A Sensitive Aβ Oligomerization Assay for Identification of Small Molecule Inhibitors

Amanda M. Gonzales and Robert A. Orlando*

Department of Biochemistry and Molecular Biology, University of New Mexico, School of Medicine, MSC08 4670, 1 University of New Mexico, Albuquerque, New Mexico 87131, USA

Abstract: Amyloid deposits found in Alzheimer's disease result from aggregation of Aβ peptide which leads to loss of synaptic function, chronic microglial activation and cognitive impairment. Because of this, identification of small molecule inhibitors of Aβ aggregation as potential therapeutics is a topic of current interest. The majority of inhibitor screening approaches rely on *in vitro* assays that lack the necessary sensitivity to distinguish low-molecular weight Aβ oligomers from larger, more advanced-stage fibrillar structures. Differentiating between these two structures is of vital concern since recent studies indicate that small, early-stage Aβ oligomers are the most neurotoxic form of peptide aggregate. To address this limitation, we have explored the adaptability of a recently described ELISA-based assay for discovery of small molecule inhibitors of Aβ oligomerization. Results show that this assay is highly sensitive as it is able to quantify Aβ oligomers with as little as 80 nM input peptide. In addition, data were obtained re-confirming the function of curcumin as a potent inhibitor of Aβ aggregation (IC_{50} = 2 μM) and defining its inhibitor:peptide functional stoichiometry. Further examination of other known anti-aggregation compounds showed that this assay is able to discriminate between inhibitors of early-stage, low-molecular weight oligomers and later-stage, high-molecular weight fibrillar structures. These findings indicate that this new ELISA-based assay is capable of identifying novel small molecule inhibitors that function during the initial stages of Aβ peptide assembly.

Keywords: Alzheimer, amyloid, oligomer, inhibitor, curcumin, EGCG.

INTRODUCTION

Histological examination of hippocampal and neocortical tissues from individuals afflicted with Alzheimer's disease reveals a hallmark feature of this progressive disorder, β-amyloid plaques. It is now well established that Aβ peptide is a major component of these focal deposits. The molecular events leading to amyloid deposition are still under intense investigation; however, evidence is accumulating that suggests plaque formation is a result of a perturbation in the normal equilibrium of Aβ peptide formation and peptide clearance [1-3]. The Aβ peptide originates from what is believed to be normal processing of the amyloid precursor protein (APP). APP, a transmembrane protein, is cleaved in two successive proteolytic reactions to release Aβ peptide, which is either 40 or 42 amino acids in length depending on its intramembrane cleavage site. Once formed, it is thought that Aβ is cleared through normal drainage function of the cerebral spinal fluid (CSF) [4-6]. Aβ-related pathologies develop when free peptide, once reaching a critical concentration, forms insoluble oligomers which seed further aggregation eventually leading to the formation of characteristic amyloid lesions. Although amyloid plaques certainly contribute to neuronal damage in AD patients, recent evidence has suggested that early stage, low molecular weight forms of Aβ oligomers inhibit hippocampal long-term potentiation and may in fact be the most neurotoxic form of Aβ aggregates [7-10]. Because of this, reducing Aβ oligomer load to maintain or restore cognition has become a clinically important objective.

Reducing or preventing deposition of Aβ oligomers can be approached from several directions: by reducing the processing of APP to slow the generation of Aβ peptide, by increasing Aβ peptide clearance, or by inhibiting, or slowing, the rate of peptide aggregation. The final proteolytic step to generate Aβ peptide involves the cleavage of APP by gamma-secretase. A number of secretase inhibitors have been identified through extensive drug screening and are now being examined for efficacy in late-Phase clinical trials [11]. Increasing the rate of Aβ peptide clearance has been explored through the use of low-flow, ventriculoperitoneal shunts [12], originally designed to increase drainage of CSF for individuals with hydrocephalus [13]. Although a practical approach in theory, recent evidence has cast doubts if any benefits can be obtained for Alzheimer patients with this therapy [14]. Alternatively, prevention of Aβ aggregation has been explored through the identification of small molecule inhibitors [15]. From these efforts, a number of useful lead compounds have been identified such as sulfonated anions, benzofuran derivatives, as well as other polyphenol-based compounds [16-20]. Evaluations of these lead compounds have utilized various methodologies including differential centrifugation, light scattering, fluorescent spectral shifts and SDS-PAGE mobility shifts [21], all of which measure large molecular aggregates of Aβ peptide. Although these assays have provided useful data for novel lead compound identification, they also harbor a number of critical

