ACS Chemical Neuroscience
Research Article
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Comparison of Three Amyloid Assembly Inhibitors: The Sugar *scyllo*-Inositol, the Polyphenol Epigallocatechin Gallate, and the Molecular Tweezer CLR01

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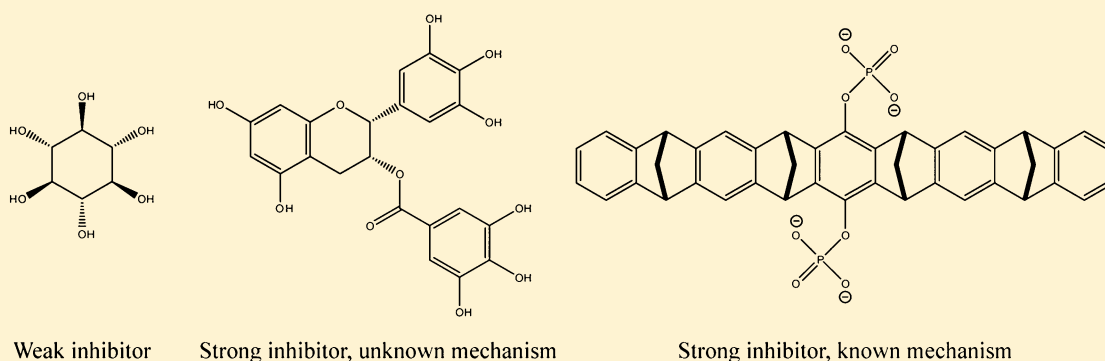

ABSTRACT: Many compounds have been tested as inhibitors or modulators of amyloid β-protein (Aβ) assembly in hope that they would lead to effective, disease-modifying therapy for Alzheimer's disease (AD). These compounds typically were either designed to break apart β-sheets or selected empirically. Two such compounds, the natural inositol derivative *scyllo*-inositol and the green-tea-derived flavonoid epigallocatechin gallate (EGCG), currently are in clinical trials. Similar to most of the compounds tested thus far, the mechanism of action of *scyllo*-inositol and EGCG is not understood. Recently, we discovered a novel family of assembly modulators, Lys-specific molecular tweezers, which act by binding specifically to Lys residues and modulate the self-assembly of amyloid proteins, including Aβ, into formation of nontoxic oligomers by a process-specific mechanism (Sinha, S., Lopes, D. H., Du, Z., Pang, E. S., Shanmugam, A., Lomakin, A., Talbiersky, P., Tennstaedt, A., McDaniel, K., Bakshi, R., Kuo, P. Y., Ehrmann, M., Benedek, G. B., Loo, J. A., Klarner, F. G., Schrader, T., Wang, C., and Bitan, G. (2011) Lysine-specific molecular tweezers are broad-spectrum inhibitors of assembly and toxicity of amyloid proteins. *J. Am. Chem. Soc. 133*, 16958−16969). Here, we compared side-by-side the capability of *scyllo*-inositol, EGCG, and the molecular tweezer CLR01 to inhibit Aβ aggregation and toxicity. We found that EGCG and CLR01 had comparable activity whereas *scyllo*-inositol was a weaker inhibitor. Exploration of the binding of EGCG and CLR01 to Aβ using heteronuclear solution-state NMR showed that whereas CLR01 bound to the two Lys and single Arg residues in Aβ monomers, only weak, nonspecific binding was detected for EGCG, leaving the binding mode of the latter unresolved.

KEYWORDS: *Alzheimer's disease, amyloid β-protein, epigallocatechin gallate, inhibitor, molecular tweezers, protein aggregation, scyllo-inositol*

Alzheimer's disease (AD) is the most common neurodegenerative disease. At present, ∼36 million people worldwide suffer from dementia, primarily caused by AD, and this number is predicted to rise to 115 million in 2050.[1] Despite tremendous research efforts, to date AD has no cure. Main challenges in AD research include identification of the neurotoxic agents that lead to neuronal injury and synaptic failure in the brain of affected individuals and understanding the mechanisms by which these agents work. The cause of AD is believed to be abnormal self-assembly of amyloid β-protein (Aβ) into neurotoxic oligomers and fibrillar polymers. Amyloid plaques and neurofibrillary tangles, the two hallmark lesions in the AD brain, comprise mainly Aβ and hyperphosphorylated tau fibrils, respectively.

