Comparative Study of Inhibition at Multiple Stages of Amyloid-β Self-Assembly Provides Mechanistic Insight

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ABSTRACT

The “amyloid cascade hypothesis,” linking self-assembly of the amyloid-β protein (Aβ) to the pathogenesis of Alzheimer’s disease, has led to the emergence of inhibition of Aβ self-assembly as a prime therapeutic strategy for this currently unpreventable and devastating disease. The complexity of Aβ self-assembly, which involves multiple reaction intermediates related by nonlinear and interconnected nucleation and growth mechanisms, provides multiple points for inhibitor intervention. Although a number of small-molecule inhibitors of Aβ self-assembly have been identified, little insight has been garnered concerning the point at which these inhibitors intervene within the Aβ assembly process. In the current study, a julolidine derivative is identified as an inhibitor of Aβ self-assembly. To gain insight into the mechanistic action of this inhibitor, the inhibition of fibril formation from monomeric protein is assessed quantitatively and compared with the inhibition of two distinct mechanisms of growth for soluble Aβ aggregation intermediates. This compound is observed to significantly inhibit soluble aggregate growth by lateral association while having little effect on soluble aggregate elongation via monomer addition. In addition, inhibition of soluble Aβ aggregate association exhibits an IC₅₀ with a somewhat lower stoichiometric ratio than the IC₅₀ determined for inhibition of fibril formation from monomeric Aβ. This quantitative comparison of inhibition within multiple Aβ self-assembly assay suggests that this compound binds the lateral surface of on-pathway intermediates exhibiting a range of sizes to prevent their association with other aggregates, which is required for further assembly into mature fibrils.

Alzheimer’s disease (AD) is currently the most common type of dementia, affecting an estimated 5.2 million Americans (Alzheimer’s Association, 2008). As the life expectancy in the United States and other postindustrialized nations increases, AD presents a burgeoning epidemic. AD initially affects short-term memory and progresses to include pervasive cognitive and emotional dysfunction. These manifested symptoms are hypothesized to result from a cascade of events initiated by the self-assembly of monomeric amyloid-β protein (Aβ), leading first to the formation of soluble aggregates and later progressing into larger insoluble fibrils, which ultimately deposit in the extracellular space of the brain parenchyma. This “amyloid cascade hypothesis” is supported by experimental evidence (Walsh and Selkoe, 2007), including genetic correlations, transgenic animal models, and cell culture studies, and has established the inhibition of Aβ self-assembly as a prime therapeutic strategy in the fight against AD.

Several small molecules that inhibit the in vitro formation of amyloid fibrils from monomeric Aβ have been identified (Findeis, 2000; Hamaguchi et al., 2006; LeVine, 2007). Studies using quantitative measures of inhibition assembled from light scattering measurements, thioflavin T (ThT) fluorescence, or immunoassays have facilitated comparisons of inhibitory potential among different molecular structures (Howlett et al., 1999a,b; Ono et al., 2003, 2004; Ferrão-Gonzales et al., 2005; Byeon et al., 2007; Reinke and Gestwicki, 2007; Dolphin et al., 2008, 2009). The majority of these studies, however, have quantified the

ABBREVIATIONS: AD, Alzheimer’s disease; Aβ, amyloid-β protein; DLS, dynamic light scattering; DMF, dimethylformamide; DMSO, dimethylsulfoxide; HFIP, hexafluoroisopropanol; Rᵥ, hydrodynamic radius; TEM, transmission electron microscopy; ThT, thioflavin T; SEC, size-exclusion chromatography.
effect of small-molecule inhibitors on the overall extent of fibril formation without recognition that Aβ self-assembly is a complex process involving multiple pathways and numerous on-pathway assembly intermediates, including oligomers, protofibrils, and other soluble aggregates (LeVine, 2007; Necula et al., 2007b). Some small-molecule inhibitors selectively halt the formation of mature Aβ fibrils from monomer without stopping the formation of soluble aggregates (Bohrmann et al., 2000) or the growth of preformed fibrils (LeVine, 2007). Alternatively, small-molecule inhibitors have been observed to promote fibril formation but inhibit ongoing fibril growth (Williams et al., 2005) and to selectively inhibit different mechanisms of soluble aggregate growth (Moss et al., 2004). In addition, although some small molecules prevent both oligomer and fibril formation (Howlett et al., 1999a; De Felice et al., 2004; Yang et al., 2005; Bastianetto et al., 2006; Necula et al., 2007b), other inhibitors halt the appearance of oligomers without altering mature fibril formation (Necula et al., 2007a) or, conversely, block the appearance of mature fibrils while permitting oligomer formation (Lashuel et al., 2002; Ferrão-Gonzales et al., 2005; Necula et al., 2007b). These qualitative studies demonstrate that small-molecule inhibitors of Aβ self-assembly can selectively act on various assembly pathways.

