoproteins found colocalized with the amyloid marker, serum amyloid P, in atherosclerotic plaques,[13,15] and apoC-II fibrils activate a CD36-mediated macrophage response, suggesting that fibrillar apoC-II may play a role in the pathogenesis of atherosclerosis.

Amyloid fibril formation by apoC-II is modulated by methionine oxidation,[16] amino acid muations,[16,17] and a range of solution and environment conditions including the presence of lipids.[18,19] The role of lipids in amyloid formation has been demonstrated for a range of proteins, including $\alpha$-synuclein, amyloid $\beta$ (A$\beta$) peptide, islet amyloid polypeptide (IAPP), prion protein, transthyretin, lysozyme, and calcitonin.[20] Both lipid surfaces and free, soluble lipid molecules exert significant effects on fibril formation by apoC-II.[21] The effects of soluble lipid and lipid-like molecules at the various stages of the apoC-II amyloid aggregation pathway have been extensively characterized.[22,23] A study using a range of lipids and lipid mimetics showed that the majority of these amphipathic molecules had significant modulatory effects on the rate of

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apoC-II amyloid fibril formation.[23] Furthermore, submicellar phospholipids identified as activators of apoC-II fibril formation were found to selectively enhance the nucleation phase of fibril formation but not the elongation phase.[22] Taken together, these results suggest that a broad range of small molecules may play important roles in modulation of amyloid formation *in vitro* and *in vivo*.

We screened a set of small molecule drugs to determine their effect on amyloid fibril formation by apoC-II. These compounds have been approved as therapeutics for human use or for phase I–III clinical trials in a range of disease contexts. Our studies identified a number of activators and inhibitors of apoC-II amyloid formation. Detailed investigation of the most potent inhibitor and activator demonstrate that these molecules can have significant effects on the biophysical and structural properties of amyloid aggregates.

## MATERIALS AND METHODS

**Materials.** ApoC-II was expressed and purified as described previously.[24] Purified stocks of apoC-II were stored in 5 M guanidine hydrochloride and 20 mM Tris-HCl, pH 8.0, at approximately 40 mg/mL. ThT was purchased from Sigma (St. Louis, MO). Small molecule drug compounds were selected from the National Institutes of Health (NIH) Clinical Collections plate arrays (CA, USA). These compounds are supplied at approximately 10 mM in 100% DMSO. Epigallocatechin gallate (EGCG) and fluphenazine·HCl used for further analyses were purchased from LKT Laboratories (St. Paul, MN) and Sigma (St. Louis, MO), respectively.

**EGCG and Fluphenazine·HCl Stock Preparation.** EGCG and fluphenazine·HCl stocks were prepared in DMSO, and their concentration was confirmed spectrophotometrically using a DU-800 UV/vis spectrophotometer (Beckman Coulter, CA, USA), at 274 and 257 nm, respectively.[25,26]

**Thioflavin T Fluorescence Assays.** ApoC-II stocks were diluted into freshly prepared 0.1 M sodium phosphate buffer, pH 7.4, 0.1% sodium azide (refolding buffer) containing 10 $\mu$M ThT to initiate fibril formation at the desired final concentration. ThT fluorescence was monitored continuously at 30 °C using a Paradigm plate reader (Beckman Coulter, CA, USA) with 445/20 nm excitation and 485/20 nm emission filters or a FLUOstar OPTIMA plate reader (BMG LABTECH, Germany) with 440/10 nm excitation and 480/10 nm emission filters. To minimize dilution effects in ThT assays with drug compounds, concentrated stocks of the compounds were added to the plate prior to the addition of refolded apoC-II such that the DMSO concentration was limited to 1–3% v/v. DMSO added to apoC-II alone did not significantly affect the rates of fibril formation when using standard conditions of 40 $\mu$M compound, corresponding to 1% DMSO. During concentration dependence studies where 3% DMSO was used, the rate of fibril formation was reduced (see Table S3). Absorbance contributions from EGCG and fluphenazine·HCl at 440 and 480 nm were less than 0.1 in all samples (Figure S1), indicating that inner filtering effects on ThT fluorescence due to the presence of the drugs were negligible. Control experiments showed that addition of the drugs resulted in a change in pH of less than 0.05 units. Hill plots were fitted to ThT data to estimate the time to half-maximal ThT fluorescence ($t_{1/2}$) and the inverse of $t_{1/2}$ was used to provide a measure of the apparent rate of fibril formation. In cases where ThT fluorescence reached a maximum followed by a drop in fluorescence signal over time, only data recorded prior to maximal fluorescence was used to determine rate parameters.