Most researchers agree that effective therapy for AD must target the disease early, before overt neurodegeneration and brain atrophy develop. The current thought in the AD field is

Received: December 27, 2011
Accepted: March 7, 2012
Published: March 7, 2012

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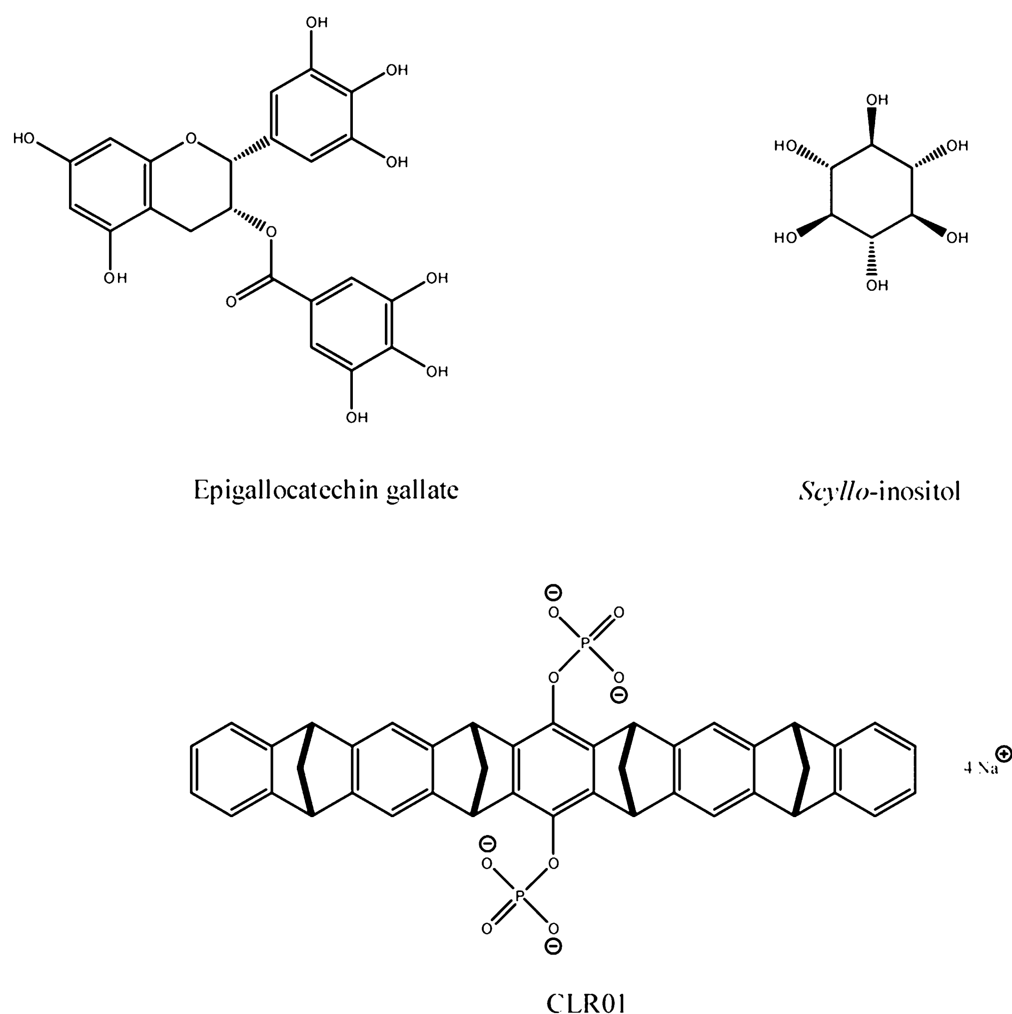

Figure 1. Schematic structures of EGCG, *scyllo*-inositol, and CLR01.

that the earliest pathogenic events are formation of toxic Aβ oligomers that disrupt synaptic communication before significant cell death occurs.[2−4] The role of tau oligomers is less well characterized, but recent data suggest that they are elevated in AD, appear in early pathological inclusions, such as neuropil threads and pretangle neurons, and colocalize with other early markers of tau pathogenesis.[5] Thus, inhibiting the formation of toxic Aβ and tau oligomers early may leave the brain with sufficient resources to restore lost synapses, providing a positive outlook for early treatment. Consequently, various inhibitors targeting Aβ and tau oligomerization have been developed in recent years, including curcumin,[6] amyloid-binding dyes,[7] polyphenols,[8,9] catechols,[10] and flavonoids.[11] In many cases, these compounds originated from common foods, such as turmeric, green tea, or red wine, and a major motivation for their selection has been their known safety. The disadvantage of this strategy is that the mechanism of action of such compounds and their mode of interaction with their targets are unknown, complicating further development. Interestingly, some of the inhibitors, for example, methylene blue, have been shown to accelerate Aβ fibrillogenesis,[9,12] whereas other compounds, including the sugar derivative *scyllo*-inositol,[13] the polyphenols (−)-epigallocatechin-3-gallate (EGCG)[14] and resveratrol,[8] C-terminal fragments of Aβ42,[15] and molecular tweezers (MTs)[16] were found to stabilize nontoxic oligomers.

