Curcumin blocks amyloid aggregation

Curcumin inhibits formation of Aβ oligomers and fibrils, binds plaques and reduces amyloid in vivo

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SUMMARY

Alzheimer's disease (AD) involves amyloid (Aβ) accumulation, oxidative damage and inflammation; and risk is reduced with increased antioxidant and anti-inflammatory consumption. The phenolic yellow curry pigment curcumin has potent anti-inflammatory and antioxidant activities and can suppress oxidative damage, inflammation, cognitive deficits, and amyloid accumulation. Since the molecular structure of curcumin suggested potential Aβ-binding, we investigated whether its efficacy in AD models could be explained by effects on Aβ aggregation. Under aggregating conditions in vitro, curcumin inhibited aggregation (IC$_{50}$ =0.8 µM) as well as disaggregated fibrillar Aβ40 (IC$_{50}$ =1 µM), indicating favorable stoichiometry for inhibition. Curcumin was a better Aβ40 aggregation inhibitor than ibuprofen and naproxen, and prevented Aβ42 oligomer formation and toxicity between 0.1-1.0 µM. Under electron microscopy, curcumin decreased dose-dependently Aβ fibril formation beginning with 0.125 µM. Curcumin's effects did not depend on Aβ sequence but on fibril-related conformation. AD and Tg2576 mice brain sections incubated with curcumin revealed preferential labeling of amyloid plaques. In vivo studies showed that curcumin injected peripherally into aged Tg mice, crossed the blood brain barrier and bound plaques. When fed to aged Tg2576 mice with advanced amyloid accumulation, curcumin labeled plaques and reduced amyloid levels and plaque burden. Hence, curcumin directly binds small β-amloid species to block aggregation and fibril formation in vitro and in vivo. These data suggest that low dose curcumin effectively disaggregates Aβ as well as prevents fibril and oligomer formation, supporting the rationale for curcumin use in clinical trials preventing or treating AD.
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INTRODUCTION.

The 4 kD (40-42 aa) amyloid-β peptide (Aβ) is derived from the amyloid precursor protein (APP) through sequential proteolysis by the aspartyl protease BACE (β-secretase) and presenilin-dependent γ-secretase cleavage (1). Mutations at the cleavage sites in APP or in presenilin that increase production or aggregation of Aβ provide a compelling argument for a central role for Aβ aggregation in the pathogenesis of Alzheimer's disease (AD). The progressive accumulation of Aβ aggregates is widely believed to be fundamental to the initial development of neurodegenerative pathology and to trigger a cascade of events such as neurotoxicity, oxidative damage, and inflammation that contribute to the progression of AD (2-5). Therefore, many therapeutic efforts are targeted at reducing Aβ production including inhibiting secretase, increasing Aβ clearance with amyloid vaccines, or blocking Aβ aggregation (with antibodies, peptides or small organic molecules that selectively bind and inhibit Aβ aggregate and fibril formation).

Aβ fibrillization involves formation of dimers and small oligomers followed by growth into protofibrils and fibrils via a complex multistep-nucleated polymerization. Polymerizing Aβ fibrils and intermediates can be stained by amyloidophilic dyes such as Congo Red (CR) (6). Congo Red can inhibit the toxic and inflammatory activity of polymerizing Aβ (7,8) and prevent the natural oligomer formation found at low Aβ concentrations (9). However, CR is toxic and negatively charged, and therefore poorly brain penetrant (10).

Curcumin (diferulomethane) is a low molecular weight molecule with potent antioxidant and anti-inflammatory activities that has a favorable toxicity profile and is under development as a potential cancer chemotherapeutic agent (11). Our previous results demonstrated that chronic dietary curcumin lowered Aβ deposition in 16 month old APPsw transgenic mice (Tg2576) (12).
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However, it remain unresolved whether curcumin was reducing plaques in vivo in part by effects on aggregation. As shown in Figure 1, curcumin has a structure similar to Congo Red but with the charge replaced by polar groups like the brain-permeable compound chrysamine G (10,13). It is also similar to RS-0406, a novel compound selected from a screen of 113,000 compounds as a potent inhibitor of Aβ oligomer formation (14). We hypothesized that, like these polar Aβ binding compounds, curcumin might be able to cross the blood brain barrier and bind to amyloid and related aggregates. Therefore, in these studies, we used an in vitro model of Aβ fibrillization to show that curcumin can bind amyloid to inhibit Aβ aggregation as well as fibril and oligomer formation with dosing at achievable levels. We also demonstrate that curcumin can label plaques in vitro and in vivo, block toxicity of oligomers in vitro, and significantly reduce amyloid levels in aged Tg2576 mice (22 month old) fed a curcumin diet beginning at 17 months-after established amyloid deposition.
EXPERIMENTAL PROCEDURES

Materials Aβ40 and Aβ42 peptides were purchased from the laboratory of Dr. Charles Glabe, University of California (Irvine, CA) and American Peptide (Sunnyvale, CA). Aβ fragment peptides 1-13, 1-28, 25-35, 34-40 and 37-42 were purchased from Bio-Synthesis (Lewisville, TX), and Aβ 12-28 was purchased from American Peptide Company. Aβ 8-17, 14-24, 17-24, 17-28 was purchased from Quality Control Biochemicals (Hopkinton, MA). Curcumin, naproxen and ibuprofen were purchased from Cayman Chemicals (Ann Arbor, MI). Stocks of curcumin, naproxen and ibuprofen (5 mM) were dissolved in 100% ethanol and stored at -80°C. A 5 mM stock of Congo Red was prepared in dH2O immediately before use. The 6E10 antibody (Aβ1-17) was purchased from Signet Labs (MA) and the A11 antibody was generously provided by C. Glabe (UC Irvine). All other reagents were from Sigma (St. Louis, MO).

