

A Safe, Blood-Brain Barrier Permeable Triphenylmethane Dye Inhibits Amyloid- β Neurotoxicity by Generating Nontoxic Aggregates

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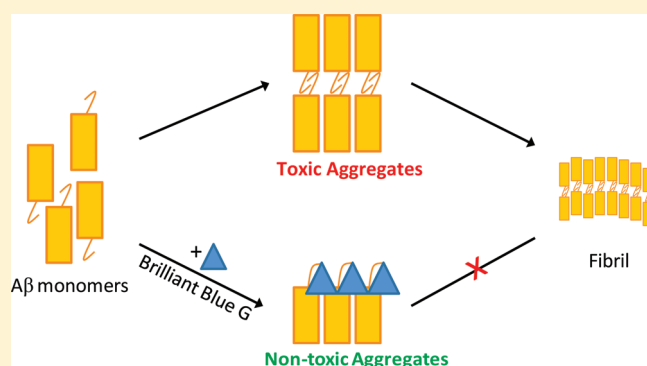
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S Supporting Information

ABSTRACT: Growing evidence suggests that on-pathway amyloid- β ($A\beta$) oligomers are primary neurotoxic species and have a direct correlation with the onset of Alzheimer's disease (AD). One promising therapeutic strategy to block AD progression is to reduce the levels of these neurotoxic $A\beta$ species using small molecules. While several compounds have been shown to modulate $A\beta$ aggregation, compounds with such activity combined with safety and high blood-brain barrier (BBB) permeability have yet to be reported. Brilliant Blue G (BBG) is a close structural analogue of a U.S. Food and Drug Administration (FDA)-approved food dye and has recently garnered prominent attention as a potential drug to treat spinal cord injury due to its neuroprotective effects along with BBB permeability and high degree of safety. In this work, we demonstrate that BBG is an effective $A\beta$ aggregation modulator, which reduces $A\beta$ -associated cytotoxicity in a dose-dependent manner by promoting the formation of off-pathway, nontoxic aggregates. Comparative studies of BBG and three structural analogues, Brilliant Blue R (BBR), Brilliant Blue FCF (BBF), and Fast Green FCF (FGF), revealed that BBG is most effective, BBR is moderately effective, and BBF and FGF are least effective in modulating $A\beta$ aggregation and cytotoxicity. Therefore, the two additional methyl groups of BBG and other structural differences between the congeners are important in the interaction of BBG with $A\beta$ leading to formation of nontoxic $A\beta$ aggregates. Our findings support the hypothesis that generating nontoxic aggregates using small molecule modulators is an effective strategy for reducing $A\beta$ cytotoxicity. Furthermore, key structural features of BBG identified through structure–function studies can open new avenues into therapeutic design for combating AD.

KEYWORDS: Alzheimer's disease, amyloid- β , oligomers, Brilliant blue, nontoxic aggregates, fibrils



With an ever increasing number of new cases each year, Alzheimer's disease (AD) has become the most common form of senile dementia. The disease is primarily diagnosed in persons over the age of 65.¹ Symptoms manifest in a slow and progressive manner, but are ultimately debilitating and fatal. Currently, 5.3 million people in the United States are affected by AD, with the number projected to rise to 13.5 million by 2050.² Although several United States Food and Drug Administration (FDA)-approved drugs temporarily reduce symptoms, no treatment exists that slows or stops the progression of AD.

A pathological hallmark of AD is the accumulation of insoluble protein aggregates, composed primarily of neurotoxic amyloid- β ($A\beta$). $A\beta$ is created from sequential proteolytic cleavage of the amyloid precursor protein (APP) by the β - and γ -secretases.³ Although a number of $A\beta$ isoforms, with lengths from 39 to 43 residues, are generated, $A\beta$ 40 and $A\beta$ 42 are the most physiologically relevant. In AD patients, $A\beta$ 40 and $A\beta$ 42 are found in an approximate 9:1 ratio, but $A\beta$ 42 is more aggregation-prone.⁴

According to the original amyloid-cascade hypothesis, conversion of soluble $A\beta$ monomers into insoluble fibrils causes AD

onset.⁵ Recently, this hypothesis has been further refined. Growing evidence suggests that certain types of soluble $A\beta$ oligomers and protofibrils are more toxic than $A\beta$ fibrils, and their presence correlates strongly with dementia.^{6–9} Therefore, reduction of neurotoxic $A\beta$ oligomers and protofibrils represents a promising strategy to inhibit $A\beta$ -associated neurotoxicity.

Numerous small molecules have been studied for their ability to modulate $A\beta$ aggregation and reduce neurotoxicity.^{10–13} Congo red, an amyloid-structure specific dye, has the ability to modulate fibril formation and reduce $A\beta$ neurotoxicity.^{14–18} Besides amyloid-specific dyes, several lipid-based modulators and polyphenols, such as scyllo-inocitol, nordihydroguaiaretic acid, curcumin, epigallocatechin gallate (EGCG), and resveratrol, have been reported to modulate $A\beta$ aggregation and reduce $A\beta$ -associated toxicity.^{10,19–25} Although the results from these molecules are encouraging and validate $A\beta$ aggregation modulation

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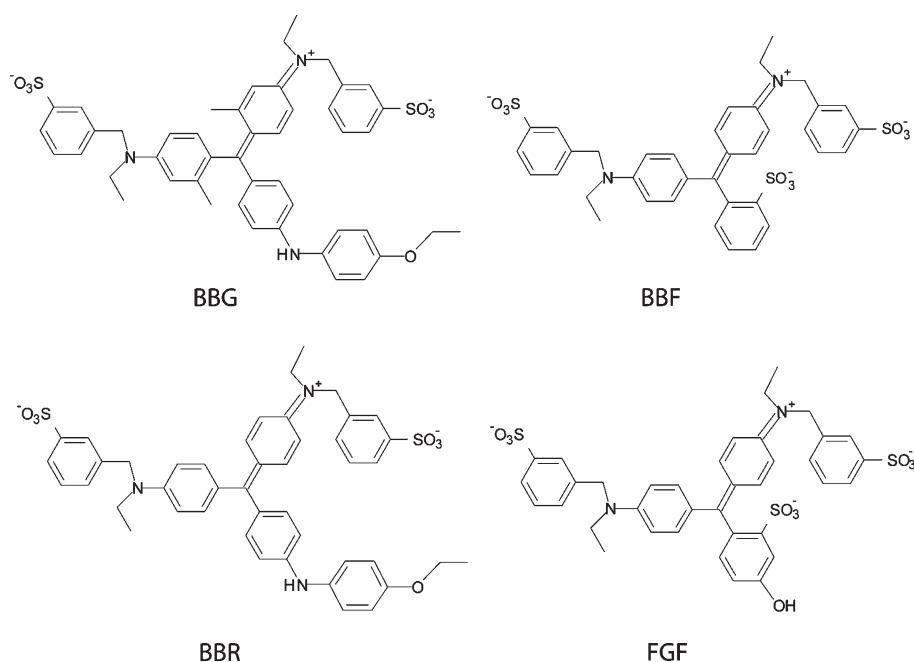


Figure 1. Chemical structures of Brilliant Blue G (BBG), Brilliant Blue FCF (BBF), Brilliant Blue R (BBR), and Fast Green FCF (FGF) at neutral pH.

as a promising strategy, a practical and effective therapeutic has yet to be identified. Most $A\beta$ aggregation small-molecule modulators identified are not suitable for AD therapeutic leads because they lack low toxicity or blood-brain barrier (BBB) permeability. Crossing the blood-brain barrier is a big challenge in AD drug development; 98% of small molecule drugs and almost 100% of large molecule drugs cannot cross the BBB.²⁶ For example, therapeutic application of Congo red has been hindered by poor BBB permeability as well as carcinogenicity.²⁷ Although several polyphenol-based $A\beta$ modulators including tannic acid and EGCG are very effective in reducing $A\beta$ neurotoxicity in cell-based assays,^{21,28} tannic acid and EGCG does not cross the animal BBB and the human BBB, respectively.^{29–31} As a result, there remains a strong driving force to identify new small-molecule AD therapeutic candidates that modulate $A\beta$ aggregation and are also safe and BBB-permeable.

In this Article, we identify a new $A\beta$ modulator with all of these properties. To our knowledge, $A\beta$ modulating capacities of triphenylmethane dyes have not yet been reported. Here we show that Brilliant Blue G (BBG), a triphenylmethane dye (Figure 1) with a demonstrated safety profile³² and BBB-permeability,³³ also substantially removes $A\beta$ cytotoxicity even at below stoichiometric concentrations. Brilliant Blue FCF (BBF), a close structural analogue of BBG, is approved by the FDA as a blue food dye (Figure 1) and has one of the highest safety profiles among the seven currently approved synthetic food dyes. In tests, it exhibits no observable toxicity up to a daily consumption of 600 mg/kg body mass in healthy animals.³⁴ In the United States, more than six thousand pounds of BBF is produced annually, and daily intake of up to 12.5 mg/kg is tolerable in humans.³⁵ BBG has recently garnered prominent attention in neuroscience regarding its therapeutic potential to treat acute spinal cord injury. In animal models, systemic administration of BBG reduced damage and expedited recovery after spinal cord injury.³³ BBG also confers neuroprotection to the brain by inhibiting adverse inflammatory reactions and mitigates multiple

sclerosis symptoms.^{36,37} With the possibility for systemic administration into the nervous system with no known adverse side effects, BBG is an attractive $A\beta$ aggregation modulator candidate.