Several small molecule inhibitors/modulators of Aβ and tau assembly are in clinical or preclinical development for AD.[17] Recent failure of a number of clinical trials emphasizes both the challenge and the urgency to develop a better understanding of the molecular mechanisms that cause disease and design more compounds and improved trials.[18] Two assembly modulators, *scyllo*-inositol (ELN005) and EGCG (Sunphenon) (Figure 1), presently are in phase 2 clinical trials for AD. Each compound has been shown to be efficacious in animal models, yet the mechanism of action of these compounds is not well understood and safety concerns have been raised for each.[19−21]

Recently, we have reported that the MT derivative CLR01 (Figure 1) was an effective inhibitor of the self-assembly and toxicity of several disease-associated amyloid proteins.[16] In addition, CLR01 was found to protect primary neurons from Aβ42-induced synaptotoxicity and to ameliorate brain pathology, including Aβ and tau burden, in the brain of transgenic AD mice.[22] Relative to other inhibitors, most of which have been found empirically, the putative mechanism of action of CLR01 is quite well understood. CLR01 binds to Lys residues with micromolar affinity[16,23] and interferes with a combination of hydrophobic and electrostatic interactions that are important in the self-assembly of most amyloidogenic proteins, including Aβ[24−26] and tau.[27−30] This mode of action is a novel, process-specific mechanism plausibly applicable to most amyloidogenic proteins. Structural investigation using electron capture

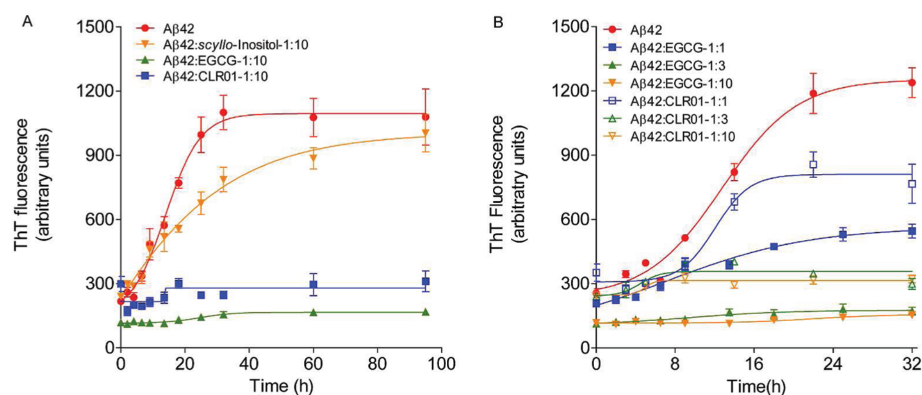

Figure 2. Inhibition of Aβ42 β-sheet formation. 10 μM Aβ42 was incubated at room temperature with mechanical agitation in the absence or presence of each inhibitor and β-sheet formation was measured using the ThT assay. (A) The effect of *scyllo*-inositol, EGCG and CLR01 was measured at 1:10 Aβ/inhibitor concentration ratio. (B) The effect of EGCG and CLR01 was measured at 1:1, 1:3, or 1:10 Aβ/inhibitor concentration ratio. The data are presented as mean ± SEM of three independent experiments.

dissociation mass spectrometry and solution-state NMR has confirmed that CLR01 indeed binds to the two Lys residues and to a lesser extent to the single Arg residue, already in Aβ monomers.[16]

Here, we asked how the activity of the artificial Lys-receptor, CLR01, which was explored as an inhibitor of amyloid proteins' assembly based on a mechanistic rationale, compared with those of the natural compounds, *scyllo*-inositol and EGCG. On the basis of the distinct structures of these three compounds (Figure 1), it is unlikely that they have a similar mode of interaction with Aβ. We aimed to compare these three compounds side-by-side and advance our understanding of the way they interact with Aβ, modulate its assembly, and inhibit its toxicity.