Curcumin staining of human AD and Tg2576 mouse brain sections (in vitro) Just before use, curcumin was diluted from 2.5 mM to 50 nM in 0.1 M TBS (pH 7.4) containing 3% BSA with 0.5% Tween-20. Thioflavin S (1%) was freshly prepared in dH2O, stirred for 0.5 hr and filtered. Human AD hippocampus was fixed in buffered formalin and snap-frozen. Tg2576 mice (22 months old) were perfused with Hepes buffer and protease inhibitors (15), and brains were removed, fixed in buffered formalin and snap frozen. Both brains were cryosectioned at 12 µm and stored at –70°C. Sections were warmed to room temperature for 10 minutes and dipped in 75% ethanol, treated with 0.3% Triton X-100 and 0.1 M TBS (pH 7.4) containing 3% BSA with 0.5% Tween-20 for 10 minutes each. Different concentrations of curcumin were applied to sections for 1 hr at 37°C in a humidified chamber. Sections were washed in TBS 3 times, rinsed once in dH2O and coverslipped with fluorescent mounting media. Adjacent sections were stained with 1% thioflavin S for 10 min at RT and dipped for 1 min in 75% ethanol, 95%
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ethanol, 100% ethanol, 95% ethanol, and 75% ethanol before rinsing in dH2O. All sections were examined and photographed with a fluorescence microscope using FITC optics.

**In vivo curcumin treatments and labeling in Tg2576 mice.** Groups of APPsw Tg2576 transgenic mice raised on Purina 5015 breeder chow were placed on 500 ppm curcumin (Sabinsa, Piscataway, NJ) or control safflower oil based test diets (TD#02347 and #02346, respectively, Harlan Teklad) and aged until 22 months. A curcumin stock solution was diluted 1:100 into sterile PBS. Three 22 month old Tg2576 mice (50 gm body weight) were anesthetized with 25 mg/ml Nembutal (i.p.). One mouse on the chronic 500 ppm curcumin diet was injected with 200 µl of curcumin/PBS into the right carotid artery over a 5 min period. Because blood volume for a 50 gm mouse was estimated to be about 4 ml (80 ml/kg x 0.05 kg), we expected blood levels of curcumin to reach ~2 µM. The other two mice were injected with PBS only as a control and one of these mice had also received the 500 ppm curcumin diet. After 1 hr, the mice were perfused as previously described (15). Brains were removed, snap frozen in 2-methylbutane chilled by liquid nitrogen. Freshly cut cryosections (12 µm) were air dried for 10 min in the dark and coverslipped with VECTASHIELD mounting media for fluorescence protection (Vector Laboratories, Burlingame, CA). Sections were examined and photographed with a Nikon Microphot-Fx fluorescence microscope using a FITC filter set.

**Measurement of in vivo amyloid levels**

**Image analysis of plaque pathology.** Plaque burden was assessed using a characterized polyclonal antibody against Aβ1-13 (DAE) (15). Coronal sections were made through anterior (Bregma -1.00-1.46 mm), middle (Bregma -1.58-2.30 mm) and posterior hippocampus (Bregma -2.46-3.16 mm) of control (n=6) and curcumin treated mice (n=8). Immunolabeling was
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examined in various cortical and hippocampal areas of animals and image analysis and image analysis was performed as previously described (12,15).

**Amyloid extracts and assays.** Amyloid levels were evaluated in the cortex of control (n=6) and curcumin-fed (n=4) Tg2576 mice. Samples were processed in TBS and lysis buffer (detergent) as previously described (16). Detergent-insoluble pellets from cortex were sonicated in 8 volumes of 5M guanidine, 50 mM Tris-HCl and solubilized by agitation at room temperature for 3-4 hr. Guanidine-soluble extracts were diluted 1:10,000 with TBS containing 5% BSA and 1x protease inhibitor cocktail (Calbiochem) and assayed for total Aβ by ELISA as previously described (17).

**Disaggregation and inhibition of Aβ aggregation with curcumin**

**Inhibition of aggregation.** Aβ40 was dissolved at 1 mg/ml in dH2O, and curcumin stock was diluted in 0.1 M TBS with 0.02% Tween-20. Aβ40 (100 µg/ml) was mixed a 1:1 volume ratio with curcumin in Eppendorf tubes and incubated for 6 days at 37 °C. Final concentrations of Aβ40 were 50 µg/ml (11.6 µM), and curcumin concentrations were 0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 µM.

**Disaggregation of preformed fibrils** Aβ40 (100 µg/ml) was incubated for 3 days at 37 °C to generate fibrils. Pre-formed fibrils were mixed with the same concentrations of curcumin as above for an additional 3 days at 37 °C.

At the end of both incubations, an aliquot (3 µL) was taken from each tube for electron microscopy analysis. The remaining Aβ solution was assayed for aggregates using the 6E10/6E10 sandwich ELISA.

**6E10/6E10 aggregation ELISA** To determine the relative amount of aggregated Aβ, the monoclonal antibody 6E10 was used for both detection and capture as described by Howlett et al 1999 (17). Briefly, 96 well plates were coated with 3 µg/ml of 6E10 in 55 mM sodium
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bicarbonate buffer (pH 9) at 4°C overnight before wells were blocked for 2 hr at RT with TBS-Tween 20 (TBS-T, 0.1 M TBS, pH 7.4, 0.02% Tween-20) with 1% BSA. Samples were transferred to the wells and plates were placed on an orbital shaker (30 rpm) for 2 hrs at RT. After washing 4 times with TBS-T, biotinylated 6E10 antibody diluted 1:1200 in TBS-T containing 1% BSA was applied to the plate for 1 hr at RT with shaking. After washing, alkaline phosphatase streptavidin (Vector Labs., Burlingame, CA) diluted at 1:4000 in TBS-T with 1% BSA was applied to the plates, followed by the addition of the fluorescent substrate, Attophos (Promega). Fluorescence was measured using a CytoFluor II fluorescence plate reader (excitation 450 nm and emission 580 nm; Applied Biosystems, Foster City, CA).

**Efficacy of curcumin compared with other NSAIDs** Aβ 1-40 was dissolved at 1 mg/ml in dH$_2$O. A 50 µg/ml solution was made with 0, 0.5, 2 or 8 µM of curcumin. Naproxen and ibuprofen were tested at 0, 8, 16 and 32µM. Samples were sealed in Eppendorf tubes and incubated for 6 days at 37°C. Aβ aggregates were assayed with the 6E10/6E10 ELISA as above. Separate drug only controls were used to assess possible drug fluorescence interference in the assay.