Quantitative measures can provide additional information about inhibitor action but have been infrequently used to assess the ability of a single compound to interrupt different steps along the self-assembly pathway. Some small molecules have been shown to inhibit the distinct processes of fibril formation and fibril extension with similar IC50 values (Ono et al., 2003, 2004; Dolphin et al., 2008). However, these studies examined mature Aβ fibrils, which are end products of the assembly process, but did not consider soluble Aβ aggregates, currently speculated to play a principal role in AD progression (Kirkitude et al., 2002; Walsh and Selkoe, 2007). In the current study, a julolidine aldehyde is investigated quantitatively for its ability to prevent Aβ1-40 fibril formation from monomer and to slow the growth of soluble Aβ1-40 aggregates via two distinct mechanisms: elongation by monomer addition, and direct lateral association (Nichols et al., 2002). Although soluble aggregate elongation is unaffected, inhibition of both the extent of aggregate formation from monomeric protein and the growth of soluble aggregates by lateral association is observed, suggesting an interaction with an on-pathway intermediate and indicating the contribution of lateral aggregate association to final aggregate content. IC50 values determined for these two processes suggest that this inhibitor is capable of binding a range of aggregate sizes, including aggregate species that appear early in the self-assembly pathway. Together, these results illustrate the mechanistic insight provided by quantitative comparisons of inhibitor action at different stages of Aβ self-assembly.

Materials and Methods

Materials. Aβ1-40 peptide was purchased from AnaSpec, Inc. (San Jose, CA) or W. M. Keck Biotechnology Resource Laboratory at Yale University (New Haven, CT). Hexafluoroisopropanol (HFIP), ThT, phosphorous oxychloride, 8-hydroxy-2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinoline-9-carbaldehyde (compound 2) was identified as a promising inhibitor of Aβ self-assembly after the screening of a large library of aromatic compounds (D. D. Soto-Ortega, Q. Wang, M. A. Moss, unpublished results). To synthesize this compound (Scheme 1), phosphorous oxychloride (0.11 mg, 0.70 mmol) was added drop-wise to a precooled solution of DMP (2 ml) at 0°C, and the mixture was stirred at this temperature for 30 min. To this mixture, a solution of 8-hydroxyxylolidine (compound 1) (120 mg, 0.63 mmol) in DMP (2 ml) was added drop-wise in a period of 45 min at 0°C as the color of the reaction mixture turned to green from colorless. The reaction was heated gradually to 80°C. Then, the reaction was quenched by pouring into 20 g of ice, and the pH was adjusted to 7–8 using 10 M NaOH. The mixture was stirred for 15 min and filtered. The filtrate was concentrated by rotary evaporation, and the residue was purified by flash column chromatography (4:1 hexane/ethyl acetate). The compound was recrystallized from ethyl acetate/hexane to give the pure product.

Preparation of Aβ1-40 Monomer and Soluble Aggregates. Lyophilized Aβ1-40 peptide was dissolved in 0.1% NaOH at a concentration of 2 mg/ml, and pre-existing aggregates were removed by size-exclusion chromatography (SEC) on a Superdex 75 HR10/30 column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) pretreated with bovine serum albumin. Aβ1-40 monomer concentrations were determined from UV absorbance at 276 nm using an extinction coefficient of 1450·M·1·cm−1 (Nichols et al., 2002). Isolated Aβ1-40 monomer was used fresh or stored at 4°C for up to 48 h.

Soluble Aβ1-40 aggregates were prepared from isolated monomeric Aβ1-40 as described previously (Gonzalez-Velasquez and Moss, 2008; Kotarek et al., 2008). In brief, monomeric Aβ1-40 (100–200 μM) was agitated vigorously at 25°C for 4 to 15 h in the presence of 2 to 10 mM NaCl and 40 mM Tris-HCl, pH 8.0. Aggregation was monitored by ThT fluorescence as described below. Soluble Aβ1-40 aggregates were separated from fibril via centrifugation and from unreacted monomer via SEC on a Superdex 75 column. Soluble Aβ1-40 aggregate concentrations, expressed in monomer units, were determined from UV absorbance at 276 nm corrected for light scattering (Nichols et al., 2002), and equivalent ThT measurements were determined. Isolated soluble aggregates were stored at 4°C and used for experimentation within 36 h of purification. After storage, ThT fluorescence measurements were used to correct for any change in aggregate concentration, and dynamic light scattering (DLS) measurements were assessed to ensure that aggregates maintained their initial size.