**Pelleting Analysis by SDS-PAGE.** ApoC-II was refolded and incubated at 30 °C in the presence of final concentrations of 40 $\mu$M EGCG or 40 $\mu$M fluphenazine·HCl. At specified time points, an aliquot was taken for the total sample, and the remaining fibrils were isolated by centrifugation at 100 000 rpm (436 000*g*) in an OptimaMax centrifuge using a TL-100 rotor (Beckman Coulter Instruments, Inc., Fullerton, CA, USA) for 30 min at 20 °C. The supernatant was immediately removed, and the pellet was resuspended in the original volume of refolding buffer. The resuspended pellet, supernatant, and total were then run on a 16.5% Tris-Tricine SDS-PAGE gel.

**Optical Absorbance Pelleting Assays.** ApoC-II alone and in the presence of 40 $\mu$M EGCG and 40 $\mu$M fluphenazine·HCl were incubated for 72 h at 30 °C, in addition to compounds alone at 40 $\mu$M in refolding buffer. The samples were then pelleted at 100 000 rpm using the TL-100 rotor for 30 min at 20 °C. Optical absorbance of the supernatants and resuspended pellets was measured using a DU-800 UV/vis spectrophotometer (Beckman Coulter, CA, USA). Absorbance at 320 and 310 nm, where apoC-II does not contribute significantly, was used to measure concentrations of EGCG and fluphenazine·HCl, respectively (Figure S1). The absorbance from supernatants of each compound pelleted alone and with apoC-II was used to calculate the concentration of compound remaining in solution and, subsequently, the concentration found to sediment bound to fibrils. The supernatant measurements were used for calculations in order to avoid error due to scattering contributions in the pellet fraction.

**Transmission Electron Microscopy.** ApoC-II was refolded and incubated at 30 °C in the presence of final concentrations of 40 $\mu$M EGCG or 40 $\mu$M fluphenazine·HCl. After 24 h, these samples were diluted to 0.1 mg/mL with Milli-Q water. Carbon-coated copper grids were glow-discharged for 15 s, and an aliquot of the sample was placed on the grid and left to adsorb for 1 min. The grid was then blotted, stained twice with 2% potassium phosphotungstate, pH 6.8, and air-dried. Negatively stained grids were examined at the Bio21 Electron Microscopy Unit using a FEI Tecnai G2 TF30 transmission electron microscope (FEI-Company, Eindhoven, The Netherlands), and images were acquired digitally using a Gatan US1000 2k × 2k CCD Camera (Pleasanton, CA, USA).

**Sedimentation Velocity Analysis.** ApoC-II was refolded and incubated at 30 °C in the presence of final concentrations of 40 $\mu$M EGCG or 40 $\mu$M fluphenazine·HCl for 24 h. Sedimentation velocity experiments were performed using an XL-I analytical ultracentrifuge (Beckman Coulter Instruments, Inc., Fullerton, CA, USA), an An-60Ti rotor, double-sector 12 mm path length cells with quartz windows, and charcoal-filled Epon centerpieces. Radial absorbance data were collected at 280 nm at a rotor speed of 8000 rpm (5160*g*). Sedimentation velocity profiles were analyzed using the program SEDFIT to obtain continuous sedimentation coefficient [c(s)] distributions, assuming a worm-like chain model described previously.[27,28]

## RESULTS

**Small Molecule Drug Compounds Modulate the Rate of Amyloid Fibril Formation by ApoC-II.** We sought to determine the effects of common small molecule drugs on the process of amyloid fibril formation by apoC-II. A subset of the National Institutes of Health (NIH) Clinical Collections drug



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libraries was selected for analysis based on prior knowledge of the effects of these molecules on the rate of $A\beta_{1-42}$ fibril formation (unpublished data).

The rate of amyloid fibril formation by 0.5 mg/mL (56 $\mu$M) apoC-II was measured in the absence and presence of each drug compound at a nominal concentration of 100 $\mu$M, providing approximately a 2:1 molar ratio of drug compound to protein. The time taken to reach half-maximum ThT fluorescence ($t_{1/2}$) was calculated by fitting the ThT fluorescence time courses to Hill plots (Figure S2 and Table S1). Several of the compounds modulated the rate of ThT fluorescence development, indicating that these molecules altered the rate of amyloid fibril formation by apoC-II. The compounds pantoprazole and esomeprazole, both containing azole groups, and fenoldopam mesylate and dihydrexidine, containing catechol groups, generated unusual ThT fluorescence signals with sigmoidal increases in fluorescence, indicating fibril growth, followed by distinct decreases in fluorescence (Figure S2B). This observation is most likely due to the lateral aggregation of mature fibrils, leading to settling of a proportion of the fibrils in the microtiter plate during the time course of the experiment.