**Electron microscopy analysis of fibril formation** Electron microscopy (EM) was used to observe inhibition of Aβ fibril formation in disaggregation and aggregation inhibition experiments (Figure 5). To determine if filaments had formed, 3 µl of Aβ peptide solution was applied to 150 mesh copper grids coated with Formvar/carbon film (EM Sciences, Fort Washington, PA) for 30 sec. Excess solution on the other side of grids was absorbed with filter paper and grids were stained with 1 drop of 0.5% filtered uranyl acetate for 20 sec and staining solution absorbed with filter paper again. After air drying for 4 hrs or overnight, grids were examined with an electron microscope (TECNAI 10/12, Phillips) at 80 kV.
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**Curcumin binding experiments with Aβ peptides** Aβ40 and Aβ42 were solubilized in HFIP, dried overnight at RT and speed vacuumed for 10 minutes (18). Aliquots were stored at -80°C until they were needed when they were resolubilized in 20 µl of DMSO and dissolved in dH2O (1 mg/ml). Additional samples of Aβ40, Aβ42 and shorter Aβ peptides were dissolved in dH2O, while Aβ17-24 was dissolved in DMSO. Peptides were aggregated in TBS (0.05 M Tris-HCL, pH 7.4, 75 mM NaCl, 0.025% NaN3) at 500 µg/ml. Two hundred µl of each peptide solution was sealed in Eppendorf tubes and incubated without shaking at 37 °C for 10 days. After 10 days, an aliquot was removed for EM analysis of structure before solutions from each tube were mixed with curcumin at a final concentration of 1 µM. Samples were incubated at 37°C for 1 hr, and then spun at 16000 g for 10 min. To assess curcumin staining of synthetic peptide aggregates, pellets were mixed with 10 µl of dH2O, smeared to slides and air-dried for 1hr. Supernatant, spun down after mixing Aβ peptides with 1 µM curcumin, were applied to cryosections of Tg2576 mouse brain (22 months) at 37°C for 1 hr. Slides were cover-slipped with anti-quenching mounting media before examination under a fluorescent microscope (Table 1).

**Inhibition of Aβ oligomer formation**

**Immunoblot of curcumin and Congo Red treated Aβ42 oligomers.** An aliquot of Aβ42 (0.045 mg) was dissolved in 20 µl of DMSO and diluted in HAM’s F-12 media without phenol red (Biosource, Camarillo, CA). Aβ42 (5 µM) was incubated with curcumin (0, 0.25, 1, 4, 16, and 64 µM), or Congo Red (16 and 64 µM) in 37°C water bath for 4 h. After the incubation, the samples (72 µL) were spun at 14,000 x g (4°C for 10 min) and the supernatant (65 µL) was mixed with an equal part of Tricine sample buffer without reducing agents (Bio-Rad). The
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unaggregated Aβ42 control was not incubated at 37°C and after dilution in F-12 media, it was mixed with sample buffer (no centrifuging) and stored at -20°C before it was electrophoresed. Samples (30 µL, no boiling) were electrophoresed at 100V on a 10-20% Tris-tricine SDS gel, transferred at 100V for 1 h, and blocked overnight (4°C) with 10% nonfat milk in PBS + 0.1% gelatin. The blots were probed with 6E10 (1:2000, 3 h at RT), followed by goat anti-mouse-HRP (1:10,000, 1h at RT), and developed with ECL.

**Dot blot assay:** Aβ40 oligomer was prepared from HFIP solubilized Aβ (4 mg/ml, 10-20 min in HFIP at 25°C). An 80 µl Aβ aliquot was diluted 1:10 with 800 µl of sterile H2O. The final Aβ concentration was 400 µg/ml or 88 µM. Curcumin was added to Aβ solutions to give final 0, 2, and 16 µM curcumin in 0.01% methanol. After the pH was adjusted (pH 3), the samples were incubated for 2.5 hr at 42°C followed by a 48 hr incubation at RT with stirring. Samples (500 ng of oligomer) were applied to nitrocellulose membrane in a Bio-Dot apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 10% non-fat milk in TBS-T at RT for 1 hr, washed with TBS-T, and probed with anti-oligomer A11 antibody (19) solution (1:10,000) or 6E10 (1:10,000) in 3% BSA-TBS-T overnight at 4°C. After washing, it was probed with anti-rabbit-HRP or anti-mouse-HRP conjugated antibody (Pierce Biotechnology, Inc., Rockford, IL) solution (1:12,000) for 1h at RT. The blot was developed with SuperSignal (Pierce) for 2-5 min. Dots were scanned and analyzed with a Model GS-700 densitometer using Molecular Analyst software (Bio-Rad).

**Toxicity assays:** Confirmation of Aβ oligomer toxicity was performed in human APPSwe neuroblastoma (N2a) cells which were cultured in 50% DMEM/50% Opti-MEM (GIBCO Invitrogen Corporation)/5% fetal bovine serum/200 µg/ml Glutamax. Cells were plated at equal densities (8000 cells/well) with 1.5% bovine calf serum (BCS) and maintained at 37°C in an
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To determine if curcumin could block this oligomer toxicity, SH-SY5Y human neuroblastoma cells were grown and maintained as previously described (20). Cells were plated at 10,000 cells/well in 96 well plates and were differentiated in low serum DMEM media with N₂ supplement and 1x10⁻⁵M all-trans retinoic acid for 7 days. The media was removed and replaced with fresh maintenance media containing 0.1% BSA. Aβ42 oligomer (100 nM) was added to cells for 48 h at 37°C with or without 0.1, 1, 2.5 and 5 µM curcumin. After treatment, LDH assay was performed as mentioned above. Cell viability was assayed using the MTT reduction assay (21), where absorbance was measured at 550 nm. Each data point was determined in triplicate and the standard deviation did not exceed 5%.
RESULTS

Because curcumin is a fluorochrome, we first compared plaque-associated curcumin fluorescence with that of thioflavin S, using sections from APPsw (Tg2576) mouse brain (Figure 2A, B and E) and AD hippocampus (Figure 2C and D). Like thioflavin S (Figure 2A and C), curcumin brightly labeled amyloid plaques (1 µM, Figure 2B, D and E). In AD brain, curcumin labeled plaques with a yellow fluorescence (Figure 2D) similar to thioflavin S in an adjacent section (Figure 2C). Thioflavin S labeled tangles very strongly (Figure 2C) while curcumin labeled tangles only weakly or not at all (Figure 2D). Curcumin fluorescence was initially yellow-orange, but with prolonged (>2 minutes) light exposure, the fluorescence gradually shifted to yellow/green fluorescence. This phenomenon is illustrated in Figure 2E, where the right side of the field was illuminated for 3 minutes before shifting the field back to make the photo exposure. Color change depended on the intensity of plaque staining and the fluorescent exposure time, and was stable for several weeks at 4°C. Lipofuscin age pigments in this aged mouse brain (small arrows) are orange/red under these thioflavin optics; and, unlike curcumin labeling, did not show a fluorescent shift with prolonged illumination. Optimal staining concentrations for curcumin were 0.5-2 µM.