In this Article, we explore BBG's ability to modulate $A\beta$ aggregation and reduce neurotoxicity with biochemical, biophysical, and cell-based assays. In particular, we monitored $A\beta$ oligomer formation via immunoblotting using $A\beta$ -conformation specific antibody, A11. Polyclonal A11 antibody reacts with $A\beta$ oligomers and protofibrils, including neurotoxic conformers, but not with $A\beta$ monomers and fibrils.^{8,38,39} We report that BBG promotes $A\beta$ monomer conversions into nontoxic aggregates. To begin identifying the BBG structural features responsible for this activity, we also evaluated the modulating capacities of three close structural analogues, Brilliant Blue R (BBR), BBF, and Fast Green FCF (FGF) on $A\beta$ aggregation and cytotoxicity. Our findings suggest that the structural differences of BBG and BBR from BBF and FGF along with the two additional methyl groups attached to the triphenylmethane structure of BBG are important for effective modulation of $A\beta$ aggregation and cytotoxicity. Unique interaction modes of BBG and BBR with $A\beta$ are expected to provide new insight on molecular mechanism of $A\beta$ aggregation and cytotoxicity. Our findings validate a relatively new hypothesis that generating nontoxic $A\beta$ aggregates by small molecules is an effective way to reduce $A\beta$ -associated neurotoxicity even without preventing $A\beta$ oligomer formation.^{21,28} Thus, our work provides evidence of and mechanistic details of reduced $A\beta$ -associated neurotoxicity for a novel type of $A\beta$ aggregation modulator that also has encouraging attributes as a therapeutic lead compound.

RESULTS AND DISCUSSION

Modulation of $A\beta$ Aggregation by BBG. In order to evaluate the aggregation modulating capability of BBG, we employed dot-blotting, transmission electron microscopy (TEM), and the thioflavin T (ThT) fluorescence assay. TEM and ThT fluorescence assays are widely used to monitor $A\beta$ aggregation. TEM images

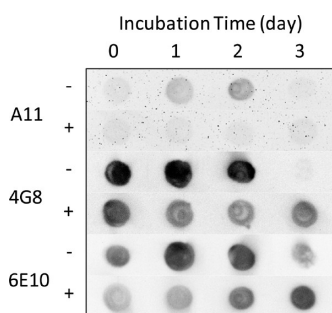


Figure 2. Monitoring $A\beta$ aggregation by dot-blotting. Oligomer-specific A11 antibody and $A\beta$ -sequence specific antibodies, 4G8 and 6E10, were used. $50 \mu\text{M}$ $A\beta$ was incubated in the absence (–) (top panels) or presence of $3\times$ BBG (+) (bottom panels) for 1–3 days at 37°C . Samples were taken on the indicated day and spotted onto a nitrocellulose membrane. Each membrane was immunostained with the A11, 4G8, or 6E10 antibody. The blot images were taken using a UVP BioSpectrum imaging system.

provide morphological information of $A\beta$ aggregates. ThT is a dye that fluoresces at 485 nm when it binds to amyloid fibrils.^{23,40,41} Therefore, ThT fluorescence measurement is an efficient tool to monitor the progression of fibril formation. However, the ThT assay is not very effective in detecting soluble oligomers that are known to be more neurotoxic than amyloid fibrils.^{6–9} Furthermore, the diversity of $A\beta$ aggregate morphologies and differing levels of neurotoxicity^{42,43} represent a challenge for correlating the aggregate morphology observed by TEM to $A\beta$ -associated neurotoxicity. Recently, dot-blotting with $A\beta$ -specific antibodies was successfully used to detect and distinguish the spectrum of $A\beta$ conformer species.^{8,38,39,44–46} In particular, A11 is useful for detecting neurotoxic $A\beta$ intermediates.^{39,45} Previously it was shown that $A\beta$ -associated toxicity could be mitigated by reducing the presence of A11-reactive species.^{8,21} Alternatively, 4G8 is an $A\beta$ -sequence-specific monoclonal antibody^{47–50} which has an epitope that lies within amino acids 17–24 of $A\beta$. The 4G8 epitope corresponds to a region of the $A\beta$ peptide that is known to form β -sheet structures. During transition from monomers and low molecular weight oligomers to fibrils, β -sheet stacking buries the 4G8 epitope and ultimately limits 4G8 antibody binding. This leads to a dramatic loss of the 4G8 signal which can thereby be used to detect extensive fibril network structure formation.⁵¹ Lastly, 6E10 is a monoclonal antibody that recognizes residues 1–16 of $A\beta$, the N-terminus of $A\beta$.^{45,51} Although the 6E10 antibody was originally thought to bind various $A\beta$ species with equal strength, recent studies indicate that the 6E10 antibody binds to different $A\beta$ species with different binding affinities.^{13,45,52} Similar to the 4G8 antibody, the 6E10 antibody binding affinity to fibrils is a few times lower than those of oligomers and monomers. According to the structural model of $A\beta$ 40 fibril proposed by Grigorieff et al., two pairs of $A\beta$ protofibrils intertwine adjacently to form a fibril.⁵³ In their model, the N-terminus of the each protofibril is interlocked to form a fibril, which can bury the 6E10 epitope upon fibril formation. We performed dot-blotting using a panel of $A\beta$ -specific antibodies, together with traditional TEM and ThT fluorescence assay, to monitor the formation of neurotoxic $A\beta$ oligomers, protofibrils, and fibrils.

Two pathologically important $A\beta$ isoforms, $A\beta$ 42 and $A\beta$ 40, have been widely used to evaluate the modulating capacities of numerous small molecules on $A\beta$ aggregation and cytotoxicity.^{54–58}

We used the more abundant isoform, $A\beta$ 40, in this study. $A\beta$ samples were prepared by incubating $50 \mu\text{M}$ of $A\beta$ 40 monomer from 0 to 3 days at 37°C without shaking in either the absence (control) or presence of BBG as described previously.^{59–61} In order to detect even a weak modulating effect of BBG on $A\beta$ aggregation, we chose $150 \mu\text{M}$ of BBG ($3\times$ BBG), which is 3 times higher than the concentration of $A\beta$ ($50 \mu\text{M}$). $A\beta$ samples were taken periodically during incubation and subjected to dot-blotting, the ThT fluorescence assay, and TEM.

In the absence of BBG, dot-blotting results indicated that the majority of A11-reactive $A\beta$ aggregates formed between day 1 and 2 (Figure 2). However, when the $A\beta$ monomer was coincubated with BBG, A11-reactivities signals remained very low until day 3. However, reduction of the A11-reactivities signals at day 1 and 2 did not result from a loss of $A\beta$ moieties, as supported by the sustained 6E10 signal at days 1 and 2 compared to day 0 (Figure 2). In the absence of BBG, the 6E10 signal was highest at days 1 and 2 and then decreased at day 3 (Figure 2). This variation can be explained by the weaker binding affinity of 6E10 for $A\beta$ monomers and fibrils compared to $A\beta$ intermediates as described previously.⁵² In the presence of $3\times$ BBG, the 6E10 signal at day 3 was very strong, suggesting the idea that $A\beta$ intermediates rather than $A\beta$ fibrils were predominant in the sample. In the $A\beta$ control sample, the majority of 4G8 signal was lost between day 2 and 3 (Figure 2), implying that the majority of 4G8 epitopes were buried due to compact stacking of β -sheet structures, a prototypical feature of amyloid fibrils.⁵¹ However, in the presence of $3\times$ BBG, a significant 4G8 signal at day 3 was observed, which also supports the idea that $A\beta$ fibril mesh networks were not formed. These findings imply that BBG effectively inhibits the formation of A11-reactive $A\beta$ oligomers and $A\beta$ fibrils, the most toxic $A\beta$ species.

Next we characterized the morphology of the $A\beta$ species formed with and without BBG using negative-stain TEM. TEM images clearly show a distinct morphological difference between $A\beta$ samples incubated in the absence and those in the presence of $3\times$ BBG (Figure 3). At day 1, in the absence of BBG, oligomers and protofibrils (~ 100 nm in length) were formed (Figure 3, top-left). Considering that high molecular weight $A\beta$ protofibrils as well as oligomers were A11-reactive in the literature,^{21,39,62} there is the possibility that the protofibrils observed at day 1 were also A11-reactive. Interestingly, even in the presence of $3\times$ BBG, protofibrils (~ 100 nm in length) were predominantly observed (Figure 3, bottom-left panel). Despite the morphological similarities, the $A\beta$ aggregates prepared with $3\times$ BBG possessed substantially lower immunoreactivity to the A11 antibody than those prepared without BBG. These findings imply that, at day 1, the low A11 reactivity of $A\beta$ samples coincubated with BBG resulted from promoted formation of A11-unreactive $A\beta$ protofibrils.