Detection of Aβ1-40 Aggregates Using ThT. The presence of amyloid aggregates was monitored via fluorescence determination as described previously (Gonzalez-Velasquez and Moss, 2008; Kotarek et al., 2008) for solutions containing Aβ1-40 in the presence of 10 or 40 μM ThT and 40 mM Tris-HCl, pH 8.0. ThT fluorescence was measured using an LS-45 luminescence spectrometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) with excitation at 450 nm and emission at 485 nm for 15 min. All ThT measurements were performed at 0°C, and the reaction was monitored using a computer controlled with a quartz cell of 1 cm with the conventional path length.

Synthesis of 8-Hydroxy-2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinoline-9-carbaldehyde. 8-Hydroxy-2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinoline-9-carbaldehyde (compound 2) was identified as a promising inhibitor of Aβ self-assembly after the screening of a large library of aromatic compounds (D. D. Soto-Ortega, Q. Wang, M. A. Moss, unpublished results). To synthesize this compound (Scheme 1), phosphorous oxychloride (0.11 mg, 0.70 mmol) was added drop-wise to a precooled solution of DMP (2 ml) at 0°C, and the mixture was stirred at this temperature for 30 min. To this mixture, a solution of 8-hydroxyxylolidine (compound 1) (120 mg, 0.63 mmol) in DMP (2 ml) was added drop-wise in a period of 45 min at 0°C as the color of the reaction mixture turned to green from colorless. The reaction was heated gradually to 80°C. Then, the reaction was quenched by pouring into 20 g of ice, and the pH was adjusted to 7–8 using 10 M NaOH. The mixture was stirred for 15 min and filtered. The filtrate was concentrated by rotary evaporation, and the residue was purified by flash column chromatography (4:1 hexane/ethyl acetate). The compound was recrystallized from ethyl acetate/hexane to give the pure product.

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adjusted to neutral using saturated sodium bicarbonate solution. Upon being stirred at 25°C overnight, the precipitated product was collected by filtration. Washing with water and recrystallization from hexane yielded 90 mg of a buff-colored solid; yield, 65%. 1H NMR (300 MHz, CDCl3): δ 9.40 (s, 1H), 6.80 (s, 1H), 3.23 (m, 4H), 2.64 to 2.70 (m, 4H), 1.84 to 1.89 (m, 4H).

Dissolution of Compound 2 for Experimentation. Compound 2 was dissolved at 2 to 20 mM in DMSO and stored at −20°C. Stock solutions of compound 2 were diluted in DMSO such that subsequent dilution into final reactions resulted in varying concentrations of compound 2 in the presence of a constant 2.5% (v/v) DMSO. DMSO 2.5% (v/v) was also added to control reactions run in parallel. This level of DMSO was confirmed to have no interference with DLS signals used to monitor association reactions. For association reactions, stock solutions of compound 2 were additionally filtered through a 0.2-µm polytetrafluoroethylene Minispike Acrosid syringe filter (Pall Life Sciences, Ann Arbor, MI) before dilution to ensure a clean light-scattering signal. UV measurements confirmed negligible loss of the compound during the filtering procedure.

ThT Detection of Aβ1-40 Aggregates in the Presence of Compound 2. ThT detection of Aβ1-40 aggregates in the presence of compound 2 was carried out to ensure that ThT fluorescence is unchanged by the simultaneous binding of both compounds to Aβ1-40 aggregates. Unpurified Aβ1-40 aggregate preparations in 40 mM Tris-HCl, pH 8.0, were combined with compound 2 and ThT. Incubations contained either 2 µM Aβ1-40 aggregates, 0 to 50 µM compound 2 with 2.5% (v/v) DMSO, and 10 µM ThT to reflect expected final concentrations in monomer aggregation assay measurements or 20 µM soluble Aβ1-40 aggregates, 0 to 500 µM compound 2 with 2.5% (v/v) DMSO, and 40 µM ThT to reflect the expected final concentrations in HFIP-induced monomer aggregation assays. Solutions were incubated at 4°C for 15 min to ensure complete binding, and ThT fluorescence was evaluated. Detection is expressed as the percentage of the total fluorescence that was observed for an equivalent concentration of soluble Aβ1-40 aggregates in the absence of compound 2.