*Address correspondence to this author at the Department of Biochemistry and Molecular Biology, University of New Mexico, School of Medicine, MSC08 4670, 1 University of New Mexico, Albuquerque, New Mexico 87131, USA; Tel: 505-272-5593; Fax: 505-272-3518;
E-mail: rorlando@salud.unm.edu

1874-0707/09

constraints that limit their application in large-scale, semi- or high-throughput screening of small molecule chemical libraries. Also, because measurements are restricted to large molecular aggregates, these methods are less apt to identify inhibitors of early stage, low-molecular weight Aβ peptide oligomers. The most notable drawback of these assays is their limited sensitivities. Aβ oligomerization is a concentration dependent reaction, and for these assays to provide reproducible detection of this reaction, starting Aβ peptide concentrations of 1-100 μM are very often needed to drive the oligomerization process. Questions have arisen asking if these *in vitro* reactions are truly reflective of Aβ oligomerization *in vivo* since it is unlikely that localized brain levels of Aβ peptide would ever reach these concentrations [22-25].

Another important consideration for inhibitor identification studies is mechanism of action. While the identity of a number of Aβ oligomerization inhibitors have been reported, few address the stoichiometric relationship between inhibitor and peptide, and when calculated, suggest inhibitor to peptide ratios of no greater than one, or in some cases less than one [26-32]. Presuming that inhibitors must physically bind to peptide to prevent oligomerization [33, 34], it is difficult to imagine a mechanism of action where inhibitor to peptide ratios are less than 1. Such questionable stoichiometric ratios could be due to the high concentrations of Aβ peptide needed in these assays to initiate detectable oligomerization. These problems could be solved with an assay that requires a lower starting concentration of peptide and bears greater sensitivity of detection.

To address these challenges, we have explored the use of a highly sensitive ELISA-based method for detecting Aβ oligomers for the purpose of identifying novel small molecule inhibitors of Aβ aggregation. Based upon previous methods developed by LeVine [35] and El-Agnaf [36], the sensitivity of this approach permits the use of sub-μM concentrations of Aβ peptide for initiation of the oligomerization reaction and more closely reflects amounts that can be expected *in vivo*. Most importantly, using lower peptide concentrations will focus inhibitor identification assays on the early stages of oligomer formation to more precisely target the most neurotoxic form of Aβ aggregates [36]. And finally, an ELISA-based approach should not only allow for more cost effective screening of large chemical libraries, but should also permit a more accurate assessment of peptide:inhibitor stoichiometry for future studies aimed at identifying mechanism of action.

MATERIALS AND METHODOLOGY

Reagents and Materials

1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), dimethylsulfoxide (DMSO), fraction V bovine serum albumin and all buffer reagents were obtained from Sigma-Aldrich (St. Louis, MO). Tetramethylbenzidine (TMB) was purchased from Roche (Indianapolis, IN). Human Aβ(1-42) was purchased from AnaSpec (San Jose, CA). NUNC MaxiSorp ELISA plates were obtained from eBioscience (San Diego, CA). Monoclonal antibody 4G8 specific for human Aβ amino acids 17–24 (Fig. **1A**) and horseradish peroxidase (HRP)-conjugated 4G8 were purchased from Signet Labs (Dedham, MA). Curcumin, ferulic acid, vanillin, rosmarinic acid, catechin, epigallocatechin gallate (EGCG) and resveratrol were purchased from Cayman Chemicals (Ann Arbor, MI).

Preparation of Monomeric Aβ(1-42) Peptide

Aβ(1-42) peptide was dissolved in HFIP [35, 37] to a final concentration of 4 mg/ml and divided into 500 μg aliquots. Aliquots were dried under a stream of sterile N_2 and stored at -20°C until use. Immediately preceding each experiment, aliquots were dissolved in DMSO to a final concentration of 1 mM. To ensure peptide was fully dissolved, solutions were sonicated for 15 min followed by heating at 60°C for an additional 15 min. Any unused peptide was discarded.

Aβ Peptide Oligomerization Reactions

Aβ peptide from DMSO stock was diluted to the indicated concentrations either into phosphate buffered saline, pH 7.2 (PBS) alone or into test compound, pre-diluted into PBS. Stock solutions of all test compounds were made with DMSO for solvent compatibility. Dilutions were large enough to ensure that final DMSO concentrations were consistently <1% in the reaction mix. Reactions were incubated at 37°C for 24 h and then processed for capture ELISA.