At day 2, a mixture of protofibrils and fibrils was observed in the absence of BBG (Figure 3, top-middle; Figure 4A), which indicated that some $A\beta$ oligomers and protofibrils were converted into longer protofibrils and fibrils. However, in the presence of $3\times$ BBG, protofibrils (less than 100 nm) were still predominantly observed similar to day 1 (Figure 3, bottom-middle).

At day 3, the $A\beta$ sample prepared in the absence of BBG exhibited only long mature fibrils in a meshed network (Figure 3, top-right), which is consistent with dot-blotting and ThT fluorescence results. Here, ThT fluorescence sharply increased beginning at 48 h (Figure 5), which was interpreted to be the

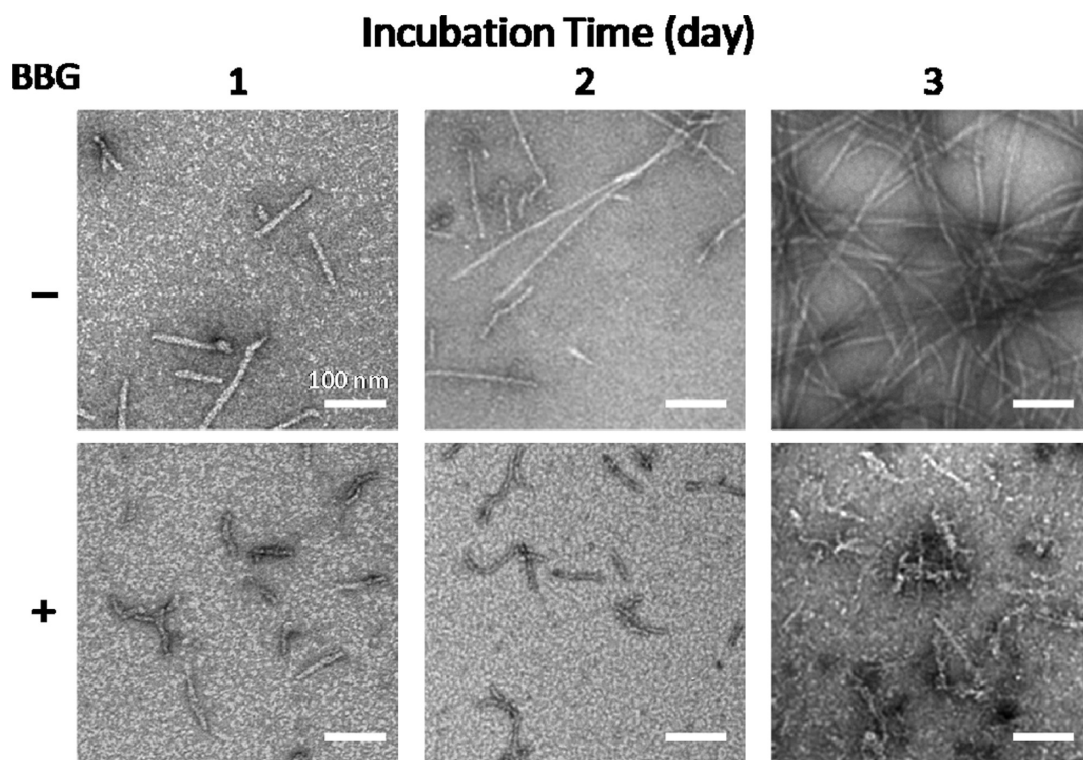


Figure 3. Monitoring $A\beta$ aggregate and fibril formation by TEM. $50\ \mu\text{M}$ $A\beta$ was incubated in the absence (–) (top panels) or presence of $3\times$ BBG (+) (bottom panels) for 1–3 days at $37\ ^\circ\text{C}$. Presence of oligomers and protofibrils (top-left); protofibrils and isolated fibrils (top-middle); fibril mesh network (top-right); oligomers and protofibrils (bottom-left); protofibrils (bottom-middle); protofibrils (bottom-right). Scale bar is 100 nm.

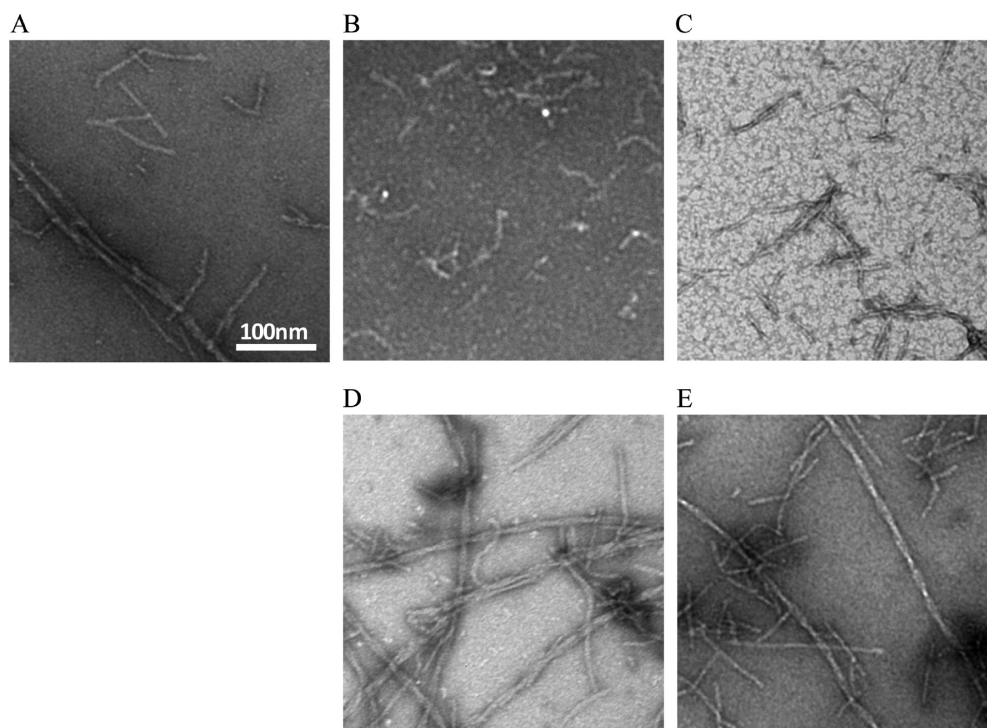


Figure 4. TEM images of $50\ \mu\text{M}$ of $A\beta$ incubated for 2 days at $37\ ^\circ\text{C}$ in the absence of any dye (A), or in the presence of $10\times$ BBG (B), $10\times$ BBR (C), $10\times$ BBF (D), or $10\times$ FGF (E). Scale bar is 100 nm.

onset of amyloid fibril formation. In dramatic contrast, in the presence of $3\times$ BBG, TEM showed that protofibrils ($\sim 100\ \text{nm}$)

remained the predominant species (Figure 3, bottom-right), which supports the idea that coincubation of $3\times$ BBG stops or

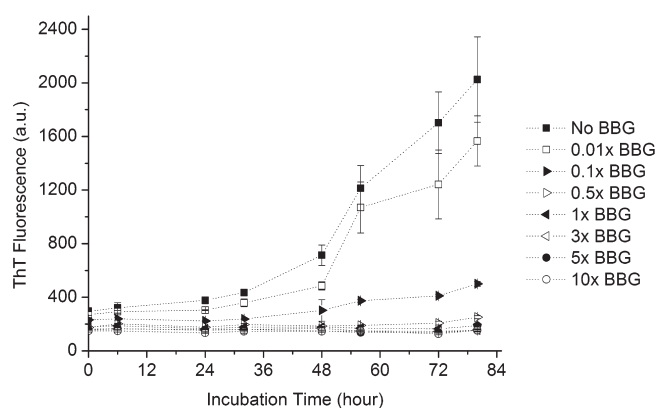


Figure 5. Time course of ThT fluorescence of $A\beta$ samples with varying concentrations of BBG. $50 \mu\text{M}$ of $A\beta$ monomer was incubated at 37°C in the absence (no BBG) or presence of the indicated concentrations of BBG (from $0.001\times$ to $10\times$) for up to 80 h. $5 \mu\text{L}$ of $A\beta$ sample was taken at 0, 6, 24, 32, 48, 54, 72, and 80 h for ThT fluorescence analysis. ThT fluorescence was measured in arbitrary units (a.u.). Values represent means \pm standard deviation ($n = 3$).