Unlike thioflavin S and Congo Red, curcumin is highly hydrophobic and should readily enter the brain to bind to plaques in vivo. In order to evaluate this idea, we injected curcumin (50 µM in 200 µL) or vehicle (PBS) into the carotid artery of aged Tg2576 mice. Mice were sacrificed an hour later and unfixed cryosections were immediately prepared from snap frozen brains. Untreated mice injected with PBS only showed no plaque staining (Figure 3A) but a mouse on a chronic curcumin diet (500 ppm) showed discernable, but weak plaque staining when injected with PBS alone (Figure 3B). Plaques in the curcumin-injected mouse were
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brightly labeled (Figure 3C and D). These data suggested that curcumin can cross the blood brain barrier and bind to plaques in transgenic mice after oral feeding or peripheral injection.

Having established that curcumin can stain in vitro and in vivo amyloid, we sought to test whether it can interfere with Aβ aggregation and its disaggregation. Because curcumin fluoresces over the same wavelengths as thioflavin S, we could not use thioflavin-based assays to quantify amyloid fibrils in curcumin-treated samples, and thioflavin-based assays require high concentrations of Aβ. Therefore, we employed a sandwich ELISA for aggregated Aβ using the same N-terminal Aβ antibody (6E10) for both capture and detection. Aβ40 (final conc 50 µg/ml) was incubated under fibril forming conditions with 0-8 µM curcumin. As shown in Figure 4A, Aβ aggregation was significantly inhibited with increasing doses of curcumin, with an approximate IC₅₀=0.81 µM (gray circles; *P*<0.001). When Aβ was pre-aggregated for 3 days before incubation with curcumin, increasing doses of curcumin were capable of inducing the disaggregation of pre-aggregated Aβ40, with an IC₅₀=1 µM (filled circles, *P*<0.005; Figure 4B).

In control experiments, curcumin over this range did not interfere with the Aβ aggregate ELISA and did not interfere with an ELISA with 6E10 as the capture and anti-Aβ 34-40 as the detection (not shown), implying that curcumin did not simply block 6E10 antibody binding.

We also sought to test whether curcumin was better than other NSAIDs at inhibiting Aβ aggregation. Aβ40 (final conc. 50 µg/ml) was incubated with curcumin (0-8µM), naproxen (0-32 µM), or ibuprofen (0-32 µM) for 6 days at 37°C. The presence of Aβ aggregates was determined with the 6E10/6E10 ELISA. In this assay, curcumin again showed dose-dependent inhibition of Aβ aggregate formation over the low dose range (Figure 4C), in contrast to naproxen and ibuprofen (Figure 4D, E). These data indicate that although high concentrations of
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naproxen and ibuprofen can inhibit Aβ aggregation (20), curcumin is a better aggregation inhibitor.

Electron microscopic analysis of inhibition of fibril formation

To determine whether curcumin could inhibit the formation of fibrils, electron microscopy was used to examine samples of Aβ40 incubated with and without curcumin as described in methods. Aβ40 (50 µg/ml) incubated for 6 days at 37°C formed extensive, but less mature fibrils (Figure 5A) than fibrils formed with a higher concentration of Aβ40 (100 µg/ml., Figure 5D). These fibrils were extensive and more uniform in thickness than those at the lower concentration, with less branching, bumps, or nodules. In order to test whether low physiologically relevant concentrations of curcumin might inhibit fibril formation, we tested curcumin's impact on Aβ assembly at the lower Aβ40 (50 µg/ml) concentration. If a low dose of curcumin (0.125 µM) was included in the initial incubation, fibril formation appeared reduced (Figure 5B). A higher dose (2 µM) resulted in similar, but more potent inhibition of fibril formation (Figure 5C). Droplets of curcumin/Tween accumulated with curcumin dose as fibril formation was inhibited.

In order to determine whether curcumin could also be inhibitory when added after initial aggregation events, we incubated 100 µg/ml Aβ40 for 3 days at 37°C and then incubated another 3 days at 37°C without (Figure 5D) or with 0.125 µM (Figure 5E) and 2 µM curcumin (Figure 5F). The results show that curcumin limits fibril formation even when added midway through the incubation, consistent with an impact on fibril maturation or dissolution.

Curcumin prevents formation of oligomers

Soluble oligomers of Aβ or "ADDLs" are a neurotoxic species implicated in AD pathogenesis (22-25). In order to test whether curcumin can block the formation of these
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neurotoxic oligomers, we dissolved Aβ40 and Aβ42 in HFIP to reconstitute "seedless" monomer and then incubated with and without curcumin in an oligomer formation protocol modified from Kayed et al 2003 (19) and Chromy et al., 2003 (18). The resulting oligomer enriched supernatants were taken for analysis by dot blot and immunoblot with an oligomer-specific antibody A11 and 6E10. Dot blots showed dose-dependent inhibition of oligomer formation (Figure 6A). When dot blots were probed with a non-oligomer specific Aβ 6E10 antibody, there was no appreciable decrease in signal (Figure 6B), indicating that the A11 signal was specific for oligomeric Aβ. One-way ANOVA analysis of A11 dot blot scans indicated that both 2 and 16 µM curcumin can significantly reduce Aβ oligomer formation ($P<0.001$).