Aβ1-40 Monomer Aggregation Assay. SEC-isolated Aβ1-40 monomer in 40 mM Tris-HCl, pH 8.0, was combined with compound 2 in the presence of NaCl for final concentrations of 20 µM monomer, 150 mM NaCl, and 0 to 400 µM compound 2 with 2.5% (v/v) DMSO, where reactions containing 0 µM compound 2 served as the positive control. Reactions were incubated at 25°C under continuous agitation (vortex, 800 rpm) to promote nucleation. During the time course of aggregation, 20-µl aliquots were periodically withdrawn from the reaction solution, combined with 140 µl of 0 µM ThT, and evaluated for ThT fluorescence.

HFIP-Induced Aβ1-40 Monomer Aggregation Assay. SEC-isolated Aβ1-40 monomer in 40 mM Tris-HCl, pH 8.0, was combined with compound 2 in the presence of ThT and incubated at 4°C for 15 min to ensure complete binding. To initiate monomer aggregation, diluted HFIP was added for final concentrations of 2.5% (v/v) HFIP, 20 µM Aβ1-40 monomer, 0 to 500 µM compound 2 with 2.5% (v/v) DMSO, and 40 µM ThT, where reactions containing 0 µM compound 2 served as the positive control. Reactions were incubated without agitation at 25°C, and ThT fluorescence was continuously monitored. Plateau fluorescence values were taken as representative of the total aggregate formation, and aggregate formation is reported as a percentage of the positive control.

**Scheme 1.** Synthesis of compound 2.

**Soluble Aβ1-40 Aggregate Elongation Assay.** SEC-isolated soluble Aβ1-40 aggregates in 40 mM Tris-HCl, pH 8.0, were combined with compound 2 in the presence of ThT and incubated without agitation at 4°C for 15 min to allow binding. To initiate aggregate growth by elongation, SEC-isolated Aβ1-40 monomer was added for final concentrations of 20 µM Aβ1-40 monomer, 1 µM soluble Aβ1-40 aggregates, 0–80 µM compound 2 with 2.5% (v/v) DMSO, and 10 µM ThT. Reactions containing 0 µM compound 2 served as the positive control. Reactions were incubated without agitation at 25°C. Incorporation of monomer into growing aggregates, or aggregate elongation, was monitored by periodically evaluating ThT fluorescence. Experiments in which ThT fluorescence of soluble Aβ1-40 aggregates was monitored in the absence of added monomer or the ThT fluorescence of Aβ1-40 monomer was monitored in the absence of added soluble aggregates served as negative controls and reflected the stability of soluble aggregates and monomer, respectively. Results are reported as the change in ThT fluorescence with time. Elongation rates were determined by regression of the linear portion of this data. The percentage inhibition was calculated from the ratio of the experimental elongation rate to the elongation rate observed in the absence of inhibitor.

**Soluble Aβ1-40 Aggregate Association Assay.** SEC-isolated soluble Aβ1-40 aggregates in 40 mM Tris-HCl, pH 8.0, were combined with filtered compound 2 and incubated without agitation at 4°C for 15 min to allow binding. To initiate aggregate growth by association, concentrated NaCl was added for final concentrations of 150 to 300 mM NaCl, 1 or 2 µM soluble Aβ1-40 aggregates, and 0 to 40 µM compound 2 with 2.5% (v/v) DMSO, where reactions containing 0 µM compound 2 served as the positive control. Concentration ranges for both soluble Aβ1-40 aggregates and NaCl were used as a result of the inherent variation in association rates among soluble Aβ1-40 aggregate preparations. Parallel positive control and inhibition reactions performed within a single experiment were designed to contain identical concentrations of both soluble Aβ1-40 aggregates and NaCl. Reactions were incubated without agitation at 25°C. Association of aggregates was monitored via measurement of increases in aggregate R1 using DLS. Experiments in which R1 of soluble Aβ1-40 aggregates was monitored in the absence of added NaCl served as a negative control and reflected the stability of the soluble Aβ1-40 aggregates. Results are reported as the change in R1 with time. Association rates were determined by regression of the linear portion of this data and are reported as a percentage of positive control. The percentage inhibition was calculated from the ratio of the experimental association rate to the association rate observed in the absence of inhibitor.