Aβ oligomer reactions were fractionated by 12% polyacrylamide gel electrophoresis (PAGE) by mixing reactions with an equal volume of 2X native sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 0.02% bromophenol blue) and employing a standard Tris-Glycine buffer system without sodium dodecyl sulfate. After separation, gels were silver stained using the Morrissey method [38] and photographed with a Nikon D90 SLR digital camera.

Capture ELISA for Aβ Oligomer Detection

NUNC Maxisorp high-binding ELISA plates were coated with mAb 4G8 diluted to 2 μg/ml in PBS for a minimum of 16 h at 4°C. After rinsing plates with PBS and blocking non-specific sites with PBS-T/B (PBS containing 0.1 % Tween-20, 1% bovine serum albumin) for 1.5 h, Aβ peptide oligomerization reactions were added to wells and incubated with immobilized capture mAb for 2 h. Wells were rinsed three times with TBS-T (20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween-20) using a Biotek ELx50 automated plate washer. HRP-conjugated mAb 4G8 was added to wells at 1 μg/ml diluted into PBS-T/B and incubated at 23°C for 1 h. Unbound secondary antibody was removed by rinsing three times with TBS-T and bound antibody was measured following addition of TMB reagent. TMB reaction was terminated after ~10 min with the addition of an equal volume of 1 M H_2SO_4. Absorbance was recorded at 450 nm with a reference wavelength of 650 nm using a Molecular Devices SpectraMax 384 Plus plate reader.

Statistical Analyses

All experimental protocols were carried out in at least triplicate points to determine mean values. Error bars represent standard deviation from mean values.

RESULTS

The Aβ oligomerization assay presented here is directly adapted from the method published by LeVine [35]. The principle of this assay employs the use of one anti-Aβ monoclonal antibody as both capture- and detection-antibody for

single epitope recognition (Fig. **1A**). With this format, monomeric peptide will be captured by the immobilized mAb, but not detected by the same mAb since the epitope is already occupied (Fig. **1B**). By contrast, the detection mAb will identify multimeric peptide complexes due to the availability of unoccupied epitopes. Inhibitors of oligomerization are identified simply by a reduction or loss of detection-antibody binding.

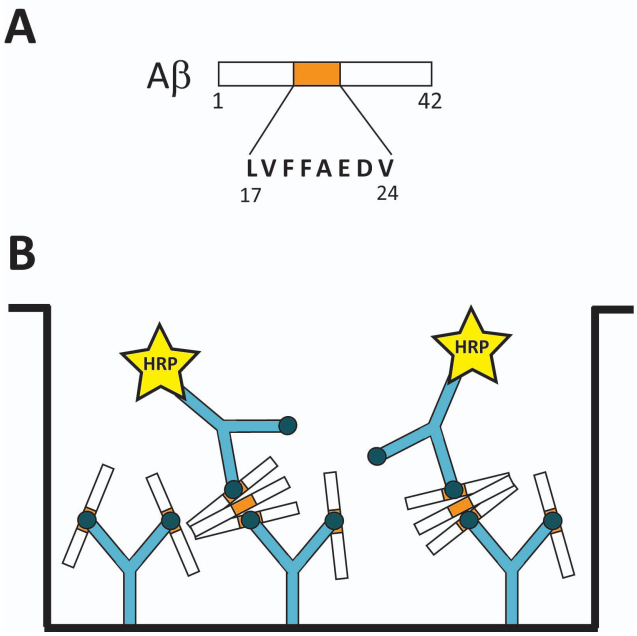


Fig. (1). Principle of the ELISA-based Aβ oligomer detection assay. (**A**) Human Aβ(1-42) and location of the mAb 4G8 epitope (amino acids 17-24). (**B**) Total Aβ peptide mixture (monomers and oligomers) are incubated with immobilized capture mAb 4G8. Both monomers and oligomers are able to bind to immobilized mAb 4G8. Detection of bound peptide is made with horseradish peroxidase (HRP)-conjugated mAb 4G8 recognizing the same epitope as the capture antibody. With this configuration, the 4G8 epitope within the monomeric peptide will be occupied by the 4G8 capture antibody and only multimeric complexes with additional 4G8 epitopes that are not engaged by the capture antibody will be bound by the detection antibody and provide a signal.