We then compared curcumin and Congo Red for their ability to block oligomer formation. Aβ42 (5 µM) was included to show the unaggregated monomeric form of Aβ (3.5 kDa, Figure 6C, lane 1). When Aβ42 was allowed to aggregate without curcumin or CR, two bands between 14.3-20 kDa representing the 4-5-mer and an aggregated oligomer smear at 44-127 kDa were also present (lane 2). As the curcumin dose increased from 0 to 4 µM, the amount of higher MW aggregated smear decreased as the amount of monomer Aβ increased (Figure 6C-D; lane 2-5). When curcumin was present at 16 and 64 µM, the oligomeric Aβ bands (14.3-127 kDa) disappeared while the monomeric Aβ remains (lane 6 and 7) similar to the unaggregated Aβ in lane 1. At 16 and 64 µM, CR could reduce the oligomeric smear but not the 4-5-mer Aβ band. Together, these data imply that non-toxic curcumin can inhibit Aβ42 oligomer formation as well or better than toxic CR.

Oligomeric-dependent toxicity was first determined using the oligomeric specific antibody A11 to block Aβ42 toxicity in APPSwe N2a cells (Figure 7a). LDH release was measured after 72 h incubation and one-way ANOVA demonstrated a significant increase in
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LDH in cells incubated with oligomer Aβ alone \( (P<0.0001) \). Incubating cells in conjunction with the A11 antibody resulted in a 42.7\% reduction in toxicity, indicating that cell toxicity was dependent on the Aβ42 oligomers \( (P<0.0001) \). To further evaluate whether curcumin can inhibit oligomer toxicity, Aβ42 oligomer (100 nM) with and without curcumin (0—5 µM) were added to differentiated SH-SY5Y neuroblastoma cells for 48 h before cell viability and toxicity were determined. One-way ANOVA analysis revealed a significant increase in cell viability with all curcumin doses (Figure 7B). Moreover, this treatment effect was most effective at 0.1 and 1 µM curcumin, since the inhibitory effect appeared to plateau with 2.5 and 5 µM curcumin. Thus, these data indicate that curcumin inhibited cell toxicity of Aβ42 oligomers at 0.1 and 1 µM doses.

**Fibril conformation required for curcumin binding**

We have shown that curcumin can bind to plaques and block Aβ aggregation, as well as fibril and oligomer formation. The molar ratios for successful Aβ fibril and aggregate inhibition by curcumin in the assays we used were clearly greater than 1:1, consistent with the idea that curcumin does not bind monomeric Aβ, but to some secondary structure involved in later stages of assembly. Thus, we evaluated whether fibril formation or a specific sequence is necessary for curcumin binding. Various fragments of Aβ were incubated for 10 days at 37ºC to allow fibrils to form. These sequences are listed in Table 1. An aliquot was removed for EM analysis before solutions were incubated with 1 µM curcumin. After centrifugation, supernatants were also applied to Tg2576 brain sections to label plaques. EM analysis demonstrated that with our conditions full length Aβ40 and Aβ42 formed fibrils or protofibrils depending on whether they were dissolved in dH₂O or HFIP (Table 1). Shorter peptide sequences such as Aβ1-28, 12-28, and 25-35 also had the ability to form fibrils that were fluorescently labeled by curcumin on
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slides (not shown). Plaque-labeling was markedly reduced or absent by pre-absorption of curcumin with fibril forming Aβ peptides (Aβ12-28 and 25-35). In contrast, non-aggregating 17-28, a sequence containing the overlap of 12-28 and 25-35 did not absorb out or compete with curcumin plaque-labeling. In fact, even full length Aβ, when HFIP-monomerized, did not absorb out or compete with curcumin plaque-labeling. A range of other shorter Aβ peptides (1-13, 8-17, 14-24, 17-24, 34-40, 37-42) did not form fibrils and pre-absorption of the curcumin with these non-fibril forming Aβ peptides had no effect on curcumin's plaque labeling. Taken together, these experiments argue that, like other amyloid dyes, curcumin binds Aβ only if it is aggregated. That is, curcumin Aβ labeling does not depend on primary sequence alone but is dependent on aggregate-related structure.

Although we have shown that curcumin could limit amyloid accumulation in Aβ-infused rats and in Tg2576 mice when administered before extensive amyloid accumulation in Tg2576 mice (10-16 months old), other approaches that were successful in young APP transgenics have not been effective against the large amounts of deposited amyloid in older APP transgenics (26). Therefore, we tested curcumin's amyloid suppressing efficacy in a small cohort of Tg2576 with 500 ppm curcumin in chow beginning at 17 months, an age when amyloid accumulation is as great or greater than in human AD brain (27). Animals were sacrificed at 22 months of age and brains were removed for histological and biochemical analysis. Coronal sections from control (n=6) and curcumin mice (n=8) were stained with an antibody against Aβ1-13 (DAE). Plaque burden was visibly reduced throughout cortical and hippocampal areas in curcumin mice (Figure 8D-F) compared to control mice (Figure 8A-C). Statistical analysis revealed a significant reduction in plaque burden (-32.5%, P<0.0001, Figure 8G) and levels of the detergent-insoluble
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Aβ (-85%, P=0.02; Figure 8H). Thus, these data indicate that curcumin has potent anti-amyloidogenic activity, even in aged animals.
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**DISCUSSION**

The association of NSAID consumption with decreased AD risk is a consistent epidemiological finding (28-34). Potential mechanisms underlying apparent protection are being explored. A subset of NSAIDs (including ibuprofen) can directly lower Aβ42 production independently of cyclooxygenase inhibition and slow amyloid accumulation *in vivo* (35,36). Curcumin has NSAID activities independent of cyclooxygenase-inhibition (37-39) and reduces amyloid accumulation *in vivo* (12), but fails to reduce Aβ42 production *in vitro* (40). Thus, curcumin’s amyloid suppressing function must stem from another activity. A post-production mechanism inhibiting Aβ aggregation is consistent with curcumin's suppression of Aβ deposition in rat models with chronic intraventricular infusion of exogenous human Aβ (41).