The reciprocal of $t_{1/2}$ was calculated as a measure of the apparent rate of fibril formation in the presence of each compound (Figure 1). Analysis of these apparent rate values

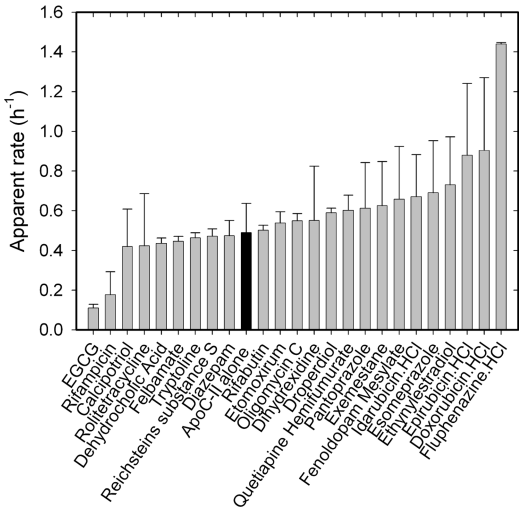


**Figure 1.** Effect of low molecular weight drug compounds on the apparent rate of fibril formation by apoC-II. ThT fluorescence for 0.5 mg/mL apoC-II alone was monitored continuously at 30 °C in the presence of compounds at a nominal concentration of 100 $\mu$M. Time to half-maximal ThT fluorescence ($t_{1/2}$) was calculated by fitting the ThT timecourse to a Hill plot, and the apparent rate was calculated by inverting the $t_{1/2}$. The rate of fibril formation in the presence of each compound was assayed in duplicate, and the error bars indicate standard error corresponding to the range of the two measurements. Standard error, where $n$ = 3, is presented for apoC-II alone.

revealed that EGCG was the most potent inhibitor of apoC-II fibril formation and that fluphenazine·HCl provided the greatest activation of fibril formation. Parallel experiments showed that EGCG and fluphenazine·HCl had similar modulatory effects on the rate of amyloid formation by the $A\beta_{1-42}$ peptide (Table S2). While rifampicin produced a substantial decrease in the average apparent rate of apoC-II fibril formation, this change was not significant due to variability in the apparent rates observed in replicate measurements of fibril formation. Expression of the calculated apparent rates relative to that of apoC-II alone (Table S1) indicated that EGCG reduced the rate of fibril formation over 4-fold, whereas fluphenazine·HCl accelerated fibril formation by approximately 3-fold. Thus, these two compounds were selected for further investigation

**EGCG and Fluphenazine·HCl Do Not Alter the Extent of Fibril Formation at Equilibrium.** Inspection of raw ThT fluorescence data (Figure S2) and parameters resulting from the fits to these data (Table S1) revealed that, in many cases, the small molecule drug compounds reduced the final maximal ThT fluorescence signal plateau, with respect to that of apoC-II alone. Notably, both EGCG and fluphenazine·HCl significantly reduced the maximal ThT signal to approximately 42 and 68% of that of apoC-II alone, respectively (Table S1). This observation may be due to a reduction in the total amount of fibrils formed after long periods in the presence of these compounds. In this case, an increased proportion of soluble apoC-II and a reduced amount of apoC-II incorporated into fibrils would be expected. Alternatively, the compounds may be reducing the ThT fluorescence yield for a given amount of fibrils, perhaps by inhibiting the interaction of ThT with the fibrils.

In order to distinguish these two possibilities, the extent of fibril formation in the presence of EGCG and fluphenazine·HCl was determined using a pelleting assay. As both compounds have significant optical absorbance in the UV/vis spectrum overlapping with that of apoC-II, direct quantification of the protein in pellet and supernatant fractions was not possible. Thus, the fractions were subjected to SDS-PAGE, allowing direct qualitative comparison of the extent of fibril formation over time (Figure 2) by providing the proportion of protein in the supernatant fraction and pellet fraction, corresponding to soluble apoC-II and apoC-II incorporated into fibrils, respectively.