RESULTS AND DISCUSSION

We compared first the capability of the three compounds to inhibit Aβ42 aggregation using the thioflavin T (ThT) fluorescence assay. ThT is a dye which shows enhanced fluorescence upon binding to β-sheet rich aggregates.[31] Ten μM aggregate-free Aβ42 were allowed to aggregate in the absence or presence of different concentrations of each inhibitor. In Aβ42 samples incubated in the absence of inhibitors, following a ~3 h lag phase, ThT fluorescence increased gradually until it reached a plateau by ~30 h and remained unchanged up to 96 h (Figure 2A). In contrast, in the presence of 10-fold excess EGCG or CLR01, little or no change in ThT fluorescence intensity was observed, indicating inhibition of β-sheet formation. Under the same conditions, in the presence of 10-fold excess *scyllo*-inositol, the ThT fluorescence increased monotonously without an apparent lag phase, though the rate of the fluorescence increase was slower than that of Aβ42 alone. This behavior suggested that, under the conditions used, *scyllo*-inositol might have accelerated nucleation but interfered with Aβ42 fibril elongation. Dose-dependence experiments using EGCG and CLR01 (Figure 2B) showed that both compounds inhibited Aβ42 β-sheet formation completely at 3-fold excess and partially at a 1:1 concentration ratio. EGCG was more effective than CLR01 at 1:1 concentration ratio (Figure 2B). Interestingly, the initial ThT fluorescence at t = 0 in samples containing Aβ42/EGCG mixtures at 1:3 or 1:10 concentration ratios was lower than that in all other samples, suggesting that excess EGCG might quench ThT fluorescence in addition to its effect on Aβ assembly.

As the most toxic species involved in AD pathogenesis are believed to be soluble Aβ oligomers, we compared next the capability of the inhibitors in arresting Aβ oligomerization. Aβ42 oligomers prepared according to Necula et al.[7] and incubated in the absence or presence of each inhibitor were applied to nitrocellulose membranes at different time points and probed by a dot-blot assay using the oligomer-specific polyclonal antibody (pAb) A11.[32] Identical membranes were probed using the Aβ-specific monoclonal antibody (mAb) 6E10 as a loading control. In the absence of inhibitors, A11 immunoreactivity was observed already at t = 0 h and increased up to 8 days (Figure 3). In contrast, Aβ42 samples incubated in the presence of EGCG or CLR01 did not show A11 reactivity at any of the time points, suggesting that each of these compounds inhibited formation of the toxic oligomers recognized by A11. As opposed to CLR01 or EGCG, the immunoreactivity of samples incubated with *scyllo*-inositol followed a similar trend to the control samples (Figure 3) suggesting that formation of toxic oligomers was not inhibited.

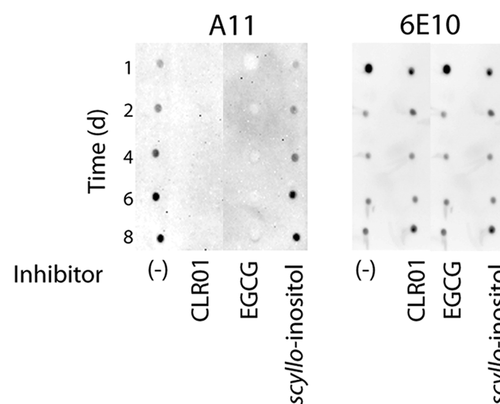

Figure 3. Inhibition of Aβ42 oligomerization. Aβ oligomerization in the presence or absence of *scyllo*-inositol, EGCG, or CLR01 was probed using dot blots with polyclonal antibody A11. Identical membranes were probed using monoclonal antibody 6E10 as a loading control.

To compare the ability of the three compounds to block Aβ42 toxicity, we treated differentiated rat pheochromocytoma

dx.doi.org/10.1021/cn200133x | *ACS Chem. Neurosci.* 2012, 3, 451−458

(PC-12) cells, primary hippocampal neurons, or mixed primary hippocampal neuronal/microglial cultures with 10 μM Aβ42 in the absence or presence of each compound and measured cell death using the lactate dehydrogenase (LDH) release assay. Aβ42 was found to induce, 30.5 ± 2.2%, 28.0 ± 1.8%, and 19.7 ± 2.1% cell death in the PC-12 cells, primary neurons, and mixed culture, respectively (Figure 4). In PC-12 cells, CLR01