Selected NSAIDs such as ibuprofen, naproxen, ketoprofen, and indomethacin and various compounds can bind to amyloid and inhibit Aβ aggregation (42,43). Aggregation inhibitors include Congo Red, its derivative chrysamine G, and RS-0406, a β-sheet breaker that inhibits Aβ42 fibrillogenesis and oligomer formation (14). CR can also bind to amyloid and block oligomer formation at low Aβ concentrations (9). Its fibril-binding properties are a result of a symmetrical sulfonated azodye structure with charge spaced across a hydrophobic bridge. This allows an alignment of CR molecules along the fibril axis with electrostatic interactions between the negative charges on CR and the positive charges on protonated anti-parallel Aβ. This charge can be replaced by polar groups such as those present in chrysamine G, making it more brain permeable than CR (10) (Figure 1). RS-0406, which was selected by high throughput screening, has analogous polar groups spaced by a hydrophobic bridge. Curcumin is similar to CR since it can also bind to plaques (Figure 2), prevent oligomer formation at similar low ID$_{50}$, and recognizes secondary structure in fibrillar and oligomeric Aβ. But like chrysamine G,
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curcumin’s symmetrical phenol groups make it more brain permeable than CR and able to cross the blood brain barrier to bind to plaques \textit{in vivo} (Figure 3).

Our ELISA and aggregation studies show that curcumin can inhibit aggregation or promote its disaggregation at low concentrations (IC$_{50}$=0.81-1 µM, Figure 4). Monomeric Aβ formed fewer aggregates in the presence of curcumin while increasing doses of curcumin promoted disassembly of pre-formed Aβ aggregates. Ultrastructural evidence, illustrating fewer amyloid fibrils in curcumin treated samples, supports anti-oligomer antibody and aggregation ELISA data with a completely different method. Sequences of Aβ that are instrumental in fibrillogenesis (eg. full length Aβ, Aβ1-28, Aβ12-28, and Aβ25-35) are known (44-46). Our experiments also suggested that curcumin could bind and inhibit fibrils formed from these fibrillogenic sequences. Despite the common overlapping sequence of these fibrillogenic peptides being aa 25-28, curcumin cannot bind non-aggregating peptides that span this region (Aβ17-28) (Table 1). This would argue that curcumin’s effects did not depend on Aβ sequence but on fibril-related conformation.

The aggregation ELISA demonstrates that curcumin inhibited Aβ aggregation better than the two NSAIDs, ibuprofen and naproxen. Ibuprofen has been shown to inhibit more than 50% of the β-sheet conformation when 100 µM ibuprofen was incubated with 12.5 µM Aβ25-35 (43). In our studies, doses as high as 16 µM of ibuprofen or naproxen did not effectively inhibit aggregation. Levels for naproxen to directly block aggregation (32 µM) are probably not achievable in brain in the absence of toxicity.

Soluble Aβ oligomers are more diffusible than amyloid fibrils, highly toxic, and increasingly viewed as playing an important role in AD pathogenesis (22-25,47). The observation that low or even submicromolar curcumin effectively blocks soluble Aβ oligomer
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formation and toxicity suggests curcumin's clinical potential to protect against oligomer-mediated synaptic or other neurotoxicity similar to RS-0406 *in vitro* (14). Consistent with this, oral curcumin reduced synaptic marker loss of CNS infused exogenous Aβ (41). It is unclear whether a subset of other phenolic antioxidants found in plant extracts, including gingko biloba (48) and resveratrol from red wine might be similarly protective and potentially contribute to AD risk reduction (49) because of effects on Aβ aggregation.

A clinical trial of another drug known to inhibit Aβ aggregation has been performed using a compound (clioquinol) that inhibits zinc and copper ions from binding to Aβ (50). This raises the question as to whether the direct binding to β-amyloid, metal chelation, is another possible mechanism to account for curcumin-mediated reduction in Aβ aggregates and oxidative damage. Metals can promote Aβ aggregation and clioquinol; a metal chelator of copper, zinc and iron; has been shown to dramatically reduce Aβ deposits in the same Tg2576 line used in our study (51). Because metals like copper can bind Aβ at picomolar (Aβ1-40) and even attomolar (Aβ1-42) concentrations and promote aggregation, high affinity metal chelators can limit Aβ aggregation in vitro where most buffers have trace (~0.1 µM) copper (52). Thus, curcumin chelation of both iron and copper (but not zinc) has been proposed as one mechanism potentially contributing to amyloid reduction in animal models (53). In the latter report, spectrophotometrically detected curcumin copper binding reached half-maximum at ~3-12 µM copper with positive cooperativity, with Kd1~ 10-60µM and Kd2~1.3µM, well above the ~0.1µM trace copper expected with in vitro buffers. As reviewed by Baum and Ng, it is not clear whether curcumin's avidity for copper and potential concentration in the brain will be high enough to directly alter CNS Aβ metal binding.
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An efficacious drug must also be bioavailable and safe at needed dose. We demonstrate that curcumin can prevent Aβ fibril formation at low doses in the 0.1-1 µM range which is consistent with a recent in vitro report from Ono et al (54). These doses can likely be achieved physiologically. Curcumin is rapidly glucuronidated after oral dosing so plasma levels remain low, despite high intake. However, high oral curcumin dosing appears safe; for example, 4-8 gram doses in human patients produce no toxic effects, with peak serum curcumin levels of 0.51-1.77 µM (55). Mouse brain curcumin levels of 0.41 µg/g (~1.1 µM) were measured one hour after dosing that produced 0.6 µg/g (1.6 µM) in the plasma (56), suggesting that achievable brain levels may be close to blood levels. Curcumin-iron interaction reached half-maximum at ~2.5-5 µM iron and exhibited negative cooperativity, with Kd1~0.5-1.6 µM and Kd2~50-100 µM, suggesting that some iron chelation may be achieved at brain curcumin concentrations in the submicromolar range (53). We have found that oral curcumin treatment reduces CNS iNOS, inflammatory cytokines and lipid peroxidation (57). Brain levels of curcumin in the 0.1-1 µM range are similar to those required to inhibit CNS AP-1 mediated transcription in vivo (58) and related suppression of iNOS (59) and antioxidant activities. Curcumin can inhibit lipid peroxidation better than vitamin E (60) and is a more potent antioxidant. Its IC<sub>50</sub> for Aβ aggregation is slightly below its IC<sub>50</sub> for lipid peroxidation (1.3µM) (61,62), indicating that curcumin would be effective at concentrations required for desirable antioxidant and anti-inflammatory activities. While higher (>5 µM) doses of curcumin have been reported to inhibit multiple kinases and enzyme activities and bind metals, these phenomena are not likely to occur in brain with oral dosing. On the other hand, in vitro efficacy may not predict in vivo results. For example, while curcumin and another polyphenolic, rosmarinic acid, were found to inhibit Aβ aggregation in vitro (54), we find that rosmarinic acid actually increases Aβ accumulation in Aβ
Curcumin blocks amyloid aggregation infused rodent brain (63), which was in sharp contrast to curcumin’s effect in the same model (41) and in our current in vivo results.