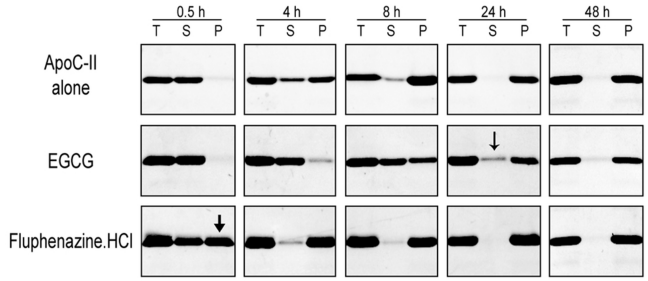


**Figure 2.** SDS-PAGE analysis of the effect of EGCG and fluphenazine·HCl on fibril formation by apoC-II. 0.5 mg/mL apoC-II was incubated at 30 °C alone or in the presence of 40 $\mu$M EGCG or 40 $\mu$M fluphenazine·HCl for 0.5, 4, 8, 24, and 48 h. Protein in the total noncentrifuged sample (T) and after centrifugation in the supernatant (S) and pellet (P) was analyzed by SDS-PAGE. ApoC-II incubated in the presence of EGCG shows an increased proportion of nonfibrillar protein in the supernatant fraction after 24 h (italic arrow). ApoC-II incubated in the presence of fluphenazine·HCl shows an increased amount of fibrils in the pellet fraction after 0.5 h (bold arrow).

In the presence of 40 $\mu$M EGCG, the proportion of apoC-II remaining in the supernatant fraction after 4, 8, and 24 h was higher than that for apoC-II alone, confirming that this compound inhibits fibril formation. After 48 h in the presence of EGCG, the proportion of apoC-II pellet and supernatant fractions was similar to that of apoC-II alone. In the presence of


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40 $\mu$M fluphenazine·HCl, a higher proportion of apoC-II was found in the pellet fraction after 30 min when compared to that for apoC-II alone, confirming that fluphenazine·HCl accelerates fibril formation. After 24 h in the presence of fluphenazine·HCl, the proportions of apoC-II found in both the supernatant and pellet fractions were similar to those of apoC-II alone, suggesting that fibril formation progressed to a similar extent in both samples. Taken together, these results indicate that a similar total amount of fibrils is formed after long periods by apoC-II alone and apoC-II in the presence of EGCG and fluphenazine·HCl, suggesting that the observed reduction in maximal ThT fluorescence is due to suppression of ThT fluorescence signal by these molecules.

**Modulation of Fibril Formation Rate and Reduction of ThT Fluorescence by EGCG and Fluphenazine·HCl Are Concentration-Dependent.** Inhibition of apoC-II fibril formation was examined in the presence of up to 160 $\mu$M EGCG (Figure 3A). Increasing concentrations of ECGC up to 160 $\mu$M progressively increases the $t_{1/2}$ of fibril formation, indicating concentration-dependent inhibition of apoC-II fibril formation (Figure 3C and Table S3). Examination of the ThT time courses and fitting parameters from these experiments also reveals a large and concentration-dependent reduction in the maximum ThT fluorescence values. Similar measurements of apoC-II fibril formation in the presence of fluphenazine·HCl show that $t_{1/2}$ decreased as a function of fluphenazine·HCl concentration, indicating activation of fibril formation (Figure 3C and Table S3). Similar to EGCG, maximum ThT values were reduced in a concentration-dependent manner by the presence of fluphenazine·HCl (Figure 3B).

The suppression of ThT fluorescence by both EGCG and fluphenazine·HCl was confirmed by adding the compounds, at a concentration of 40 $\mu$M, to preformed, mature apoC-II fibrils. These measurements revealed a reduction in ThT fluorescence of approximately 67 and 39% for EGCG and fluphenazine·HCl, respectively. Addition of an equivalent amount of DMSO to preformed apoC-II fibrils results in a decrease in ThT fluorescence of approximately 18%.

**Fluphenazine·HCl and EGCG Bind to ApoC-II Fibrils with High Affinity.** The observed reduction in ThT fluorescence in the presence of EGCG and fluphenazine·HCl may be either due to interaction of these compounds directly with ThT in solution or binding of the compounds to the fibrils, which could interfere with the binding of ThT to the fibrils. EGCG and fluphenazine·HCl have distinctive UV/vis absorbance spectra (Figure S1), thereby providing specific absorbance signals with which the concentration of each compound can be measured. Thus, the pelleting assay described above was combined with UV/vis absorbance measurements to quantify the association between the compounds and apoC-II fibrils. Fibrils grown for 72 h in the presence of compounds at 40 $\mu$M and compounds in refolding buffer alone were pelleted, and the concentration of each of the compounds in the supernatant and pellet fraction was measured. The results indicated that approximately 89% of EGCG and 57% of fluphenazine·HCl sedimented with the fibrils and were present in the pellet fraction following centrifugation (Table S4). This suggests that under the conditions used the compounds bind strongly to apoC-II fibrils, with stoichiometries of approximately 2:3 for EGCG and 2:5 for fluphenazine·HCl (compound/apoC-II).