**Transmission Electron Microscopy.** A 20-µl sample containing soluble Aβ1-40 aggregates was placed upon a formvar-supported nickel grid (Electron Microscopy Sciences). The sample solution was wicked away using a piece of filter paper placed at the bottom side of the grid. Sample application was repeated in this manner until the grid contained a sufficient quantity of sample for visualization. The gridded sample was then stained with 2% uranyl acetate for 12 min. The staining solution was wicked away from the grid edge, and the grid was allowed to air dry. The dried grid was visualized using a Hitachi H-8000 transmission electron microscope (Hitachi High Technologies America, Pleasanton, CA).

**Statistical Analysis.** Statistical analysis was performed using Prism 5 software (GraphPad Software Inc., San Diego, CA). Differences among independent groups were assessed using a one-way analysis of variance, where p < 0.05 was considered significant. Linear and nonlinear regressions were assessed using the coefficient of determination, r².

**Results**

Compound 2 Inhibits Aggregation of Aβ1-40 Monomer. To characterize the effect of compound 2 on the self-assembly of Aβ, the 40-residue isoform of Aβ, Aβ1-40, was...
selected. $A_{\beta-40}$ is the most abundant isoform in vivo (Walsh and Selkoe, 2007), and the compositional analysis of amyloid plaques suggests that $A_{\beta-40}$ deposits at more advanced stages of plaque formation (Güntert et al., 2006). These characteristics render $A_{\beta-40}$ practical for studying later stages of $A\beta$ self-assembly that are considered in more detail in this study. $A_{\beta-40}$ monomer aggregation was induced by continuous agitation in the presence of salt, and the appearance of $\beta$-sheet aggregates was monitored using ThT fluorescence. This assay format facilitated the rapid characterization of aggregation behavior without regard to growth mechanisms. Similar aqueous-based assay formats have been used to identify other small-molecule inhibitors of $A\beta$ self-assembly (Ono et al., 2003, 2004; Kanapathipillai et al., 2005; Dolphin et al., 2008). Extensions of the lag time, reductions in the rate of fluorescence increase, and decreases in the plateau fluorescence have all been used as positive measures of inhibition.

Aggregation of 20 $\mu$M $A_{\beta-40}$ monomer displayed a time course of ThT fluorescence characteristic of $A\beta$ self-assembly, which exhibited a lag time that was followed by a period of rapid growth and concluded with a plateau as equilibrium was reached (Fig. 1). The presence of 2.5% DMSO, used to facilitate solubilization of compound 2, had a negligible effect on aggregation of 20 $\mu$M $A_{\beta-40}$ monomer (data not shown). When compound 2 was present at concentrations equimolar with $A_{\beta-40}$ monomer, the lag time was increased, indicating the inhibition of $A_{\beta-40}$ self-assembly by compound 2. At a concentration 2-fold in excess of $A_{\beta-40}$ monomer, compound 2 additionally reduced the rate of growth observed after the lag period, indicating a more pronounced inhibition of $A_{\beta-40}$ self-assembly at a higher concentration of this small molecule. When the concentration of compound 2 was further increased to a level 20-fold in excess of $A_{\beta-40}$ monomer, nearly complete inhibition was observed over the 1.5-h period. These results demonstrate that the presence of compound 2 results in a dose-dependent decrease in the formation of $A_{\beta-40}$ aggregates from monomeric protein.

**ThT Fluorescence of Preformed $A_{\beta-40}$ Aggregates Is Unchanged in the Presence of Compound 2.** An alternative explanation for the data presented in Fig. 1 might be that compound 2 impedes the detection of $A\beta$ aggregates via disruption of the binding of ThT to the amyloid $\beta$-sheet structure. Such a complication has been observed for other small molecules that recognize aggregated forms of $A\beta$, including the ThT analog BTA-1 (LeVine, 2005) and the unrelated compound nordihydroguaiaretic acid (Moss et al., 2004). To discount this possible explanation for the observed reductions in fluorescence, ThT detection of preformed $A_{\beta-40}$ aggregates was assessed in the presence of compound 2. $A_{\beta-40}$ aggregates formed in the absence of compound 2 were incubated with both ThT and compound 2, and the decrease in fluorescence detection relative to that observed for aggregates incubated with ThT alone was determined. As shown in Fig. 2, the fluorescence detection of 2 $\mu$M $A_{\beta-40}$ aggregates in the presence of 10 $\mu$M ThT was decreased by at most 15% in the presence of concentrations of compound 2 as high as 50 $\mu$M. These concentrations are representative of the diluted samples used for measurements in the aqueous-based $A_{\beta-40}$ monomer aggregation assay, in which excess concentrations of compound 2 led to reductions in ThT fluorescence of 58 to 96%. This result confirms that reductions in fluorescence observed during $A_{\beta-40}$ monomer aggregation assays are reflective of the dose-dependent inhibition of $A_{\beta-40}$ monomer aggregation by compound 2 and not the disruption of $A_{\beta-40}$ aggregate detection by ThT.