The first objective was to test the sensitivity of this assay by determining the lowest input amount of Aβ peptide necessary to form measurable levels of Aβ oligomers. For this, Aβ peptide was diluted to concentrations across 3-log units (10 nM to 10 μM) to assess the dynamic range of the assay. Oligomers were identified with as little as 80 nM input Aβ peptide (Fig. **2**). Oligomer formation was clearly dependent upon peptide concentration and reached maximal levels over a single order of magnitude of input peptide indicating a rapid first-order reaction. Based on these data, 200 nM input Aβ peptide concentration was chosen for all subsequent assays. Formation of low molecular weight oligomers with this concentration was confirmed by polyacrylamide gel electrophoresis followed by silver staining (Fig. **2**, inset).

The capacity of this ELISA-based assay to identify small molecule inhibitors of Aβ oligomerization was next tested using the polyphenolic natural product, curcumin. Curcumin

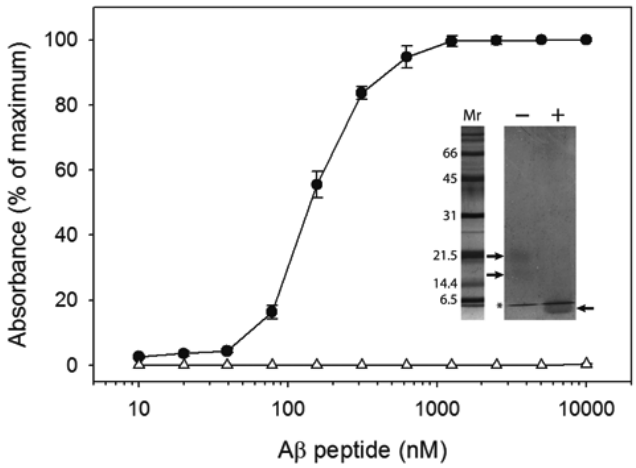


Fig. (2). Aβ oligomerization quantified by capture ELISA. Aβ(1-42) peptide was diluted to the indicated concentrations in the absence (●) or presence (Δ) of 0.1% Tween-20 and incubated at 37°C for 24 h. Tween-20 has been shown to inhibit Aβ oligomerization and provide a negative control [35]. Aβ oligomers were detected by capture ELISA; unconjugated mAb 4G8 was used as immobilized capture antibody, HRP-conjugated mAb 4G8 was used as detection antibody. Absorbance values shown (% of maximum) were calculated as percent of absorbance measured with 10 μM input Aβ(1-42) peptide; this being the maximum absorbance recorded for this assay. Mean values were determined from six replicates. Error bars represent standard deviation from mean values. Inset, 200 nM Aβ peptide was incubated as above in the absence (−) or presence (+) of 0.1% Tween-20 and separated by PAGE followed by silver staining. Arrows indicate presence of low molecular weight oligomers (−) or monomeric peptide (+). Asterisk indicates the bromophenol dye front.

has recently been shown to be a potent inhibitor of Aβ oligomerization both *in vitro* and *in vivo* [29]. Aβ peptide was diluted directly into various concentrations of curcumin to a final concentration of 200 nM and incubated at 37°C for 24 h. Oligomers were then quantified by capture ELISA. Curcumin was highly effective in inhibiting Aβ oligomerization in a dose-dependent manner (Fig. **3**). The IC_{50} value was calculated as 2 μM, which is in agreement with previously published values [29, 39].

The therapeutic benefits of curcumin as a dietary supplement are still debated largely because of its poor bioavailability [40, 41] and its propensity to be chemically unstable in an aqueous environment; degrading into ferulic acid and vanillin [42, 43]. Inquiries have been made into whether these products are able to mimic the anti-aggregation activity of curcumin. Byeon, *et al.* showed that ferulic acid can bind with reasonably high affinity to large β-amyloid fibrils [44]. Ferulic acid is also capable of destabilizing fibrillar structures, in addition to preventing fibril extension [45]. In contrast to results with ferulic acid, vanillin has been shown to inhibit formation of lower-molecular weight oligomers [46]; although concentrations of Aβ peptide (45 μM) and vanillin (300 μM) used in this study far exceeded what would be expected *in vivo* making a physiologic interpretation difficult. To re-examine the inhibitory capacity of ferulic acid and vanillin using an assay with greater sensitivity, Aβ oli-