**EGCG and Fluphenazine·HCl Affect Fibril Size Distribution and Morphology.** In order to determine whether

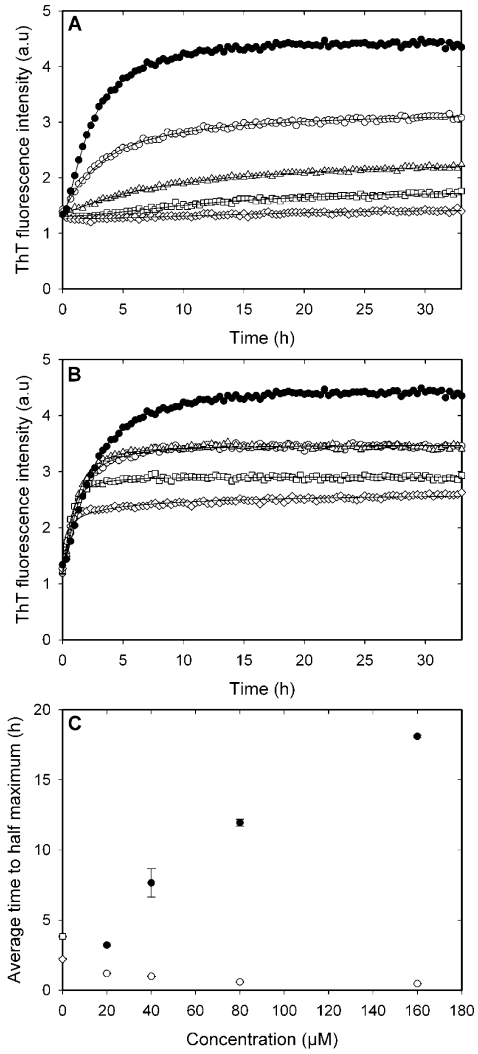


**Figure 3.** Concentration-dependent effects of EGCG and fluphenazine·HCl on fibril formation by apoC-II. ThT fluorescence was monitored continuously at 30 °C using 0.5 mg/mL apoC-II alone (black circles) and in the presence of EGCG (A) or fluphenazine·HCl (B) at concentrations of 20 $\mu$M (open triangles), 40 $\mu$M (open circles), 80 $\mu$M (open squares), and 160 $\mu$M (open diamonds). The fits to Hill plots are shown (solid lines). (C) Average time to half-maximum ($t_{1/2}$) plotted against concentration of EGCG (filled circles) and fluphenazine·HCl (open circles). The $t_{1/2}$ for apoC-II alone (open diamond) and apoC-II alone in the presence of 3% v/v DMSO (open square) are provided for reference. 3% v/v DMSO corresponds to the amount of DMSO present when compounds were added at 160 $\mu$M. Error bars represent standard error where $n$ = 2 and indicate the range of the measurements.

the presence of EGCG or fluphenazine·HCl altered apoC-II amyloid morphology, fibrils were examined by transmission electron microscopy (TEM). ApoC-II fibrils grown in the presence of EGCG revealed twisted ribbon fibrils that are characteristic of apoC-II fibrils (Figure 4C).[24] In addition to individual fibrils, a significant population of large fibril tangles and clumps was observed (Figure 4D), suggesting that EGCG induced lateral association of the ribbon-type fibrils.

Fibrils grown in the presence of fluphenazine·HCl showed primarily twisted ribbon fibrils (Figure 4E). However, in some fibril preparations formed in the presence of fluphenazine·HCl, a minor population of rod-like fibrils was identified in some areas of the TEM grid (Figure 4F). These rod-like fibrils were