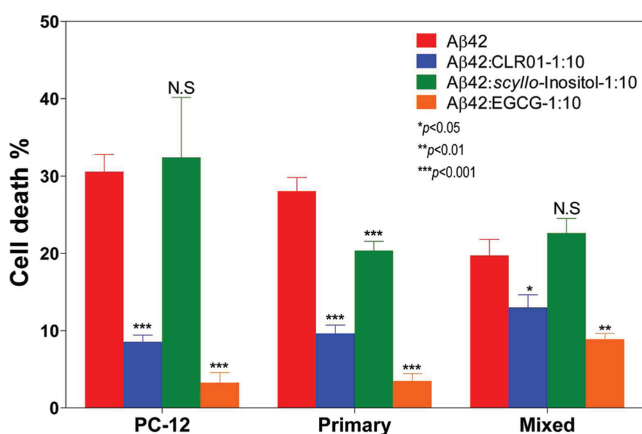

Figure 4. Inhibition of Aβ42-induced cell death in different cell types. 10 μM Aβ42 was added to differentiated PC-12 cells, primary rat hippocampal neurons, or primary rat hippocampal neurons mixed with glial cells in the absence or presence of 10-fold excess of each inhibitor. Cells were incubated with the peptide/inhibitor mixtures for 48 h, and cell death was measured using the LDH release assay. The data are presented as mean ± SEM for three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the Aβ42 in each group.

and EGCG reduced cell death to 8.5 ± 0.9% and 3.3 ± 1.3%, respectively, whereas *scyllo*-inositol did not show a protective effect and slightly increased the toxicity to 32.4 ± 9.5% (Figure 4). Similarly, in the primary neurons, 10-fold excess CLR01 or EGCG reduced the cell death level to 9.6 ± 1.1% and 3.5 ± 1.0%, respectively, whereas *scyllo*-inositol offered a weak rescue effect and reduced cell death to 20.4 ± 1.2% at the same concentration. In the mixed cultures, CLR01 and EGCG reduced Aβ42-induced cell death to 13.0 ± 1.6% and 8.9 ± 0.7%, respectively, whereas samples incubated in the presence of *scyllo*-inositol showed 22.6 ± 1.9% cell death, slightly higher than the control cultures incubated with Aβ42 alone. Overall, EGCG was the strongest inhibitor of Aβ42-induced toxicity in the three cell types and *scyllo*-inositol the weakest. All three cell types showed the same trend. Differentiated PC-12 cells appeared to be the most sensitive and the mixed culture the least sensitive to Aβ-induced toxicity. The lower toxicity of Aβ42 in the mixed culture was significantly different ($p < 0.05$) from both the PC-12 cells and the primary neurons, whereas the difference between the PC-12 cells and primary neurons was statistically insignificant.

Previously, we showed that CLR01 bound to Aβ40 at distinct sites, Lys16, Lys28, and to a low extent Arg5,[16] consistent with its putative mechanism of action. Because EGCG showed similar or stronger inhibitory effects, we asked whether it bound to similar sites on Aβ. To answer this question, we probed the binding of these two compounds with Aβ40 using solution-state NMR. We left *scyllo*-inositol out in the NMR experiments because it was substantially less effective than CLR01 or EGCG in inhibiting Aβ42 self-assembly and toxicity. The NMR experiments were conducted with full-length Aβ40 because of its higher aqueous solubility and increased sample stability relative to Aβ42.[33] The concentration of Aβ40 was kept at 60 μM and ^{1}H−^{15}N heteronuclear single quantum coherence (HSQC) 2D-NMR spectra were measured in the absence or presence of EGCG or CLR01 concentrations increasing from 30−240 μM. At this concentration, Aβ40 exists as a mixture of monomers and small oligomers;[34,35] nonetheless, the NMR signals reflect monomers only.[33]

As reported previously, CLR01 caused major chemical shift changes at all concentration ratios (ref 16 and Figure 5). At low CLR01 concentrations, these changes occurred predominantly around the three cationic bindings sites, and as the concentration of CLR01 increased, gradually the entire spectrum was affected (Figure 5B), likely due to Aβ self-assembly into nontoxic oligomers.[16] In contrast, only slight resonance perturbation was found in Aβ40:EGCG spectra at ratios up to 1:4 compared to Aβ40 alone (Figure 5A and B). The resonances affected the most were in the regions Aβ(11−15), Aβ(16−23), and Aβ(31−33), yet due to the low magnitude of the perturbation these data are difficult to interpret.