**Dose-Dependent Inhibition of $A_{\beta-40}$ Monomer Aggregation in Dilute HFIP by Compound 2 Is Quantitatively Assessed.** Incubation of monomeric $A_{\beta-40}$ in dilute HFIP leads to the rapid formation of aggregates, which is attributed to the presence of microdroplets of HFIP within the aqueous solution. These aggregates display increased ThT fluorescence and a high content of $\beta$-structure characteristic of $A_{\beta-40}$ aggregates formed when aggregation is induced by agitation and high ionic strength (Nichols et al., 2005). However, relative to the formation of $A_{\beta-40}$ aggregates induced by agitation and high ionic strength, $A_{\beta-40}$ aggregate formation induced in the presence of dilute HFIP exhibits an abolished lag phase, during which stochastic...
nucleation events occur, and yields a more uniform aggregate population consisting of predominantly soluble aggregate structures (Nichols et al., 2005). In combination with a time course that is several orders of magnitude shorter, these reaction conditions result in a highly reproducible Aβ1–40 monomer aggregation that is more amenable to quantitative evaluation. As a result, to quantify the effect of compound 2 on the overall extent of Aβ1–40 self-assembly, aggregation of Aβ1–40 monomer was carried out in the presence of dilute HFIP and monitored in situ using ThT fluorescence.

When 20 μM Aβ1–40 monomer was incubated in the presence of 2.5% HFIP, an immediate and rapid increase in ThT fluorescence was observed, and a plateau in fluorescence was reached within 20 min (Fig. 3A). This time course for aggregate formation in the presence of dilute HFIP was similar to that observed by Nichols et al. (2005). As for monomer aggregation induced by agitation and high ionic strength, the presence of 2.5% DMSO, used to facilitate solubilization of compound 2, had a negligible effect on aggregation of 20 μM Aβ1–40 monomer in dilute HFIP (data not shown). When Aβ1–40 monomer was preincubated with compound 2 before the addition of HFIP, a decrease in both the rate of increase in ThT fluorescence and the plateau fluorescence level were observed (Fig. 3A), indicative of the ability of compound 2 to inhibit, respectively, the rate at which monomeric Aβ1–40 is converted into β-sheet aggregates and the overall extent of aggregate formation. Again, insignificant changes in ThT detection of preformed Aβ1–40 aggregates were noted under these assay conditions (Fig. 2, 20 μM Aβ1–40), confirming that the observed reductions in fluorescence are indicative of inhibition. Reduction in fluorescence became more pronounced as the concentration of compound 2 was increased from 60 to 200 μM, illustrating the dose-dependence of the inhibition. The ability of compound 2 to dose-dependently reduce Aβ1–40 aggregate formation was quantitatively assessed via comparison of experimental plateau ThT fluorescence values to those of the control (Fig. 3B). Using 10 different stoichiometric ratios of compound 2 to Aβ1–40 monomer, each using an Aβ1–40 monomer concentration of 20 μM, an IC50 value of 6.8 was determined, indicating that a nearly 7-fold excess of compound 2 is required to observe 50% inhibition of Aβ1–40 aggregate formation from monomeric protein.

**Compound 2 Fails to Alter Soluble Aβ1–40 Aggregate Elongation via Monomer Addition.** Assays that examine the extent of aggregate formation from monomeric protein are effective at identifying inhibitors and assessing their relative efficacy. However, mechanistic-specific assays are needed to ascertain the effect of an inhibitor on specific stages of aggregate growth that occur along the assembly pathway between monomer and mature fibril. In one known growth mechanism, existing Aβ1–40 aggregates grow via the addition of monomeric protein. This growth process creates new amylloid material, increases aggregate size, and results in aggregates with a mass-per-unit length similar to the starting material, suggesting that monomeric units are incorporated at the ends of growing aggregates (Nichols et al., 2002). The growth of soluble Aβ1–40 aggregates via the addition of monomer was resolved by incubation of isolated soluble aggregates in low-ionic-strength buffer and in the presence of excess monomeric protein. The incorporation of monomer into growing aggregates was monitored as the change in ThT florescence.