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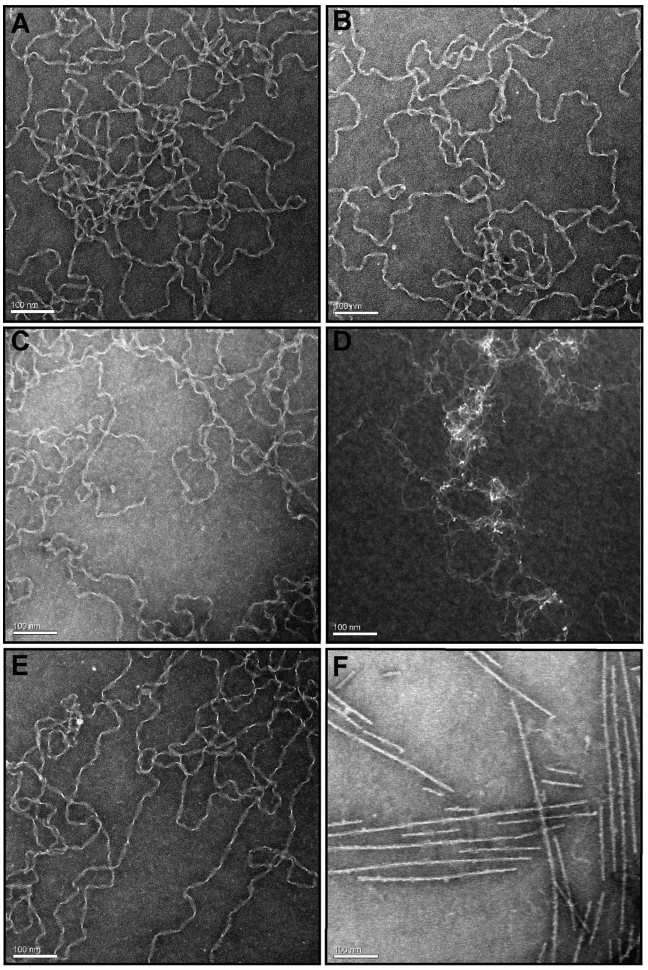


**Figure 4.** Electron micrographs of apoC-II fibrils grown in the presence of EGCG and fluphenazine·HCl. 0.5 mg/mL apoC-II was incubated at 30 °C for 24 h alone (A, B) or in the presence of 40 $\mu$M EGCG (C, D) or 40 $\mu$M fluphenazine·HCl (E, F). Scale bar represents 100 nm.

observed in only 50% of replicate samples prepared for TEM ($n$ = 4). Rod-like fibrils are a novel morphology of apoC-II fibrils that were first identified when apoC-II was incubated for long periods in the presence of low concentrations of phospholipid micelles and bilayers.[29] In contrast to this previous study, the proportion of rod-like fibrils formed in the presence of fluphenazine·HCl did not appear to increase over time.

To further investigate the effects of the compounds on the structure of apoC-II amyloid fibrils, the size distributions of the fibrillar aggregates were determined by sedimentation velocity analytical ultracentrifugation. Raw sedimentation velocity data for apoC-II fibrils grown in the presence of EGCG indicate a significantly higher rate of sedimentation than that of apoC-II fibrils alone (Figure S3). In contrast, the sedimentation profile of fibrils grown in the presence of fluphenazine·HCl was similar to that of apoC-II fibrils alone.

Continuous sedimentation coefficient [c(s)] distributions calculated for apoC-II fibrils alone showed a major peak with a modal sedimentation coefficient of approximately 190 S (Figure 5). Fibrils grown in the presence of fluphenazine·HCl showed a very similar sedimentation coefficient distribution to apoC-II fibrils alone, with only minor changes in the shape of the distribution. This suggests that fluphenazine·HCl does not alter the length of the ribbon type fibrils or induce significant lateral

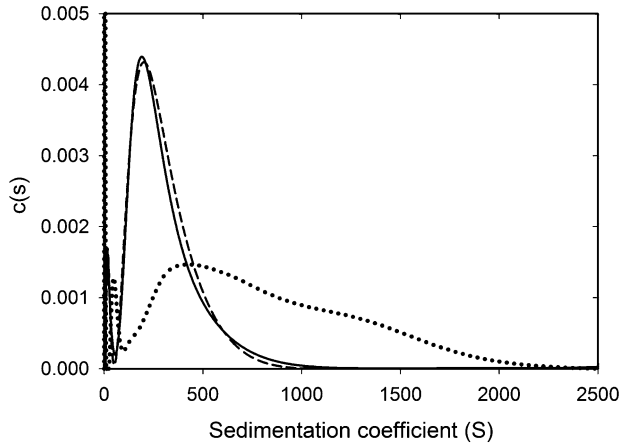


**Figure 5.** Sedimentation coefficient distributions calculated for apoC-II fibrils alone and apoC-II fibrils grown in the presence of EGCG and fluphenazine·HCl. 0.5 mg/mL apoC-II was incubated at 30 °C for 24 h alone (black line) or in the presence of 40 $\mu$M EGCG (dotted line) or 40 $\mu$M fluphenzine.HCl (dashed line).

association. Furthermore, as rod-like fibrils have a higher average sedimentation coefficient than ribbon type fibrils, these results suggest that the population of rod-like fibrils in this sample was below the detectable limit of the experiment.