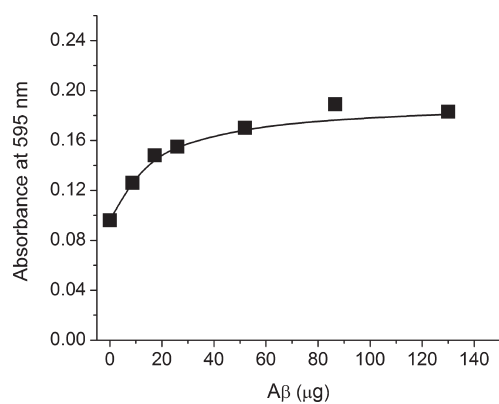


Figure 7. Saturation binding curve of BBG into $A\beta$ peptide. $49 \mu\text{M}$ BBG was incubated with varying concentrations of $A\beta$ peptide (0 – $300 \mu\text{M}$) until equilibrium was reached and the absorbance of the BBG bound to the $A\beta$ peptide was measured at 595 nm.

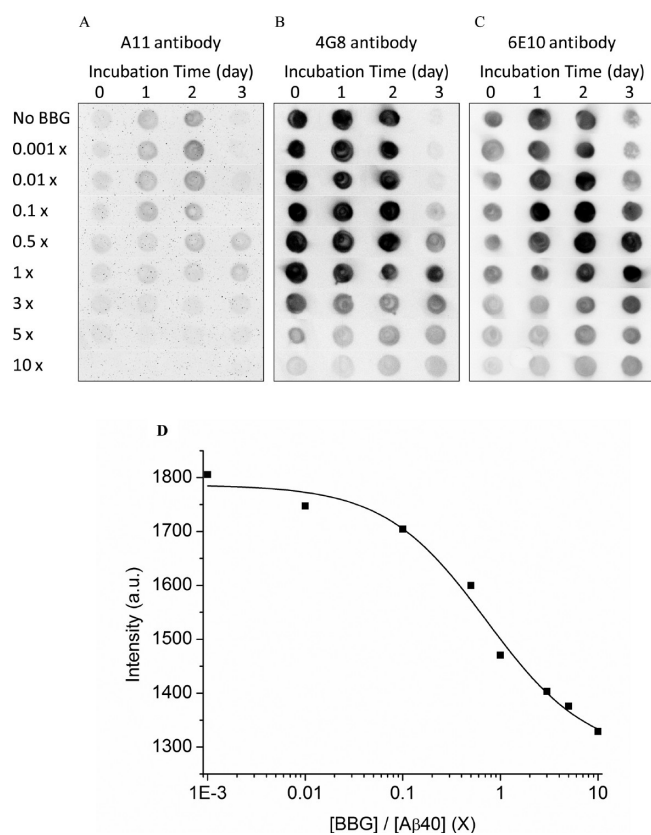


Figure 6. Dose-dependence of $A\beta$ aggregation modulation by BBG. $50 \mu\text{M}$ of $A\beta$ monomer was incubated at 37°C in the absence (no BBG) or presence of the indicated concentrations of BBG (from $0.001\times$ to $10\times$) for up to 3 days. Samples were taken on the indicated day and spotted onto a nitrocellulose membrane. Each membrane was immunostained with the A11 (A), 4G8 (B), or 6E10 (C) antibody. The blot images were taken using a UVP BioSpectrum imaging system. The blot image of the A11-reactive signal of $A\beta$ samples incubated with varying concentrations of BBG for 2 days was processed by ImageJ (NIH). The data were fitted to a sigmoid curve ($R^2 = 0.99$) (D).

at least substantially slows down the $A\beta$ aggregation process. Morphological similarities among the BBG-treated $A\beta$ aggregates

observed at days 1, 2, and 3 are consistent with very weak A11-reactivity of the BBG-treated samples at least until day 3.

These findings support that BBG is an efficient aggregation modulator and reduces the formation of A11-reactive $A\beta$ aggregates. The results also show that BBG suppresses fibril formation for at least 3 days.

Dose-Dependent Modulation of $A\beta$ Aggregation by BBG.

To further characterize the aggregation modulation capabilities of BBG, we evaluated BBG dose-dependent aggregation using dot-blotting and the ThT fluorescence assay. $A\beta$ ($50 \mu\text{M}$) was coincubated at 37°C with various BBG concentrations ranging from $0.001\times$ (50 nM) to $10\times$ ($500 \mu\text{M}$). Dot-blotting results of $A\beta$ samples using three $A\beta$ -specific antibodies (A11, 4G8, and 6E10) are shown in Figure 6. When $A\beta$ was coincubated with less than $0.1\times$ BBG ($5 \mu\text{M}$), no observable changes were found in the A11 immunoblotting patterns (Figure 6A). However, coincubation with $0.5\times$ BBG or greater resulted in a reduction in the concentration of A11-reactive species formed, over the course of the study, confirming previous results. Since A11-reactive $A\beta$ species were most abundant at day 2, we wanted to quantify the inhibition of A11-reactive $A\beta$ species formation (Figure 6D). Therefore, for day 2, integrated A11 dot-blot signal intensities were plotted against BBG concentrations. A half-maximal inhibitory concentration (IC_{50}) of $0.72\times$ BBG was derived from the data fitting to a sigmoid curve ($R^2 = 0.99$).

In the absence of BBG, the 6E10 antibody stained $A\beta$ oligomers/protofibrils, monomers, and fibrils in decreasing order of intensity, which is consistent with a few fold weaker binding to $A\beta$ fibrils by the 6E10 compared to oligomers/protofibrils as described previously.⁵² In Figure 6B and C, below $1\times$ BBG concentrations, 4G8 and 6E10 signals increase at day 3. These results are consistent with enhanced accessibility of the 4G8 and 6E10 epitopes due to BBG-induced conversion of $A\beta$ aggregates from fibril mesh networks to oligomers and protofibrils. However, as the concentration of BBG increased above $1\times$, 4G8 and 6E10 reactive signals decreased. Finally, in the presence of $10\times$ BBG, the 4G8- and 6E10-reactive signals are both very weak compared to those observed in the absence of BBG, suggesting that both 4G8 and 6E10 epitopes were substantially lost partially due to direct binding of BBG. The 4G8 epitope corresponds to a hydrophobic patch of the $A\beta$ that is known to form β -sheet structures. The 6E10 epitope is the N-terminus of $A\beta$ carrying

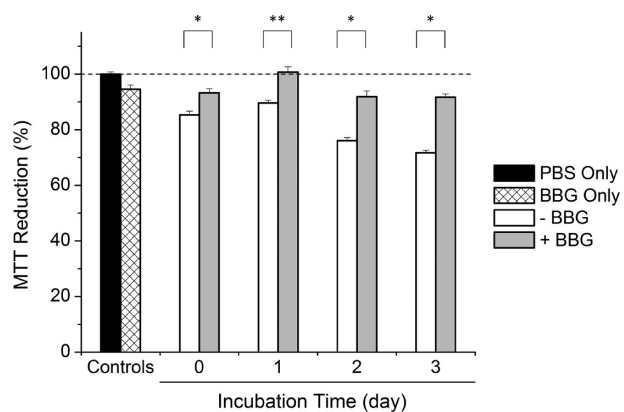


Figure 8. Viability of neuroblastoma SH-SY5Y cells incubated with preformed $A\beta$ samples in the absence or presence of BBG. Preformed $A\beta$ aggregates were prepared by incubating $50 \mu\text{M}$ of $A\beta$ monomer in the absence or presence of BBG at 37°C for 0–3 days, as indicated in the graph. Aggregates were then administered to SH-SY5Y cells at a final concentration of $5 \mu\text{M}$. After 48 h, mitochondrial metabolic activity was measured using MTT reduction. Cells administered with PBS as a control (black), $3\times$ BBG ($15 \mu\text{M}$) dye only (white with pattern), $A\beta$ incubated without BBG (white), and $A\beta$ incubated with $3\times$ BBG (gray). Values represent means \pm standard deviation ($n \geq 3$). Values are normalized to the viability of cells administered with PBS only. Two-sided Student's t tests were applied to the data. * $P < 0.001$, ** $P < 0.005$.

both positive and negative charges. Therefore, we speculate that BBG preferentially binds to the hydrophobic patch of $A\beta$ and weakly binds to the charged N-terminus of $A\beta$ via electrostatic interaction.

BBG Binding to $A\beta$. Immunoblotting assays described in the previous section suggested that BBG binds to multiple sites on $A\beta$ above $1\times$ BBG concentration. In order to determine the number of BBG binding sites on $A\beta$, a binding curve (Figure 7) was obtained according to the method described previously.⁶³ The number of binding sites (n) and the intrinsic association constant (k), 3.2 and 1.1×10^4 (M^{-1}), respectively, were determined by fitting the BBG- $A\beta$ binding data to a straight line in the double reciprocal plot (Supporting Information Figure S1) in the Supporting Information Section. Multiple BBG binding site on $A\beta$ can explain the reduced 4G8 and 6E10 reactivities of $A\beta$ aggregates at high concentrations of BBG considering the reduced accessibility of the 4G8 and 6E10 antibodies to their epitopes caused by BBG binding. The dissociation constant, the reciprocal of the intrinsic association constant, is $92 \mu\text{M}$.