Most of the compounds tested as potential inhibitors or modulators of Aβ self-assembly and/or toxicity have been found empirically, and their mode of action largely is unknown. The importance of understanding the mechanism of inhibition has been highlighted[36,37] following evidence suggesting that many small molecule inhibitors of fibrillogenesis might act nonspecifically, likely making them unsuitable for treating amyloid-related disorders.[38] Inhibition of fibril formation or dissociation of existing fibrils actually may yield toxic oligomers under certain circumstances.[39] Importantly, understanding the details of target−drug interaction is essential not only for subsequent drug development but also for preventing potential side effects.

EGCG and *scyllo*-inositol presently are in clinical trials for AD. These compounds are “nutraceuticals,” suggesting that they would be safe for human use, though concerns do exist. *scyllo*-Inositol was reported to cause nine deaths in the high-dose groups in a recent phase 2A trial, and the trial continues now with the low-dose groups only.[19] EGCG doses needed for efficacy in mouse models were close to toxic doses,[20] and recently the compound was reported to promote formation of toxic tau oligomers.[21]

Our in vitro assembly studies indicate that both EGCG and CLR01 inhibit formation of the toxic Aβ42 oligomers recognized by antibody A11 (Figure 3) and of β-sheet (Figure 2). Under the same conditions, *scyllo*-inositol does not inhibit formation of A11-positive oligomers (Figure 3), and appears to accelerate the nucleation and delay the elongation step of Aβ42 fibrillogenesis (Figure 2), without completely stopping the process. Each of the three compounds is believed to modulate the assembly of Aβ into formation of nontoxic structures.[40] Our data suggest that the concentration of *scyllo*-inositol required for this modulation is substantially higher than those of CLR01 and EGCG. Thus, consistent with the oligomerization and aggregation data, cell death experiments using three different types of cell cultures showed significant inhibition of Aβ42-induced toxicity by CLR01 and EGCG but only mild effects of *scyllo*-inositol under the same conditions (Figure 4).

In contrast to the strong effect of CLR01 on resonances around the predominant binding sites previously shown for the compound by multiple NMR experiments, the two Lys and the Arg residues already at CLR01/Aβ40 concentration ratio 1:10,