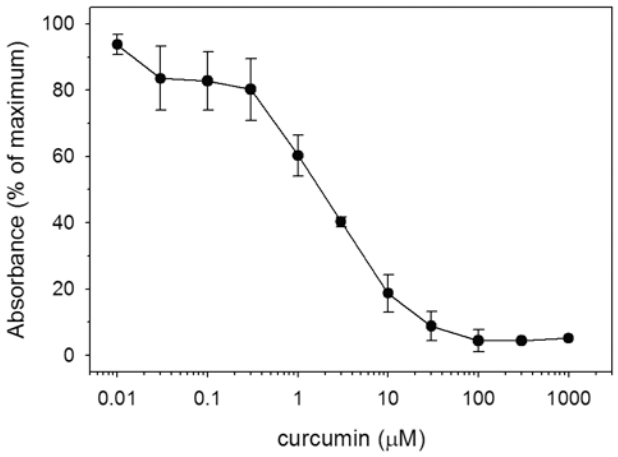


Fig. (3). Curcumin inhibits Aβ oligomer formation in a dose-dependent manner. Aβ(1-42) peptide (200 nM final concentration) was mixed with the indicated concentrations of curcumin and incubated at 37°C for 24 h. Aβ oligomers were quantified as in Fig. (**2**).

gomerization was challenged with various concentrations of each compound, in parallel with curcumin, and measured by capture ELISA. Even with concentrations as high as 20 μM, no reduction in Aβ oligomerization was measured by either compound (Fig. **4**). These data indicate that the complete, diphenolic structure of curcumin is necessary to prevent early-stage oligomer formation.

As shown in Fig. (**4**) and by others [39, 47, 48], a structure-activity relationship exists between curcumin and Aβ oligomerization with the polyphenolic platform being a necessary structural requirement. In this regard, other polyphenolic compounds have been tested and identified as inhibitors of Aβ oligomerization [49, 50], but few of these have been examined in parallel with curcumin. Moreover, a range of IC_{50} values have been reported for each of these polyphenols, but this may be due to the fact that these studies relied upon assays that require relatively high starting concentrations of Aβ peptide because of their reduced sensitivities. To gain a better understanding of the potential of some of these compounds in relation to the activity of curcumin, the effects of rosmarinic acid, epigallocatechin gallate (EGCG), catechin and resveratrol (Table **1**) on Aβ oligomerization were tested in parallel with curcumin. Catechin and resveratrol demonstrated little anti-oligomerization activity, while rosmarinic acid inhibited Aβ peptide aggregation only at the highest concentration tested, this being 20 μM (Fig. **5**). EGCG was capable of reducing peptide aggregation in a dose-dependent manner with an IC_{50} value of ~8 μM. However, the anti-oligomerization activity of curcumin far surpassed that of any other test compound.