Inhibition of $A\beta$ -Associated Cytotoxicity by BBG. Next, we sought to determine whether the conversion of A11-reactive aggregates into off-pathway aggregates reduces $A\beta$ -associated neurotoxicity. In order to evaluate the cytotoxicity of $A\beta$ species, we employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-reduction assay and neuroblastoma SH-SY5Y cells, widely used for this purpose.^{8,22,64–69} Although the MTT-reduction assay does not directly measure cell death, it detects a change in cellular redox activity that is considered an indication of cell viability.⁷⁰ Therefore, inhibition of MTT reducing activity of cells has been often interpreted as cytotoxicity. Preformed $A\beta$ aggregates were prepared by incubating $A\beta$ monomers in the absence or presence of $3\times$ BBG at 37°C for the specified time duration. The preformed aggregates were then administered to neuroblastoma SH-SY5Y cells for 48 h, and subsequently cell viability was measured by MTT reduction. Before testing the cytotoxicity of $A\beta$ samples, we first determined

whether BBG by itself is cytotoxic to neuroblastoma SH-SY5Y cells. In the presence of $3\times$ BBG dye ($15 \mu\text{M}$), SH-SY5Y cell viability was 95% of that in the absence of any BBG, which is quite consistent with the good biocompatibility and low toxicity observed in animals.^{32,33}

Next we evaluated $A\beta$ -associated toxicity. At day 0, cells treated with $A\beta$ monomers ($5 \mu\text{M}$) exhibited a mild reduction (15%) in the viability (Figure 8), which may be due to moderately toxic $A\beta$ aggregates formed during the 48 h incubation of $A\beta$ monomers with the cells. In the presence of $3\times$ BBG ($15 \mu\text{M}$), viability recovered to 93% ($P < 0.001$), which is similar to the viability of cells treated with $3\times$ BBG dye alone without $A\beta$ monomer (95%). This finding suggests that $3\times$ BBG inhibits toxic aggregate formation from $A\beta$ monomers ($5 \mu\text{M}$) during the 48 h incubation with the cells. At day 1, in the presence of $3\times$ BBG, the $A\beta$ sample exhibited cell viability of 101%, significantly higher than the cell viability (90%) of $A\beta$ samples without BBG ($P < 0.001$) (Figure 8). These findings support the hypothesis that BBG can counteract the $A\beta$ sample cytotoxicity and that BBG-induced $A\beta$ aggregates observed by TEM were nontoxic.

At day 2, in the absence of BBG, cell viability decreased to 76%. This can likely be attributed to an increased concentration of A11-reactive toxic intermediates and to the emergence of amyloid fibrils (Figure 8). Although amyloid fibrils are less toxic than A11-reactive toxic intermediates, the toxicity of amyloid fibrils is reportedly higher than that of monomers.^{8,9} In the presence of BBG, cell viability significantly improved to 92% ($P < 0.001$), consistent with the reduction of A11-reactive $A\beta$ species in the dot-blot results (Figure 2) and lack of observable fibrils in the TEM image (Figure 3). These results suggest that a decrease in the concentration of A11-reactive species correlates with a decrease in $A\beta$ -associated toxicity.

At day 3, in the absence of BBG, addition of the preformed $A\beta$ aggregates reduced the cell viability to 72%. Although the A11-reactivity dropped after day 2, the reduced viability was most probably caused by the predominant amyloid fibrils converted from both small fraction of toxic A11-reactive aggregates and probably more dominant non-A11 reactive large intermediates. Ishii and co-workers showed that $A\beta$ aggregates larger than 50 kDa were 5 times less toxic than $A\beta$ aggregates smaller than 50 kDa.⁹ Based on dot-blotting, TEM and fluorescence, the vast majority of fibrils were formed almost exclusively after day 2 (Figures 2, 3, and 5). Consequently, at day 3, fibrils were the major toxic moieties. However, when $A\beta$ was incubated with $3\times$ BBG, cell viability was significantly recovered to 92% ($P < 0.001$), which is comparable to the viability of cells incubated only with $3\times$ BBG, without $A\beta$. Based on the cell viability results, we conclude that $3\times$ BBG completely mitigates $A\beta$ -associated cytotoxicity. Moreover, these findings suggest that the majority of oligomers and protofibrils formed in the presence of $3\times$ BBG observed in TEM in fact are nontoxic and structurally distinct from toxic $A\beta$ species formed in the absence of BBG. This confirms that BBG reduces $A\beta$ -associated toxicity by promoting the conversion of $A\beta$ monomer to off-pathway, nontoxic intermediates.

Although an MTT reduction assay has been widely used to determine $A\beta$ -associated cytotoxicity on numerous cell lines,^{21,45,71–75} the MTT reduction assay results should be carefully interpreted due to a potential issue of $A\beta$ -induced expedited exocytosis of the reduced MTT. There have been several reports indicating that $A\beta$ aggregates can increase export of the reduced MTT and promote buildup of the crystalline form of the reduced MTT on the cell surface, leading to a reduced

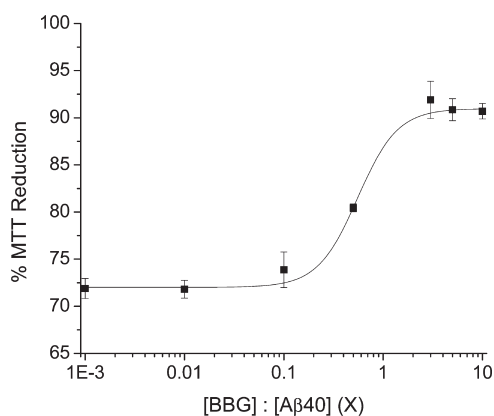


Figure 9. Dose-dependence of inhibition of $A\beta$ -associated cytotoxicity by BBG. Preformed $A\beta$ aggregates were prepared by incubating $50 \mu\text{M}$ of $A\beta$ monomer in the presence of varying concentrations of BBG ($0.001\times$, $0.01\times$, $0.1\times$, $0.5\times$, $1\times$, $3\times$, $5\times$, and $10\times$) at 37°C for 2 days. $A\beta$ aggregates were then administered to SH-SY5Y cells at a final concentration of $5 \mu\text{M}$. After incubation, mitochondrial metabolic activity was measured after 48 h using MTT reduction. Values represent means \pm standard deviation ($n \geq 3$). Values are normalized to the viability of cells administered with PBS only. The data were fitted to a sigmoid curve ($R^2 = 0.99$).

MTT uptake.^{76–78} Considering other researchers have reported a good correlation between a MTT reduction and other viability assay results, including oxidative stress⁷³ and lactate dehydrogenase (LDH) release,⁷⁵ the increased MTT exocytosis may be dependent on the cell type and $A\beta$ preparation method used.

In order to confirm the correlation of the MTT assay results with $A\beta$ -associated cytotoxicity, an alamar blue reduction assay was also performed. Alamar blue reducing cellular activity has been considered an indication of cell viability and used to measure $A\beta$ -associated cytotoxicity.^{79–81} Since alamar blue is a soluble dye, it does not cause an issue of crystal dye buildup on the cell surface. $A\beta$ samples incubated for 0–3 days at 37°C were added to SH-SY5Y cells to determine alamar blue reducing activities. Similar to the MTT reduction assay results, the cells treated with $A\beta$ aggregates incubated for 2 days exhibited significantly ($P < 0.05$) lowered alamar blue reducing activity compared to the cells without $A\beta$ treatment (control) or cells treated with $A\beta$ monomers (day 0) (Supporting Information Figure S2). Furthermore, coinubation of $3\times$ BBG with $A\beta$ for 2 days inhibited the $A\beta$ -induced loss of the alamar blue reducing activity ($P < 0.05$) (Supporting Information Figure S2), which is also consistent with the MTT reduction assay results. However, there were two factors making it difficult to detect a subtle change in the alamar blue reducing activity under the other conditions used in our studies. First, the differences in the alamar blue reducing cellular activities with different $A\beta$ samples were smaller than those in the MTT reducing activities at similar conditions. Second, the standard deviations of the measured alamar blue reducing activities (5–11%) were greater than those in the MTT reducing activities (usually less than 5%). Despite the limitations, the alamar blue assay results support the idea that the decrease in the MTT reducing activity of cells treated with $A\beta$ aggregates (at least at day 2) indicates the $A\beta$ -associated cytotoxicity and the recovery of MTT reducing activity in the presence of $3\times$ BBG means a reduction of the $A\beta$ -associated cytotoxicity under the conditions used in our studies. Therefore, we believe that the MTT reduction assay results have a good correlation with the cell

viabilities treated with the $A\beta$ samples, though we do not completely exclude the possibility that the promoted crystal buildup of the reduced MTT contributes to the MTT reduction results.