The final proof of any anti-amyloid effect is in vivo testing. Because amyloid accumulation begins decades before diagnosis, anti-amyloid therapy would ideally begin prior to clinical symptoms. Approaches that remain efficacious at advanced stages of amyloid accumulation are clearly needed; and our in vivo observations, suggest curcumin may even be beneficial even after the disease has developed.
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**Conclusion:**

Curcumin is widely used at low doses as a yellow food dye and at higher doses in traditional Indian "Ayurvedic" medicine, typically as a turmeric extract. Because of its use as a food additive and its potential for cancer chemoprevention, curcumin has undergone extensive toxicological screening and pre-clinical investigation in rats, mice, dogs and monkeys (11,64). In clinical trials, cancer patients have not shown adverse effects with doses from 2,000 to 8,000 mg per day (55). Thus, our data showing low dose inhibition of amyloid oligomer and fibril formation as well as suppression in aged animals combined with published antioxidant, anti-inflammatory and anti-amyloid activities in two animals models (15,41), provide an increasingly compelling rationale for clinical trials for curcumin in the prevention or even treatment of AD.
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FIGURE LEGENDS

Figure 1. Chemical structures of curcumin, RS-0402, Congo Red, and chrysamine G. Structures (not drawn to scale) have a common theme of two charge or polar groups separated by a hydrophobic bridge.

Figure 2. Curcumin stains amyloid plaques like thioflavin S in AD and Tg2576 brain sections. Panels A and B are adjacent sections from 22 month APPsw Tg2576 brain, while panels C and D are adjacent sections of AD hippocampus. Panel E is from the same section as Panel B, but the right half of the field was exposed for 3 minutes longer than the left half of the field. Arrows point to lipofuscin. Panels A and C are stained with 1% thioflavin S while panels B, D, and E are labeled with curcumin (1 µM). Curcumin fluorochromes produce orange to yellow/green fluorescent plaques, and thioflavin S results in much better tangle staining. Panel A and B, Bar=80 µm and Panel C-E, Bar=25 µm

Figure 3. Curcumin crosses blood brain barrier and binds to plaques in vivo. Tg2576 mice (22 months old) were injected with curcumin or vehicle as described. Mice were perfused an hour later with protease inhibitor buffer, and brains were removed and snap frozen. Unfixed cryosections (12 µM) were coverslipped and photographed with FITC filter sets (494nm/520nm). Panel A is a section from a PBS-injected mouse and shows no plaque staining. Panel B is a section from PBS-injected mouse on chronic curcumin (500 ppm) for 5 months and shows weaker plaque staining. Panels C and D are entorhinal cortex and hippocampal regions, respectively, from a mouse injected with 50µM curcumin (200 µL) as described in methods. Examples of plaque staining are illustrated in these panels. Bar = 40 µm.
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**Figure 4** Curcumin inhibits aggregation of Aβ40 and promotes its disaggregation. The presence of aggregated Aβ was analyzed by ELISA (6E10/6E10) and FSU was plotted against the dose of curcumin (log scale). **A.** Inhibition of Aβ40 aggregation. This is a plot of Aβ40 (final conc 50 µg/ml) incubated with increasing doses of curcumin (0-8 µM) for 6 days at 37°C. Samples were assayed with 6E10/6E10 sandwich ELISA in triplicate. Curcumin was found to block aggregation of Aβ40, with an IC\textsubscript{50} =~0.81 µM (gray circles; \textit{P}<0.001). **B.** Induction of disaggregation. Filled circles depict a plot of Aβ40 (100 µg/ml) pre-aggregated for 3 days and then incubated with curcumin (0-8 µM) for 3 days at 37°C. When Aβ40 was pre-aggregated, increasing doses of curcumin were able to promote disaggregation of Aβ40, where the IC\textsubscript{50} =~1 µM (closed circles, \textit{P}<0.005). **Panels C-E** illustrate that curcumin is a better Aβ aggregation inhibitor than ibuprofen or naproxen. Aβ (50 µg/ml) was incubated with curcumin (0-8µM, Panel C), naproxen (0-32 µM, Panel D), or ibuprofen (0-32 µM, Panel E). Samples were incubated for 6 d at 37°C and the presence of Aβ40 aggregates was determined with 6E10/6E10 ELISA. The bar graphs represent the average from three samples and error bars represent standard deviation.

**Figure 5** Electron microscopic analysis of curcumin preventing fibril formation and promoting its disaggregation. A small aliquot (3 µL) was applied to grids for EM analysis. **Panels A-C:** Curcumin inhibited Aβ40 aggregation. Aβ40 (50 µg/ml) was allowed to aggregate for 6 days at 37°C with vehicle alone (A) or with curcumin (B, 0.125 µM and C, 2 µM). **Panels D-E:** Curcumin disaggregated preformed Aβ40 fibrils. Aβ40 (100 µg/ml) fibrils were made for 3 day
Curcumin blocks amyloid aggregation at 37°C and then further incubated with vehicle alone (D) or with curcumin (E, 0.125 uM and F, 2 uM) for an additional 3 days at 37°C. Bar =2µm. Original magnification=8900X.