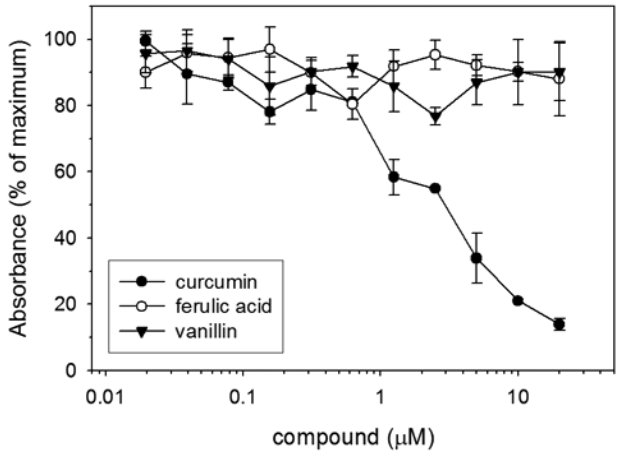


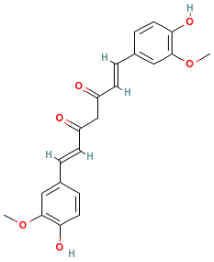
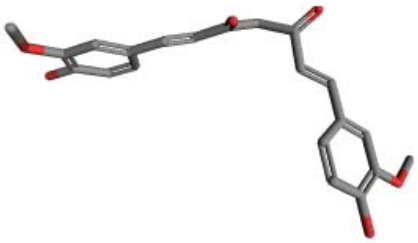
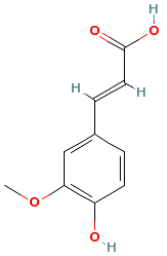
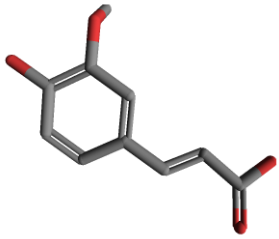
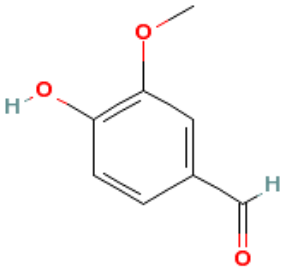
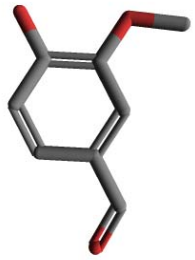
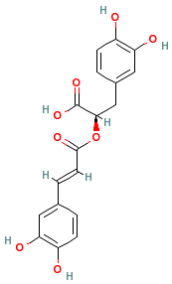
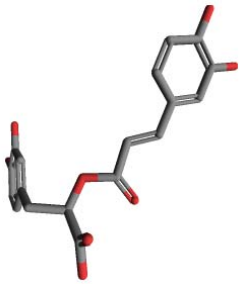
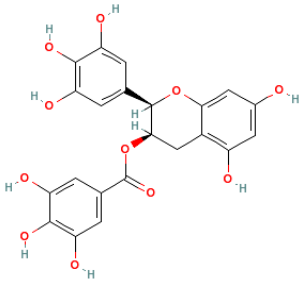
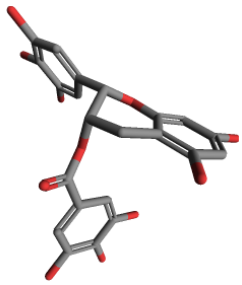
Fig. (4). Ferulic acid and vanillin have no effect on early-stage Aβ oligomer formation. Aβ(1-42) peptide (200 nM final concentration) was incubated with the indicated concentrations of curcumin (●), ferulic acid (○) or vanillin (▼) at 37°C for 24 h. Aβ oligomers were quantified as in Fig. (**2**).

DISCUSSION

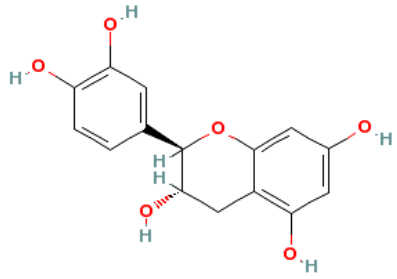
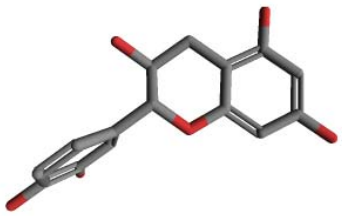
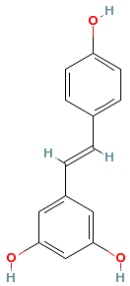
Aggregation of the Aβ peptide is a hallmark event in the pathogenesis of AD. Formation of Aβ oligomers leads to impairment of synaptic function [51], chronic microglial activation [52], and premature neuronal death. Because of this, there has been an extensive and widespread search for small molecule inhibitors to prevent formation of these toxic peptide aggregates. Previous assays designed to measure oligomerization, including spectroscopic and microscopic procedures, require large amounts of Aβ peptide to create sufficient quantities of aggregates to surpass the minimum threshold for detection. Such quantities far exceed what would be expected in AD diseased brains during initial nucleation of amyloid deposits raising questions if such reactions accurately represent the physiochemical parameters of oligomer formation *in vivo*. The majority of assays used for inhibitor identification apply dye-binding protocols that differentiate between free dye and dye bound to peptide aggregates by measuring spectral shifts. These dyes bind to peptide aggregates by non-specific, hydrophobic interactions and are unable to discern between low- versus high-molecular weight aggregates [53-58]. This has become of concern since recent studies suggest that the early stages of Aβ peptide oligomerization forming low-molecular weight aggregates may represent the most toxic form *in vivo* [7, 9, 10, 59]. For these reasons, we sought to identify a more sensitive assay for molecular discovery screening regimens that is a better representation of the early stages of Aβ oligomerization. The principle of the present study focuses on an ELISA-based capture assay for Aβ oligomer detection [35, 36]. This ELISA format uses a single Aβ-specific mAb for both antigen capture and detection and is capable of clearly distinguishing monomeric peptide from Aβ oligomers. Importantly for our objectives, this assay also discerns early-stage, low-molecular weight soluble aggregates from later-stage, amyloid fibrils [36], and in this manner is better suited to identify inhibitors of the most critical stage of Aβ peptide oligomerization.