Dose-Dependent Inhibition of $A\beta$ -Associated Cytotoxicity by BBG. In order to evaluate the ability of BBG to inhibit $A\beta$ -associated cytotoxicity at varying doses, preformed $A\beta$ aggregates were prepared by incubating $A\beta$ monomer in the absence (control) or presence of varying concentrations of BBG from $0.001\times$ to $10\times$ at 37°C for 2 days. The resulting preformed species were administered to SH-SY5Y cells, and viability was measured after 48 h using MTT reduction. From $0.001\times$ (5.0 nM) to $0.1\times$ ($0.5 \mu\text{M}$) BBG, cell viability (approximately 72%) was comparable to that of the control. However, cell viability dramatically improved when $0.5\times$ BBG ($2.5 \mu\text{M}$) or greater was coincubated with $A\beta$ (Figure 9). Consequently, from $3\times$ to $10\times$ BBG, cell viability was maintained in the range of 91 to 93%. When the data were fitted to a sigmoid dose-dependent curve ($R^2 = 0.99$), a half maximal effective concentration (EC_{50}) value of $0.55\times$ BBG was obtained. This value corresponded almost exactly to the significant reduction in the $A\beta$ -associated cytotoxicity observed at $0.5\times$ BBG. These results clearly demonstrate that BBG inhibits $A\beta$ -associated cytotoxicity in a dose-dependent manner.

Based on the results described earlier, it is obvious that BBG inhibits formation of A11-reactive $A\beta$ species and $A\beta$ -associated cytotoxicity in a dose-dependent manner. Furthermore, the EC_{50} value ($0.55\times$ BBG) derived from $A\beta$ -associated cytotoxicity sigmoidal regression (Figure 9) corresponds well to the IC_{50} values ($0.72\times$ BBG) derived from the sigmoidal regression of inhibition of A11-reactive $A\beta$ species formation by BBG (Figure 6D). Therefore, we conclude that the inhibition of $A\beta$ -associated cytotoxicity directly correlates with BBG aggregation modulation effects. Furthermore, considering that BBG-induced aggregates are nontoxic, $A\beta$ -associated cytotoxicity reduction is attributed to BBG-induced, nontoxic aggregate formation.

Bias of ThT Fluorescence Reading at High Concentration of BBG. We used ThT binding to verify the onset of fibril formation at varying BBG concentrations. As BBG concentration was increased, the ThT fluorescence of $A\beta$ samples decreased accordingly (Figure 5), consistent with our findings and observations at $3\times$ BBG. In order to quantify the inhibition of ThT fluorescence by BBG, we plotted ThT fluorescence versus BBG concentrations from $10^{-5}\times$ to $10\times$ BBG (Supporting Information Figure S3). Data fitting to a sigmoid curve ($R^2 = 0.99$) generated an IC_{50} value of $0.03\times$ BBG, which is 1 order of magnitude lower than those determined for the inhibition of A11-reactive species formation by BBG. To explain this discrepancy, we hypothesized that the ThT fluorescence reading was biased due to either spectral interference of BBG on ThT fluorescence measurement or competitive binding of BBG to ThT binding sites on amyloid fibrils. Carver et al. reported that curcumin and quercetin significantly skewed ThT fluorescence measurements via spectral interference, where alternatively resveratrol introduced a measurement bias by competing with ThT for amyloid fibril binding sites.⁸² Since BBG at neutral pH has negligible absorbance at both 450 nm (excitation wavelength of ThT fluorescence) and 485 nm (emission wavelength of ThT fluorescence),⁸³ spectral interference was ruled out as a source of the bias. Next, to determine whether BBG and ThT competitively bind to the same sites, we measured the ThT fluorescence of preformed amyloid fibrils that were momentarily mixed with varying BBG concentrations immediately prior to adding ThT

(Supporting Information Figure S4). As BBG concentration increased, ThT fluorescence decreased accordingly. When the data were fitted to a sigmoidal curve ($R^2 = 0.99$), an IC_{50} value of $0.16 \times$ BBG was obtained. From this, we conclude that the ThT fluorescence measurements of $A\beta$ samples coincubated with BBG can be biased since BBG at high concentrations can interfere with ThT binding to amyloid fibrils. Consequently, the ThT fluorescence assay should be used with caution at high concentrations of BBG.

Comparison of the $A\beta$ Aggregates Formed in the Presence or Absence of BBG. TEM, ThT fluorescence, immunoblotting, and cellular reducing activity assays have demonstrated various differences between the $A\beta$ aggregates formed with BBG and those formed without BBG. In particular, the $A\beta$ aggregates incubated for 2 days with and without $3 \times$ BBG exhibited several noticeable differences. The TEM images clearly indicate that $3 \times$ BBG inhibits long fibril formation but promotes ~ 100 nm long curvilinear protofibril formation (Figure 3 top middle and bottom middle). At day 2, $A\beta$ aggregates formed without BBG exhibited a moderate increase in ThT fluorescence compared to $A\beta$ monomers (Figure 5), which suggests that fibrillar and nonfibrillar aggregates exist together. Unfortunately, the reduction in the ThT fluorescence value of the $A\beta$ aggregates formed with $3 \times$ BBG cannot be directly interpreted as an indication of a reduction of $A\beta$ fibrils due to the interference issue. The $A\beta$ aggregates formed with $3 \times$ BBG showed very weak A11-reactivity, whereas the $A\beta$ aggregates formed without BBG exhibited a substantial A11-reactivity. The protofibrils (Figure 3 top middle) look similar to the A11-reactive $A\beta_{40}$ protofibrils that Kim et al. recently reported.³⁹ In the presence of $3 \times$ BBG, the 4G8-reactivity of the $A\beta$ aggregates decreased (Figure 2), which suggests that one of the BBG binding sites might be located to near the 4G8 epitope, the hydrophobic patch of $A\beta$ at the $3 \times$ BBG concentration.

At day 2, the $A\beta$ aggregates formed without BBG exhibited a significant decrease in the cellular reducing activity in both MTT (Figure 8) and alamar blue (Supporting Information Figure S2) assays. These two assays indicate the reduced cell viability is correlated with the A11-reactivity (Figure 2). The significantly reduced A11-reactivity of the $A\beta$ aggregates formed with $3 \times$ BBG suggests its correlation with the substantial increase in the cellular reducing activity on both MTT (Figure 8) and alamar blue (Supporting Information Figure S2). A11-reactivity is defined by immunoreactivity of $A\beta$ aggregates but not by size or morphology. Therefore, it remains unclear whether there is any correlation between the A11-reactivity and the size or morphology of $A\beta$ aggregates. Considering that the BBG-induced $A\beta$ aggregates do not form $A\beta$ fibrils or at least substantially slow down the fibril formation process, we speculate that BBG binding causes conformation changes of $A\beta$ protofibrils or blocking the nuclei leading to inhibition of fibril formation. However, further investigation is required to clarify the relationship between the BBG-induced $A\beta$ aggregate morphology change and the change in the cellular MTT and alamar blue reducing activity.

Modulation of $A\beta$ Aggregation by BBG Analogues. In order to determine whether any structural features of BBG are critical in modulating $A\beta$ aggregation and cytotoxicity, $A\beta$ aggregation modulation capacity of three close structural analogues of BBG (BBR, BBF, and FGF) were examined using dot-blotting and TEM analysis. The four compounds (BBG, BBR, BBF, and FGF) are congeners sharing the common triphenylmethane

structure. Both BBF and FGF are FDA-approved food dyes.⁸⁴ Similar to BBG, BBR is commonly used to stain proteins in protein electrophoresis. However, the chemical structure of BBR differs from BBG by the lack of two methyl groups attached to triphenylmethane (Figure 1). Both BBF and FGF have three benzenesulfonate functional groups, whereas BBG and BBR have two benzenesulfonate functional groups and one uncharged diphenylamine group. FGF differs from BBF with only one hydroxyl functional group attached to one of the benzenesulfonate functional groups (Figure 1).

Three different concentrations ($1 \times$, $3 \times$, and $10 \times$) of each BBG analogue were coincubated with $50 \mu\text{M}$ of $A\beta$ for up to 3 days, and the immunoreactivities of the $A\beta$ samples were monitored by A11, 4G8, and 6E10 $A\beta$ -specific antibodies. Similar to BBG, BBR reduced toxic A11-reactive $A\beta$ species. $3 \times$ BBR made the A11-reactivity less than half the A11-reactive signal of $A\beta$ sample incubated without any dye, though $1 \times$ BBR did not make a substantial change in the A11-reactive signal (Figure 10A). Coincubation of $10 \times$ BBR with the $A\beta$ samples led to almost complete elimination of the A11-reactive species. However, considering that even $1 \times$ BBG substantially reduced the A11-reactivity (Figure 10A), BBR is less effective than BBG in reducing A11-reactive species. Similar to $10 \times$ BBG, $10 \times$ BBR substantially reduced the 4G8-reactive signal, suggesting that both BBR and BBG bind to the $A\beta$ hydrophobic patch (4G8 epitope) in a similar fashion (Figure 10B). In contrast, even at $10 \times$ BBR, there was no substantial reduction of the 6E10-reactive signal, which suggests that the BBR interaction mode of $A\beta$ is different from that of BBG (Figures 6C and 10C). These results suggest that BBR reduces A11-reactive toxic species, but the interaction mode of BBR with $A\beta$ is different from that of BBG.