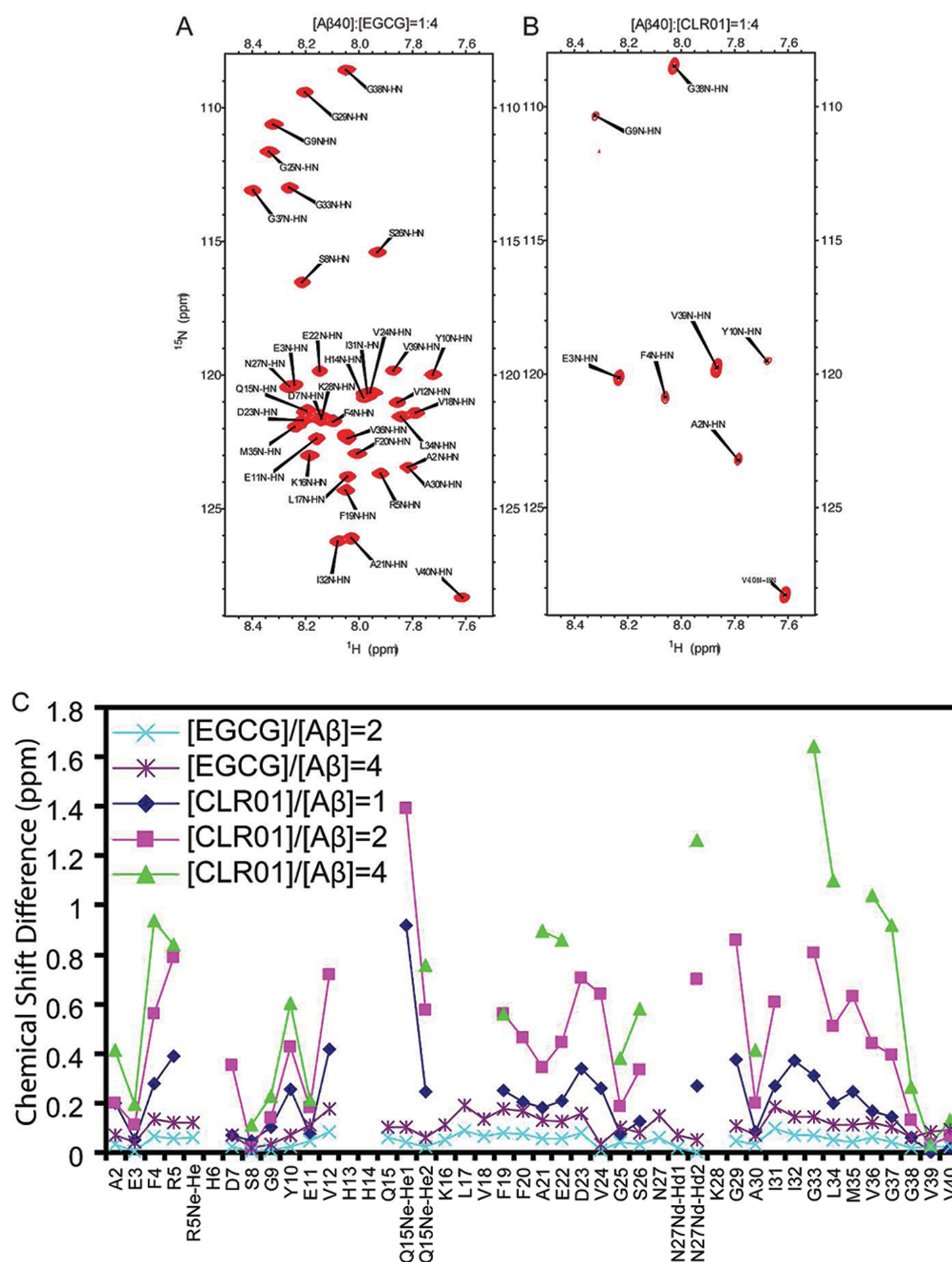

Figure 5. ^{15}N−^{1}H HSQC spectra of Aβ40:EGCG or Aβ40:CLR01 mixtures. (A) ^{15}N−^{1}H spectra of 60 μM Aβ40 in the presence of 240 μM EGCG. (B) ^{15}N−^{1}H spectra of 60 μM Aβ40 in the presence of 240 μM CLR01. (C) Degree of chemical shift change in individual backbone-amide protons and side-chain amide/guanidine protons along the sequence of Aβ40 upon addition of increasing concentrations of EGCG or CLR01.

respectively,[16] only weak interaction of EGCG with Aβ40 monomers was observed at concentration ratios up to 1:4, respectively (Figure 5). At this concentration ratio, the highest ratio studied, the maximum chemical shift induced by EGCG was ∼8 times smaller than the equivalent chemical shift changes in the Aβ40 spectrum in the presence of a similar concentration of CLR01 (Figure 5B). These findings are in line with those made for the interaction of EGCG with α-synuclein by Ehrnhoefer et al., where observation of major resonance perturbations required 5−10-fold excess of EGCG.[41] Our data suggest that, unlike CLR01, which binds to Aβ monomers, EGCG binding may occur at later stages in the assembly process. The binding site(s) for EGCG appear to be less well-defined than those of CLR01, consistent with previous reports.[42,43] It is also possible that EGCG exerts its strong protective effect via interaction with alternative cellular targets rather than with Aβ itself as the compound is known to be an antioxidant and to provide cell protection through modulation of signal transduction pathways, cell survival/death genes, and mitochondrial function[44,45] similar to other polyphenols.[46]

Our comparison of the sugar *scyllo*-inositol, the polyphenol EGCG, and the molecular tweezer CLR01 suggests that the latter two compounds are substantially more efficient inhibitors of Aβ assembly and toxicity than the former. Similar to CLR01,[16] EGCG has been shown to inhibit the assembly and/or toxicity of multiple amyloidogenic proteins, including Aβ, α-synuclein, huntingtin, islet amyloid polypeptide, and transthyretin.[41,47−49] It is therefore of great interest to decipher the mode of interaction of EGCG with these proteins. However, whereas solution-state NMR techniques provided strong

evidence for the binding mode of CLR01 to Aβ monomers, only weak interactions were found between Aβ and EGCG, leaving the mode of action of EGCG unresolved.

METHODS

Chemicals. CLR01 was prepared and purified as described previously.[16] *scyllo*-Inositol and EGCG were purchased from Sigma (St. Louis, MO).