We have explored the adaptability of this assay for discovery of small molecule inhibitors of the Aβ peptide oligomerization process. Using Aβ mAb 4G8 that is specific for residues 17-24, oligomer formation was easily measurable with as little as 80 nM input peptide. This sensitivity is far greater than can be achieved by dye-binding or light scattering assays and comparable to the results reported by LeVine [35]. Several small molecule inhibitors of Aβ

Table 1. Chemical Structures of Compounds Tested in the ELISA-Based Aβ Oligomerization Assay

Curcumin – (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione
 
Ferulic acid – (E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid
 
Vanillin – 4-hydroxy-3-methoxybenzaldehyde
 
Rosmarinic acid – (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid
 
Epigallocatechin gallate – [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate
 

(Table 1). Contd…..

Catechin – 2R-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3S,5,7-triol	
	
Resveratrol – 5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol	
	

Common name followed by IUPAC name. Shown are 2-D and 3-D representations (PubChem Compound database, National Center for Biotechnology Information).

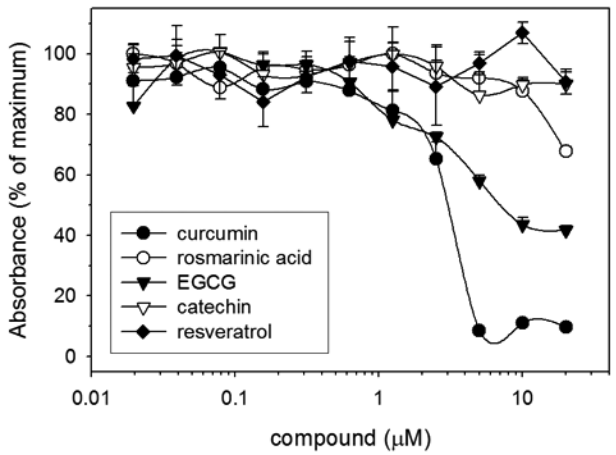


Fig. (5). Effects of rosmarinic acid, EGCG, catechin and resveratrol on early-stage Aβ oligomer formation. Aβ(1-42) peptide (200 nM final concentration) was incubated with the indicated concentrations of curcumin (●), rosmarinic acid (○), EGCG (▼), catechin (∇) or resveratrol (◆) at 37°C for 24 h. Aβ oligomers were quantified as in Fig. (**2**).

oligomerization, mostly polyphenolic compounds, have been identified using dye-binding or light scattering assays. Since these assays are unable to discriminate between low- and high-molecular weight oligomers, and thus early- versus late-stage Aβ aggregation events, several of the most promising compounds (Table **1**) were re-evaluated using the ELISA-based assay to assess their effectiveness as aggregation inhibitors during the early stages of peptide assembly. The first compound considered was curcumin. Curcumin is a natural product found in the rhizomes of the Curcuma longa family. It has a di-phenolic structure joined by α-β-unsaturated and β-diketone moieties. Curcumin has proven to be a potent inhibitor of Aβ aggregation *in vitro*, capable of reducing amyloid deposition *in vivo* and improving cognitive function in an AD mouse model [29]. In spite of these positive results, serious challenges remain to transform curcumin into a viable therapeutic option due to its poor bioavailability [40, 41] and lability in an aqueous environment [42, 43]. Because of curcumin's polyunsaturated 7-carbon spacer arm and β-diketone moiety, it readily degrades into ferulic acid and vanillin [60]. Some reports have suggested that these products maintain activity as anti-aggregates even with the loss of their polyphenolic structure [44-46, 61]. This was challenged using the ELISA-based assay and their anti-aggregation capacity was directly compared in parallel with curcumin. The results showed no measurable activity by ferulic acid or vanillin in preventing Aβ peptide oligomerization. These findings reinforce the observation that a polyphenolic structure is a minimal requirement for an inhibitor of Aβ aggregation.

Other small molecule inhibitors with polyphenolic structures have been identified that prevent Aβ peptide aggregation [49, 50], but none of these have been quantitatively compared side-by-side with curcumin. Rosmarinic acid, EGCG, catechin and resveratrol are among the most potent found with IC_{50} values reported as 1.1, 0.18, 5.3, and 5.6 μM, respectively [49]. Rosmarinic acid is a natural polyphenol found in many culinary herbs such as lemon balm, rosemary, oregano, sage, thyme and peppermint. In addition to inhibiting Aβ aggregation, a recent study has indicated that rosmarinic acid can directly bind β-amyloid fibrils and may serve as an *in vivo* imaging agent for non-invasive testing and tracking amyloid progression [62]. Catechin and its epimer, epicatechin, are the most common optical isomers found in nature. Catechins constitute about 25% of the dry weight of fresh tea leaves and are present in nearly all teas