**Figure 6** Curcumin inhibits formation of Aβ oligomers. A. Scan and bar graph of dot blot where Aβ40 oligomers were incubated with curcumin. Aβ40 (400 µg/ml) were incubated for 48 hr at 25 ºC. Curcumin was used at 0, 2 and 16 µM. Samples were immunoblotted as a dot blot and probed with A11 antibody which is specific for oligomers. Dot blot was scanned and analyzed with Molecular Analyst and one way ANOVA was used to determine significance. *** indicates a P value <0.001. Error bars represent standard error. B. Scan and bar graph of dot blots from panel A probed with 6E10 antibody against Aβ (1:10,000). Dot blots were scanned and analyzed in a similar fashion. C. Western blot of Aβ42 oligomers and various doses of curcumin (Curc) and Congo Red (CR). Aβ42 (5 µM) was incubated at 37ºC for 4 h with curcumin, Congo Red, or no compound. Samples were centrifuged at 14,000 g (4ºC for 10 min) and the supernatants were immunoblotted with 6E10 (1:2000). Bands at 3.5 kDa represent the monomeric Aβ form while the smear between 44-127 kDa are the oligomeric form of Aβ. Lane 1 is Aβ42 monomer (no aggregation) only. Lane 2-7 are Aβ42 and curcumin (0, 0.25, 1, 4, 16, and 64 µM) while lane 8 and 9 are Aβ42 and CR (16 and 64 µM). D. Western blot of 3.5 kDa monomeric Aβ band. This is a lighter exposure of the same blot seen in panel C and better illustrates the increase in monomer Aβ as curcumin dose increases.

**Figure 7** Curcumin blocks toxicity of Aβ42 oligomer in differentiated SH-SY5Y neuroblastoma cells. A. Oligomer-dependent toxicity was first evaluated in N2a cells. Aβ42 (100 nM) was incubated with and without the oligomer-specific A11 antibody (1:500) for 72 h before media was collected for LDH measurement. *** indicates P<0.0001 when compared to Aβ42 alone. B.
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Aβ42 oligomer (100 nM) was added with or without curcumin (0-5 µM) to differentiated SY5Y cells for 48 hr. MTT and LDH assay were used to determine cell viability and cell toxicity and were performed in triplicate. Data was analyzed by one-way ANOVA to evaluate treatment effect. *** indicates p-value <0.0001 when compared to Aβ42 oligomer only. ** indicates P-value<0.001 when compared to Aβ42 oligomer only.

**Figure 8** Curcumin suppresses amyloid accumulation in aged APP transgenic mice. Tg2576 mice were placed on chow with (n=4) or without 500 ppm curcumin (n=6) at 17 months of age. At 22 months of age, brains were removed for histological and immunological measurement (ELISA) of amyloid. Panels A-F depict plaque burden analyzed from coronal sections through anterior and posterior hippocampus of control mice (Panels A-C) and curcumin-treated mice (Panels D-F). Representative photos of low magnification views show fewer amyloid deposits in curcumin mice (D) compared to control mice (A). Bar = 1 mm. This suppression can be easily seen with the higher magnification photos from anterior (B and E) and posterior hippocampal sections (C and F). Bar= 40 µm. Image analysis revealed a significant treatment effect, where plaque burden was reduced by 32.5% in curcumin treated animals (P<0.0001) (Panel G). Panel H is a bar graph of ELISA measurements of total guanidine-soluble Aβ showing an 85% reduction in Aβ levels with curcumin treatment. Samples of 5M guanidine extracts were applied in triplicate and data was square root transformed to achieve quality of variance before subjection to one-way ANOVA. P values <0.05 were considered significant.
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Table 1: Curcumin binds to peptide sequences that form fibrils or protofibrils

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Presence of fibrils/protofibrils?</th>
<th>Intensity of plaque labeling with supernatant</th>
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</thead>
<tbody>
<tr>
<td>1-40 (dH2O)</td>
<td>Fibrils</td>
<td>+</td>
</tr>
<tr>
<td>1-42 (dH2O)</td>
<td>Fibrils</td>
<td>No staining</td>
</tr>
<tr>
<td>1-40 (HFIP)</td>
<td>Protofibrils</td>
<td>++</td>
</tr>
<tr>
<td>1-42 (HFIP)</td>
<td>Protofibrils</td>
<td>++</td>
</tr>
<tr>
<td>1-28</td>
<td>Protofibrils</td>
<td>+</td>
</tr>
<tr>
<td>12-28</td>
<td>Protofibrils</td>
<td>++</td>
</tr>
<tr>
<td>25-35</td>
<td>Protofibrils</td>
<td>+</td>
</tr>
<tr>
<td>1-13</td>
<td>None</td>
<td>+++</td>
</tr>
<tr>
<td>8-17</td>
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</tr>
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<tr>
<td>37-42</td>
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</tr>
<tr>
<td>Curcumin only</td>
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</tr>
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</table>

Schematic diagram and table of curcumin binding experiments. Aβ was aggregated for 10 days at 37°C before an aliquot was removed for EM analysis. Curcumin was incubated with samples before they were centrifuged. Pellets were smeared on glass slides to check for Aβ binding while supernatants were applied to Tg2576 mouse brain sections for plaque labeling. Weak staining was defined 0-++ and strong staining was +++/++++. Fibrils/protofibrils were determined by estimating thickness and length of fibers.
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REFERENCES

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Curcumin

RS-0406

Congo Red

Chrysamine G

Figure 1
Figure 3
A Inhibition of Aβ40 aggregation

B Disaggregation of Aβ40 aggregates

IC \_50 = 0.81 \mu M P = 0.0004

IC \_50 = 1.0 \mu M P = 0.0015

C Curcumin

D Naproxen

E Ibuprofen

Figure 4
Figure 6
Figure 7

**A**

Cell viability (MTT reduction % of control)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Vehicle</td>
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<tr>
<td>A11 only</td>
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</tr>
<tr>
<td>Aβ42</td>
<td>30%</td>
</tr>
<tr>
<td>Aβ42 + A11</td>
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**B**

% of LDH release

<table>
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<th>Treatment</th>
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<tbody>
<tr>
<td>Vehicle</td>
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<tr>
<td>Aβ42 (100 nM)</td>
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</tr>
<tr>
<td>Curc (0.1 μM)</td>
<td>40%</td>
</tr>
<tr>
<td>Curc (1 μM)</td>
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</tr>
<tr>
<td>Curc (2.5 μM)</td>
<td>60%</td>
</tr>
<tr>
<td>Curc (5 μM)</td>
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**C**

Vehicle

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Aβ42</td>
<td>25%</td>
</tr>
<tr>
<td>Aβ42 + A11</td>
<td>30%</td>
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</table>

Note: ***p < 0.001**
Figure 8
Curcumin inhibits formation of Aβ oligomers and fibrils and binds plaques and reduces amyloid in vivo

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