As shown in Fig. 4, whereas Aβ1–40 monomer or soluble aggregates incubated alone exhibited negligible changes in aggregate content, a steady increase in aggregate content was observed when 1 μM soluble Aβ1–40 aggregates were incubated in the presence of 20 μM Aβ1–40 monomer. Again, the presence of 2.5% DMSO had a negligible effect on the growth of 1 μM soluble Aβ1–40 aggregates initiated by the addition of 20 μM monomer (data not shown). When soluble Aβ1–40 aggregates were incubated with equimolar concentrations of compound 2 before the addition of Aβ1–40 monomer to stimulate aggregate growth, the rate of aggregate growth was similar to that observed when soluble aggregates and monomer were incubated alone, indicating no inhibition. The absence of inhibition persisted as the concentration of compound 2 was increased to 5-fold in excess of soluble Aβ1–40 aggregate. At significantly higher concentrations of compound 2 that exceeded both soluble Aβ1–40 aggregate and...
results of ThT measurements were supported by transmission electron microscopy (TEM) images acquired after 30 min of aggregate growth by elongation. Soluble Aβ1–40 (2 μM) aggregates elongated via the addition of 20 μM Aβ1–40 monomer (Fig. 5B) exhibited filament lengths significantly longer than soluble aggregates incubated alone (Fig. 5, A and D). When 2 μM soluble Aβ1–40 aggregates were preincubated and elongated in the presence of 60 μM compound 2, a similar lengthening of filaments occurred (Fig. 5C), further illustrating the inability of this compound to halt aggregate growth via monomer addition. Together, these results illustrate that compound 2 displays insignificant inhibition of soluble Aβ1–40 aggregate elongation.

**Compound 2 Inhibits the Association of Soluble Aβ1–40 Aggregates in a Dose-Dependent Manner.** Another distinct mechanism of soluble Aβ1–40 aggregate growth is that of aggregate association, in which aggregates increase in size in the absence of monomeric protein via direct aggregate-aggregate interactions. This growth process leads to an increase in aggregate size in the absence of an increase in monomeric protein via direct aggregate association. The inhibitory capabilities of compound 2 for soluble aggregate association were confirmed using TEM images acquired after 30 min after the initiation of aggregate growth by association. 2 μM soluble Aβ1–40 aggregates incubated in low ionic strength buffer existed as short individual single stranded filaments (Fig. 5, A and D), while an equivalent concentration of soluble Aβ1–40 aggregates incubated in the presence of elevated ionic strength exhibited clusters in which two or more filaments were bundled together (Fig. 5E). The staggered alignment of individual filaments led to the appearance of a network of longer, thicker fibrils. In contrast, when 2 μM soluble Aβ1–40 aggregates were preincubated and associated in the presence of 40 μM compound 2, many individual filaments remained (Fig. 5F), illustrating the inhibition of soluble aggregate association. Inhibition was not complete, however, because some grouped filaments were still observed in the presence of compound 2.

The ability of compound 2 to dose-dependently inhibit the rate of association of soluble Aβ1–40 aggregates was quantitatively assessed via a comparison of the experimental association rate with that observed for the control (Fig. 6B). Using seven different stoichiometric ratios of compound 2 to soluble Aβ1–40 aggregates, which used Aβ1–40 aggregate concentrations of either 1 or 2 μM, an IC50 value of 0.92 was determined, indicating that approximately equimolar quantities of compound 2 are required to observe 50% inhibition of the growth of soluble Aβ1–40 aggregates via association. Furthermore, IC50 fits revealed a maximal inhibition of 90%, in agreement with the incomplete inhibition observed using TEM for soluble aggregate association in the presence of excess amounts of compound 2.