made from *Camellia sinensis*, including white tea, green tea, black tea and Oolong tea. Epigallocatechin contains an additional phenolic hydroxyl group as compared with catechin and its gallic acid ester, epigallocatechin gallate (EGCG), is the most abundant form of catechin found in tea. Tea catechins, especially their gallic acid esters, are known to inhibit fibrilization of Aβ peptide and protect against Aβ oligomer induced neurotoxicity [63, 64]. Resveratrol is a polyhydroxylated stilbene found in many foods, but is especially abundant in many varietals of red wine. By itself, resveratrol is a potent anti-oxidant which is thought to be responsible for the French Paradox [65]; relating to the low incidence of cardiovascular disease in a French population with high intake of saturated fats. In addition to its demonstrated capacity to inhibit Aβ(25-35) fibril formation *in vitro* [16], resveratrol is also capable of promoting intracellular uptake and degradation of Aβ through a proteosome-dependent mechanism [66] and protecting against Aβ-induced neurotoxicity [67, 68], possibly through activation of the NAD-dependent histone deacetylase, SIRT1 [69]. In the present study using the ELISA-based assay, catechin showed no inhibitory activity during the early stages of Aβ oligomerization, whereas rosmarinic acid demonstrated some activity at the highest concentration tested. EGCG showed a clear dose-dependent inhibition of early stage oligomer formation; albeit with a 2-3 fold lower effectiveness than curcumin. These findings do not refute previous reports identifying the capacity of these compounds to inhibit Aβ oligomerization, but rather discriminate those inhibitors that are more effective in preventing early stage aggregation events as opposed to those that are more effective during later stage oligomer growth and expansion. This has important implications for the design rationale of therapeutic inhibitors, as well as developing tools for dissecting the mechanism of oligomer initiation and growth. At present, knowledge of the detailed events that transition soluble monomeric Aβ peptide into insoluble aggregates is incomplete. Evidence suggests that soluble Aβ peptide begins with an α-helical conformation that undergoes a thermodynamically driven transition into a β-sheet conformer. This β-sheet structure positions C-terminal hydrophobic amino acids along one surface permitting the stacking of multiple β-sheet orientations driven by the exclusion of water. From the data presented here, we might expect curcumin and EGCG to interrupt these early oligomerization events by stoichiometrically binding to β-sheet conformers and preventing subsequent peptide stacking. Alternatively, these compounds may shift the equilibrium from β-sheet to α-helical conformers and reduce the concentration of reactants in the oligomerization equation. Other demonstrated inhibitors, such as rosmarinic acid, catechin and resveratrol may associate with already formed low-molecular weight aggregates and prevent their further growth into the higher-molecular weight species typically measured in dye-binding or light-scattering assays.

Identifying the precise mechanism of inhibitor action is an area of active investigation; however, regardless of subtle chemical differences between inhibitors, it is commonly held that inhibitor function requires a direct binding interaction between the compound and Aβ peptide [33, 34]. This of course implies a stoichiometric relationship between inhibitor and peptide. Unfortunately, few studies address this relationship even when accurate titration data is available. In fact, using curcumin as an example, calculations made using the data from some studies [29, 70] indicate an inhibitor to peptide ratio of less than one. Assuming a direct interaction is necessary for inhibitor function, such ratios are difficult to reconcile when attempting to define mechanism of action. Perhaps the true inhibitor:peptide stoichiometries are masked in previous Aβ aggregation assays because of the high starting concentrations of peptide needed for detection. If this is indeed the case, the ELISA-based assay presented here can detect oligomers with as little as 80 nM input peptide and may provide a method to more accurately define these ratios. For example, using these lower input Aβ concentrations, a stoichiometry of 10:1, curcumin:peptide can be calculated based on an IC_{50} value of 2 μM. This value is more consistent with a proposed direct binding mechanism and provides more useful information defining curcumin's effects on the early stage oligomerization reaction.

In summary, the ELISA-based Aβ oligomer detection assay provides a sensitive, scalable methodology to identify and quantify small molecule inhibitors that might be present in available chemical libraries. Because of its sensitivity, less input peptide amounts are required making this assay more cost effective for expanded screening capabilities and accelerated molecular discovery. And, because this assay can discern small oligomeric structures representing the initial stages of peptide aggregation, more effective therapeutics can be identified targeting the low-molecular weight oligomers which are now regarded as the most neurotoxic species.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grant AG027794 to R.A.O.

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Received: June 23, 2009 Revised: August 22, 2009 Accepted: August 30, 2009