In contrast, the c(s) distribution calculated for apoC-II fibrils grown in the presence of EGCG shows a much broader distribution with a modal sedimentation coefficient of approximately 445 S and a significant population of large aggregates extending beyond 2000 S. This distribution suggests that EGCG induced lateral association of the fibrils, consistent with observations of fibrillar clumping and tangling in TEM experiments. Due to the heterogeneous nature of the fibril tangles, it is not possible to detect whether ECGC induces increased length of individual fibrils.

## DISCUSSION

We have shown that drug compounds that have been approved for human use can significantly alter the rate of amyloid formation by apoC-II, with EGCG and fluphenazine·HCl identified as the most potent inhibitor and activator, respectively. Our results also show that EGCG and fluphenazine·HCl can inhibit and activate, respectively, the rate of amyloid formation by A$\beta_{1-42}$ peptide, suggesting that the effects may be general in nature rather than specific to a particular amyloid system. EGCG is a catechin flavonoid from green tea and has antioxidant properties. Accordingly, it has attracted considerable attention for its antiviral[30] and antimicrobial potential and as a lead therapeutic for a broad range of diseases such as various cancers and neurodegenerative diseases.[31−33] Fluphenazine·HCl is a commonly used phenothiazine antipsychotic drug that blocks dopamine D2 receptors in the brain and is used primarily for the treatment of schizophrenia.

Fluphenazine·HCl has previously been investigated for its potential inhibition of prion infectivity due to its structural similarity to other reported antiprion agents such as quinacrine.[34,35] ThT fluorescence studies also suggest that the compound may be able to partially dissociate preformed A$\beta_{1-42}$ fibrils.[34] However, to the authors' knowledge, there has been no other evidence supporting the role of fluphenazine·HCl as a modulator of the process of amyloid formation. In contrast,


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EGCG has been shown to inhibit amyloid formation by a variety of amylodogenic proteins such as α-synuclein, $A\beta_{1-42}$, IAPP, huntington, prion, and transthyretin.[31,33,36−39] Studies involving α-synuclein[31,40] and $A\beta_{1-40}$[41] suggest that EGCG acts to inhibit amyloid fibril formation by promoting the formation of amorphous aggregates that are less toxic to cells and lack fibrillar structure, rendering them less likely to seed further fibril formation.[31,40] However, studies involving kappa-casein show a contrasting mechanism for inhibition where EGCG stabilizes the native state.[40] Thus, EGCG appears to inhibit amyloid formation via protein- and aggregation stage-specific mechanisms. Stage-specific effects of other small molecule inhibitors of $A\beta_{1-42}$ aggregation have been observed, where some molecules specifically inhibit formation of small oligomers or fibrils but not both.[42]

Rifampicin is an antibiotic drug that disrupts RNA synthesis and has been previously reported to inhibit amyloid formation by α-synuclein, $A\beta_{1-42}$, and IAPP.[43−45] ApoC-II fibril formation rate assays showed high variability in the presence of this compound. Meng et al. studied the effects of rifampicin on IAPP and revealed that rifampicin was unable to inhibit or disaggregate pre formed fibrils.[46] It was found instead to interfere with ThT fluorescence and, therefore, hinder quantitative measurements, similar to our findings.

Both EGCG and fluphenazine·HCl significantly reduce ThT fluorescence, both when present during apoC-II fibril formation and when added to preformed apoC-II fibrils, and this effect is concentration-dependent. ThT is used extensively for studying amyloid fibril formation *in vitro*. However, both the structural basis of ThT binding and the photophysical properties of ThT are not well-defined. Wolfe et al. cocrystallized the amyloid-like oligomer of β-2 microglobulin with ThT and demonstrated that ThT intercalates between β-sheets orthogonal to β-strands.[47] This β-sheet interface is similar to the characteristic amyloid fibril cross-β structure. The binding of ThT to this site is sterically and electronically favorable for increased ThT fluorescence.[47] Studies investigating the effect of EGCG on $A\beta_{1-40}$ and $IAPP_{8-24}$ suggest that EGCG binds to fibrils primarily through hydrophobic interaction, with enhanced binding achieved via covalent interactions through Schiff base formation.[41] The oxidation of EGCG creates EGCG-based quinones that allow for Schiff base formation by interaction with free amines or thiols adjacent to the hydrophobic binding region. Palhano et al. showed that Schiff base formation is not necessary for the ability of EGCG to remodel fibrils.[41] Some reports suggest that ThT is unable to bind after treatment with EGCG due to remodeling events that create amorphous aggregates that are not ThT reactive.[48] Our analyses confirm that apoC-II fibrils formed in the presence of both EGCG and fluphenazine·HCl are characteristic twisted ribbon amyloid fibrils, indicating that the reduction in ThT fluorescence is not due to dissociation or remodeling of fibrils into amorphous aggregates. Our results suggest that both EGCG and fluphenazine·HCl competitively inhibit ThT binding to apoC-II fibrils. The high affinity and stoichiometry of binding of EGCG and fluphenazine·HCl suggests the presence of a repetitive binding site on apoC-II fibrils. Thus, the binding sites of EGCG and fluphenazine·HCl may be similar to, or overlapping with, the binding site of ThT.