In sharp contrast to BBG, both BBF and FGF caused little or no change in the A11- and 4G8-immunoreactivity of the $A\beta$ samples compared to that of the no dye control (Figure 10). In particular, even $10 \times$ BBF and $10 \times$ FGF did not show any substantial reduction of the neurotoxic A11-reactive $A\beta$ species. At day 3, the $A\beta$ samples incubated with either BBF or FGF exhibited strong 6E10 signals, whereas $A\beta$ aggregates formed in the absence of any dye exhibited very weak 6E10 signals (Figure 10C). These 6E10 epitope accessibility results suggest that BBF- or FGF-induced $A\beta$ protofibrils and fibrils have conformations different from those of $A\beta$ protofibrils and fibrils formed in the absence of any dye.

The morphology of $A\beta$ aggregates formed after 2 days in the absence (no dye control) or presence of one of the BBG congeners was analyzed by negative-stain TEM (Figure 4). Similar to BBG, coincubation of $10 \times$ BBR promoted protofibril formation (less than 100 nm), but inhibited amyloid fibril formation (Figure 4B and C). In contrast, both BBF and FGF induced formation of the mixture of long fibrils and protofibrils, whereas the $A\beta$ aggregates incubated with BBG for 2 days were predominantly curvilinear protofibrils (Figure 4B, D, and E). The morphology of the $A\beta$ aggregates formed in the presence of either BBF (Figure 4D) or FGF (Figure 4E) was similar to that of the $A\beta$ aggregates formed in the absence of any dye (Figure 4A), which is consistent with the dot-blot results that there are little or minor differences in the immunoreactivities of the $A\beta$ aggregates formed in the absence or in the presence of BBF or FGF.

These results strongly suggest that both BBG and BBR effectively modulate $A\beta$ aggregation by promoting protofibril formation but inhibiting fibril formation. In contrast, both BBF

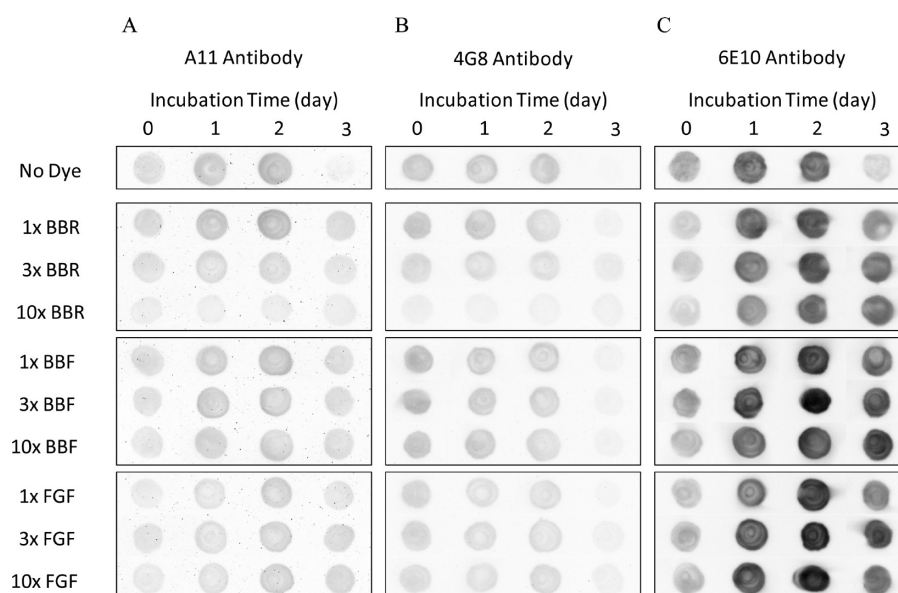


Figure 10. Modulation of $A\beta$ aggregation by BBG analogues, BBR, BBF, and FGF. $50 \mu\text{M}$ of $A\beta$ monomer was incubated at 37°C in the absence (no dye) or presence of the indicated concentrations of BBR, BBF, or FGF ($1\times$, $3\times$, and $10\times$) for up to 3 days. Samples were taken on the indicated day and spotted onto a nitrocellulose membrane. Each membrane was immunostained with the A11 (A), 4G8 (B), or 6E10 (C) antibody.

and FGF are not as effective in modulating $A\beta$ aggregation as BBG and BBR, but promote formation of the mixture of protofibrils and fibrils.

Modulation of $A\beta$ Cytotoxicity by BBG Analogues. Modulating effects of the BBG analogues on $A\beta$ -associated cytotoxicity were also evaluated by MTT reduction assay of SH-SY5Y cells (Figure 11). Similar to $3\times$ BBG, coincubation of $3\times$ BBR with $A\beta$ samples recovered the viability of SH-SY5Y cells from 76% to 97% (Figure 11), which is consistent with the reduced neurotoxic A11-reactivity of the $A\beta$ aggregates at $3\times$ BBR (Figure 10A). However, compared with $1\times$ BBG, coincubation of $1\times$ BBR exhibits less increase in the SH-SY5Y cell viability (86%) consistent with lower reduction of the A11-reactivity. These findings support the idea that BBR reduces $A\beta$ -associated cytotoxicity but is less effective than BBG.

In contrast, BBF and FGF are less effective in modulating the $A\beta$ -cytotoxicity than BBG and BBR. Even at $3\times$ BBF and FGF, the SH-SY5Y cell viability was 80% and 86%, respectively, which is higher than the cell viability without any dye but 10% lower than the cell viability with either $3\times$ BBG or $3\times$ BBR. A moderate reduction of $A\beta$ cytotoxicity produced by BBF or FGF may be due to promoted formation of less toxic fibrils than oligomers/protofibrils (Figure 4D and E). Investigations are underway to reveal the underlying mechanisms for this behavior.

Based on our findings, we identified several important BBG structural features that are important for modulating $A\beta$ aggregation and cytotoxicity. Although all four triphenylmethane-based BBG and analogues exhibit modulating ability on $A\beta$ aggregation, structural differences of BBG and BBR from BBF and FGF (one additional diphenylamine group and one less benzenesulfonate group) are responsible for the unique interaction mode of BBG and BBR on $A\beta$ leading to formation of nontoxic $A\beta$ aggregates. In particular, one sulfonate group connected to the triphenylmethane backbone contains an electron withdrawing sulfur atom that is expected to perturb π - π stacking interaction. Furthermore, considering that BBG is more effective in

modulating $A\beta$ aggregation and cytotoxicity than BBR, the two additional methyl groups in BBG should also be considered important.

Taken together, our results conclusively establish that BBG effectively reduces neurotoxic A11-reactive $A\beta$ intermediates by inducing the formation of nontoxic $A\beta$ aggregates. BBG inhibited the formation of A11-reactive $A\beta$ aggregates in a dose-dependent manner. The IC_{50} value in the inhibition of A11-reactive $A\beta$ aggregate formation by BBG after 2 day incubation was $0.72\times$ BBG. Negative-stain TEM, ThT fluorescence assay, and dot-blotting assay results strongly support the idea that BBG promotes the formation of A11-unreactive, off-pathway $A\beta$ oligomers and protofibrils, but inhibits the formation of amyloid fibrils. BBG at $15 \mu\text{M}$ ($3\times$ BBG) conferred only a minor cytotoxicity (5%) to neuroblastoma SH-SY5Y cells, which is approximately one-third of the cytotoxicity of $5 \mu\text{M}$ of $A\beta$ monomer. At $3\times$ BBG, $A\beta$ -associated cytotoxicity was completely suppressed throughout the duration of our study (3 days). BBG effectively inhibits $A\beta$ -associated cytotoxicity in a dose-dependent manner. The EC_{50} value of inhibition of $A\beta$ -associated cytotoxicity by BBG was $0.55\times$ BBG. These results strongly support the idea that the inhibition of $A\beta$ -associated cytotoxicity by BBG directly correlates with the reduction of neurotoxic A11-reactive $A\beta$ aggregates by BBG. Comparative studies of BBG and BBG analogues on modulation of $A\beta$ aggregation and cytotoxicity revealed that one additional diphenylamine group and one less benzenesulfonate group are critical for $A\beta$ aggregation modulation. Furthermore, considering that BBG is more effective in modulating $A\beta$ aggregation and cytotoxicity than BBR, the two additional methyl groups attached to the triphenylmethane structure in BBG are also important.

The inhibitory effects of BBG on $A\beta$ -associated cytotoxicity as well as highly favorable biocompatibility and BBB-permeability make BBG a promising lead compound for future AD therapeutic development.