Protein and Sample Preparation. Aβ42 was purchased from the UCLA Biopolymers Laboratory and was disaggregated by treatment with 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Sigma, St. Louis, MO) as described previously.[50] Dry peptide films were stored at −20 °C until use. For assembly inhibition experiments, the films were dissolved in a minimal volume of 60 mM NaOH followed by dilution with deionized water (18.2 MΩ produced using a Milli-Q system, Millipore, Billerica, MA) to half the final volume and then sonicated for 1 min using a model 1510 bath sonicator (Branson, Danbury, CT). Samples then were diluted to the final volume with phosphate buffer (PB: 20 mM sodium phosphate (Sigma), pH 7.4, containing 0.01% (w/v) sodium azide (Sigma) to prevent bacterial growth). Stock solutions of each inhibitor were prepared at 10 mM in Milli-Q water and diluted into the peptide solutions at the required concentration.

Thioflavin T Fluorescence. Ten μM Aβ42 solutions were incubated in the absence or presence of different concentrations of each inhibitor at room temperature with mechanical agitation, and ThT fluorescence was monitored periodically as described previously.[16]

Dot Blot Assay. Oligomers of Aβ42 were prepared, incubated in the absence or presence of each inhibitor, and probed by pAb A11 or mAb 6E10 as described previously.[16]

Cell Culture. Experiments were compliant with the National Research Council Guide for the Care and Use of Laboratory Animals, approved by the UCLA Institutional Animal Care Use Committee. Primary neurons were prepared from E18 Sprague−Dawley rat embryos as described previously.[51] Mixed cultures were grown under similar conditions without using arabinofuranoside to allow growth of glial cells. Differentiated PC-12 cells were prepared as described previously.[15]

Lactate Dehydrogenase Assay. Cell death was measured using the LDH release assay as described previously.[15] Briefly, cells were plated at a density of 20 000 cells per well using 96-well plates in 90 mL of fresh medium and incubated for 24 h. Aβ was solubilized in a minimal volume of 60 mM NaOH, diluted in F12K media in the absence or presence of different concentrations of each inhibitor, added to the cells, and incubated for 48 h at 37 °C. The final concentration of NaOH in the media was <0.6 mM. Cytotoxicity was measured using CytoTox ONE kits (Promega, Madison, WI).

NMR Spectroscopy. *Aβ40 Sample Preparation.* Lyophilized, uniformly ^{15}N-labeled Aβ40 (rPeptide, Bogart, GA) was suspended in 10 mM NaOH at a concentration of 2 mg/mL and sonicated for 1 min for disaggregation. This solution (60 μL) was diluted to 60 μM in 345 μL of 20 mM PB, pH 7.2, and 45 μL of D_2O.

NMR Spectroscopy and Titration. NMR experiments were carried out at 4 °C using a Bruker 600 MHz spectrometer equipped with a triple-resonance cryogenic probe. NMR data were processed using NMRPipe[52] and analyzed using SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, http://www.cgl.ucsf.edu/home/sparky/). ^{1}H−^{15}N HSQC spectra were acquired with 2048 (t_2) × 180 (t_1) complex data points, spectral widths of 7211 Hz in ^{1}H and 1581 Hz in ^{15}N, and 8 scans for each free induction decay.

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Author Contributions

S.S. designed and conducted experiments, analyzed data and wrote manuscript. Z.D. and P.M. conducted experiments and analyzed data. F.G.K. and T.S. supplied materials. C.W. analyzed data. G.B. conceived of research, designed experiments, and wrote manuscript.

Funding

The study was supported by American Health Assistance Foundation Grant A2008-350, Alzheimer Association Grant IIRG-07-58334, and the UCLA Jim Easton Consortium for Alzheimer's Drug Discovery and Biomarker Development.

Notes

The authors declare the following competing financial interest(s):S. Sinha, T. Schrader, F.-G. Klarner, and G. Bitan are co-inventors of International Patent Application Serial No. PCT/US2010/026419, USA Patent Application No. 13/203,962, European Patent Application 10 708 075.6, Molecular Tweezers for the Treatment of Amyloid-Related Diseases. G. Bitan is a co-founder and a director of Clear Therapeutics, Inc.

ACKNOWLEDGMENTS

We thank Dr. David Teplow for the use of his fluorescence spectrometer and plate reader.

ABBREVIATIONS

Aβ, amyloid β-protein; AD, Alzheimer's disease; EGCG, (−)-epigallocatechin-3-gallate; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; HSQC, heteronuclear single quantum coherence; LDH, lactate dehydrogenase; MT, molecular tweezer; mAb, monoclonal antibody; pAb, polyclonal antibody; ThT, thioflavin T

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