TEM analysis and sedimentation velocity studies revealed that EGCG promotes large assemblies of fibrils, suggesting that EGCG binding to the fibrils promotes their lateral association, leading to clumping and tangling. This observation is similar to previous studies on the effect of the small heat shock protein, αB-crystallin (αB-c), on fibril formation by apoC-II, where the binding of αB-c to apoC-II fibrils was found to cause lateral association and clumping in addition to creating a stabilizing effect that acted to protect against fibril fragmentation.[49] Secondary nucleation events and cytotoxic oligomers can arise due to fibril fragmentation and are widely considered to be central to the pathogenic progression in amyloid diseases.[5,50] Amyloid fibril formation has consequently been suggested as a protective mechanism, sequestering oligomeric intermediates to form large aggregates that are less toxic. EGCG induces lateral association and clumping of apoC-II amyloid fibrils, promoting their aggregation beyond 2000 S. It is therefore likely that the aggregates formed by apoC-II in the presence of EGCG would be less prone to fibril fragmentation. Together with previously published studies,[48] our results indicate that EGCG has the ability to remodel mature amyloid fibrils via diverse protein-specific mechanisms that may reduce the cellular toxicity of the fibrils. Furthermore, the acceleration of fibril formation induced by fluphenazine·HCl may act to quickly remove toxic oligomeric intermediates formed during fibril assembly.

Fibrils formed in the presence of fluphenazine·HCl showed a very similar size distribution as that of apoC-II fibrils alone, and TEM showed a primarily twisted-ribbon fibril morphology.[21] Although a small population of rod-like fibrils was observed in some TEM preparations, the size distribution data indicates that they may not comprise a significant population or may not be present in all preparations. We first observed rod-like fibrils formed by apoC-II in samples treated for long periods with submicellar phospholipids.[51] The phospholipids used in the previous study did not incorporate into or bind strongly to mature fibrils,[21,51] which is in contrast to EGCG and fluphenazine·HCl.

Our studies indicate that EGCG and fluphenazine·HCl inhibit and activate fibril formation, respectively, by both apoC-II and $A\beta_{1-42}$. While EGCG has been reported as an inhibitor for a range of amyloidogenic proteins, fluphenazine·HCl has not, to our knowledge, been previously reported as an activator of amyloid formation. Small molecule modulators of amyloid formation are of intense interest, as they can be used as tools to dissect the underlying mechanisms of amyloid fibril formation and may also be used as potential lead structures for therapeutic design. It is possible that the increased rate of amyloid fibril formation in the presence of fluphenazine·HCl and the formation of large assemblies of fibrils in the presence of EGCG may each act to reduce the concentration of toxic oligomeric species formed during the fibril assembly pathway. Our work highlights that small molecules commonly administered as drugs may play previously unrecognized roles during protein misfolding and self-assembly in amyloid disease.

## ■ ASSOCIATED CONTENT

### Supporting Information

Figure S1: UV/vis absorbance spectra for EGCG and fluphenazine·HCl. Figure S2: ApoC-II amyloid fibril formation in the presence of small molecule compounds selected from the National Institutes of Health (NIH) Clinical Collections. Figure S3: Sedimentation velocity data for apoC-II alone and in the presence of EGCG and fluphenazine·HCl. Table S1: Quantitative analysis of the effect of small molecule compounds on the rate of fibril formation by apoC-II. Table S2: Quantitative analysis of the effect of EGCG and fluphenazine·HCl on the rate of fibril formation by $A\beta_{1-42}$. Table S3:



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Apparent rates demonstrating concentration dependent modulation of apoC-II fibrilization. Table S4: Optical absorbance measurements of supernatants from the pelleting of apoC-II fibrils alone and apoC-II fibrils formed in the presence of EGCG or fluphenazine·HCl. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00399.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

ThT, Thioflavin T; apo, apolipoprotein; VLDL, very-low-density lipoprotein; A$\beta$, amyloid $\beta$ peptide; IAPP, islet amyloid polypeptide; EGCG, epigallocatechin gallate; $t_{1/2}$, time to half-maximal ThT fluorescence; TEM, transmission electron microscopy; $\alpha$B-c, $\alpha$B-crystallin

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