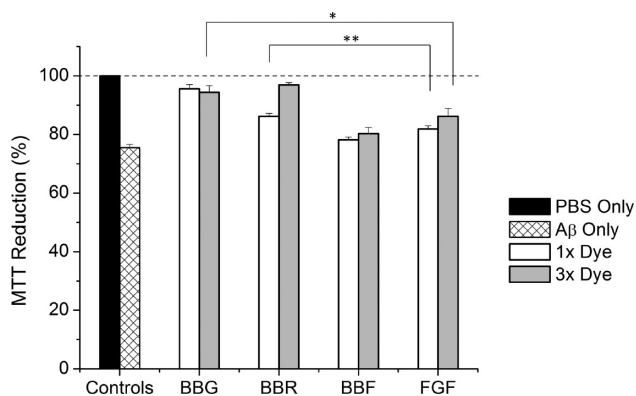


Figure 11. Viability of neuroblastoma SH-SY5Y cells incubated with preformed A β samples in the presence of BBG or BBG analogues (BBR, BBF, and FGF). Preformed A β aggregates were prepared by incubating 50 μ M of A β monomer in the presence of BBG or BBG analogues at 37 $^{\circ}$ C for 2 days, as indicated in the graph. Aggregates were then administered to SH-SY5Y cells at a final concentration of 5 μ M. After 48 h, mitochondrial metabolic activity was measured using MTT reduction. Cells administered with PBS as a control (black), A β samples incubated without BBG (white with pattern), A β samples incubated with 1 \times dye (white), or 3 \times dye (gray). Values represent means \pm standard deviation ($n \geq 3$). Values are normalized to the viability of cells administered with PBS only. Two-sided Student's t tests were applied to the data. * $P < 0.001$, ** $P < 0.01$.

METHODS

Materials. A β 40 was purchased from Anaspec, Inc. (Fremont, CA). Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Polyclonal A11 anti-oligomer and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies were obtained from Invitrogen (Carlsbad, CA). 4G8 antibody was obtained from Abcam (Cambridge, MA). Monoclonal 6E10 was obtained from Millipore (Billerica, MA). ECL advance chemiluminescence kit was obtained from GE Healthcare Life Sciences. Alamar blue dye stock solution (10 \times) was obtained from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

A β Sample Preparation. A β 40 samples were prepared as described earlier.^{59–61} A β 40 was dissolved in 0.1% trifluoroacetic acid (TFA) to obtain a 1.0 mM stock solution, which was then incubated for 1 h at room temperature without agitation. The freshly prepared 1.0 mM stock A β 40 solution was diluted with phosphate buffered saline (PBS) solution (10 mM NaH₂PO₄ and 150 mM NaCl at pH 7.4) to obtain a 50 μ M A β solution. A β samples of 50 μ M were then incubated at 37 $^{\circ}$ C for the desired time.

Dot-Blotting. A β 40 samples (2 μ L) were spotted onto a nitrocellulose membrane and were allowed to dry at room temperature. The nitrocellulose membrane was incubated in 5% skim milk dissolved in 0.1% Tween 20, Tris-buffered saline (TBS-T) solution for 1 h. The 5% milk TBS-T solution was removed, and the membrane was washed three times for 5 min each with TBS-T solution. The membrane was then incubated in antibody solution for 1 h. The 4G8, A11, and 6E10 antibodies were diluted in 0.5% milk TBS-T solution according to the manufacturer's recommendation. After incubation, the membrane was washed three times for 5 min using TBS-T solution. When a peroxidase (HRP)-conjugated antibody (4G8) was used as the primary antibody, membranes were coated with 2 mL of detection agent from the ECL Advance Detection Kit (GE Healthcare, Waukesha, WI) and the fluorescence was visualized. Otherwise, the membrane was incubated

in (1:5000 dilution in 0.5% milk TBS-T) HRP-conjugated IgG for 1 h. Then the membrane was washed three times for 5 min each with TBS-T solution, and the same detection method as previously described was used. The blot images were captured using a BioSpectrum imaging system (UVP, Upland, CA).

ThT Fluorescence Assay. An amount of 5 μ L of 50 μ M A β 40 sample solution was diluted in 250 μ L of 10 μ M ThT (dissolved in PBS) in 96-well plates. The resulting ThT fluorescence of A β samples was measured at an emission wavelength of 485 nm using an excitation wavelength of 450 nm using a Synergy 4 UV-vis/fluorescence multimode microplate reader (Biotek, Winooski, VT).

TEM. A 10 μ L A β sample was adsorbed onto a Formvar mesh grid for 1 min. The grids were then negatively stained with 2% uranyl acetate for 45 s, blotted dry, and viewed on a Jeol JEM1230 transmission electron microscope at the Advanced Microscopy Laboratory at the University of Virginia operated at 80 kV.

A β -BBG Binding Assay. A saturation curve of BBG binding to A β was obtained according to the Bradford assay protocols described previously.^{63,85,86} A 0.05 g/mL stock solution of BBG was prepared by dissolving BBG powder in water. An acidified alcohol solution was prepared by combining 95% ethanol and 85% phosphoric acid in a 1:2 volumetric ratio. Then the modified Bradford reagent was created by adding 167 μ L of the BBG stock solution to 250 μ L of the acidified alcohol and the final volume was adjusted to 1 mL. For each sample, 50 μ L of the modified Bradford reagent was added to each well in a 96-well microplate. Next, 0–30 μ L of 1 mM A β monomer was added to each well, and the final volume was adjusted to 100 μ L with double distilled water. Absorbance of the samples was measured at 595 nm using a Synergy 4 UV-vis/fluorescence multimode microplate reader (Biotek, Winooski, VT). The number of BBG binding sites and the intrinsic binding constant were derived from the saturation binding curve. The detailed procedures are described in the Supporting Information.

MTT and Alamar Blue Reduction Assays. A total of 50 mg of MTT obtained from Millipore (Billerica, MA) was dissolved overnight at 4 $^{\circ}$ C in 10 mL of PBS. The MTT solution was then sterile filtered. Human neuroblastoma SH-SY5Y cells were cultured in a humidified 5% CO₂/air incubator at 37 $^{\circ}$ C in DMEM 12:1:1 modified media with 10% fetal bovine serum and 1% penicillin-streptomycin (ThermoFisher, Waltham, MA). A total of 25 000 SH-SY5Y cells were seeded into 96-well plates and incubated for 48 h. After incubation, the culture medium was replaced with 100 μ L of fresh media, and 10 μ L of the A β sample was added to each well to obtain a final A β concentration of 5 μ M. Cells were incubated for an additional 48 h. The media was then aspirated and replaced with 50 μ L of fresh media. Then 10 μ L of the sterile MTT solution was added, and cells were incubated for 6 h at 37 $^{\circ}$ C in the dark. After incubation, 200 μ L of DMSO was added to each well to dissolve the reduced MTT, and the absorbance was measured at 506 nm using a Synergy 4 UV-vis/fluorescence multimode microplate reader.

Alamar blue reduction assay was performed according to the protocol in the literature^{79–81} and the manufacturer's protocol (Invitrogen) with some modifications. A total of 10 000 SH-SY5Y cells were seeded at each well in a 96-well plate. The cells were incubated for up to 1 week. After incubation, old media were replaced with 100 μ L of fresh media, and 10 μ L of the sample containing A β with or without BBG was added to each well. The cells were incubated for 3 days, and the media was then replaced with 100 μ L of fresh media followed by addition of 40 μ L of a 10 \times alamar blue stock solution. Next, the cells were incubated for 4–6 h to allow the cells to metabolize the alamar blue. After incubation, fluorescence was measured using an emission wavelength of 590 nm using an excitation wavelength of 555 nm using a Synergy 4 UV-vis/fluorescence microplate reader (Biotek, Winooski, VT).

■ ASSOCIATED CONTENT

S Supporting Information. Figure S1 showing the BBG- $A\beta$ binding curve data fitted into a straight-line. Figure S2 showing the alamar blue reducing activities of cells treated with $A\beta$ samples incubated without BBG for 0 to 3 days or with $3\times$ BBG for 2 days. Figure S3 showing the dose-dependence of inhibition of ThT fluorescence of $A\beta$ samples by BBG. Figure S4 showing ThT fluorescence of preformed amyloid fibrils (72 h) mixed with varying concentrations of BBG immediately prior to addition of ThT. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

H.E.W. performed the experimental work, data analysis, and manuscript writing and preparation. W.Q. developed methods. H.-M.C. and E.J.F. provided guidance and advice. I.K. provided guidance and advice and also performed data analysis and manuscript writing and preparation.

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■ ABBREVIATIONS

AD, Alzheimer's disease; $A\beta$, amyloid-beta; BBG, Brilliant Blue G; BBF, Brilliant Blue FCF; BBR, Brilliant Blue R; ThT, Thioflavin T; DMSO, dimethyl sulfoxide; EC_{50} , half-maximal enhancement value; FDA, U.S. Food and Drug Administration; HRP, horseradish peroxidase; IC_{50} , half-maximal inhibitory value; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TBS-T, 0.1% Tween 20 in Tris-buffered saline; TEM, transmission electron microscopy

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