**Discussion**

Numerous small molecules have been identified as inhibitors of Aβ self-assembly (Findeis, 2000; De Felice and Ferreira, 2002; Hamaguchi et al., 2006; Porat et al., 2006). However, elucidation of the mechanism of inhibitor action has proven challenging as a result of the complex nature of the Aβ self-assembly process. Progression from monomeric protein to the mature fibril that deposits in AD brain involves multiple reaction intermediates related by nonlinear and interconnected nucleation and growth mechanisms (LeVine, 2007; Nuclea et al., 2007b). When inhibition measurements consider only fibrillar end products of the self-assembly process, little information is gained concerning the point of inhibitor intervention within this nonlinear pathway. To provide mechanistic insight into the inhibition of Aβ1–40 assembly, we used multiple assay formats to quantitatively assess the ability of a julolidine aldehyde to prevent the formation of aggregates from monomeric protein and to at-
tenuate the growth of soluble aggregation intermediates via both monomer addition and aggregate association.

The inhibitory capability of this compound was first assessed using an aqueous-based Aβ_{1–40} monomer aggregation assay monitored via ThT fluorescence. The observed extension of the lag time and reduction in the rate of fluorescence increase (Fig. 1) were taken as evidence that compound 2 inhibits the formation of aggregates from monomeric Aβ_{1–40}. Unfortunately, the long time course and inherent variability of stochastic nucleation render this assay more effective for qualitatively identifying inhibition than in providing reproducible, quantitative information about the dose-dependence of inhibitor action. Thus, the more reproducible aggregation of Aβ_{1–40} monomer induced in the presence of HFIP (Nichols et al., 2005) was used to quantitatively examine the dose-dependent inhibition of aggregate formation by compound 2. HFIP-induced aggregation of Aβ_{1–40} monomer has been used to quantify inhibitory activity of well known Aβ aggregation inhibitors, and IC_{50} values determined using the maximum ThT fluorescence as a measure of inhibition were in close agreement with IC_{50} values established using aqueous-based assays (Cellamare et al., 2008). Using this approach, an IC_{50} at a stoichiometric ratio of 6.8 compound 2 to Aβ_{1–40} was ascertained (Fig. 3B). This IC_{50} value, which is reflective of a combination of binding affinity and stoichiometry, might suggest that compound 2 acts by binding Aβ_{1–40} monomers to prevent nucleation, as has been suggested for the inhibition of Aβ_{1–40} monomer aggregation by daunomycin (Howlett et al., 1999a) and the inhibition of Aβ_{1–42} monomer aggregation by rifamycin and hematin (Necula et al., 2007b). Alternatively, compound 2 may bind on-pathway intermediates to prevent their progression into mature fibrillar structures. This mechanism of inhibition has been inferred from the observed inhibition of Aβ_{1–40} fibril formation by benzofurans, which are incapable of binding monomeric protein (Howlett et al., 1999b), as well as the ability of 4,4’-bis(1-anilinonaphthalene 8-sulfonate) (Ferrão-Gonzales et al., 2005) and catecholamines (Lashuel et al., 2002) to prevent, respectively, the formation of Aβ_{1–42} and Aβ_{1–40} fibrils without halting the appearance of oligomeric structures.

The possibility that compound 2 may bind on-pathway intermediates was explored using assays developed previously to isolate distinct mechanisms of soluble Aβ aggregation growth (Nichols et al., 2002). This molecule was found to effectively inhibit the lateral association of soluble Aβ_{1–40} aggregates (Figs. 5, D–F, and 6A), illustrating that compound 2 is capable of binding on-pathway intermediates to prevent their progression into larger structures. By evaluating reductions in the association rate, an IC_{50} at a stoichiometric ratio of 0.92 inhibitor to monomeric units of Aβ_{1–40} was determined for the inhibition of soluble aggregate association (Fig. 6B). This somewhat lower stoichiometry, relative to the inhibition of HFIP-induced monomer aggregation, may suggest that compound 2 is capable of binding both larger aggregation intermediates and aggregate structures that appear very early in the self-assembly pathway. These early aggregates would be absent from SEC-isolated soluble aggregate preparations that are derived from self-assembly reactions at relatively late time points. Alternatively, the difference in stoichiometry might reflect the morphological differences reported for aggregates formed in dilute HFIP versus aqueous buffer (Nichols et al., 2005).

In contrast to association, compound 2 was incapable of inhibiting elongation of soluble Aβ_{1–40} aggregates via monomer addition (Figs. 4 and 5, A–C). The negligible inhibition of monomer addition to aggregated protein is similar to that observed for methylene blue (Necula et al., 2007a), Congo Red (LeVine, 2007), and nordihydroguaiaretic acid (Moss et al., 2004). However, other small molecules have been shown to inhibit Aβ fibril extension by monomer addition (Naiki et al., 1998; Ono et al., 2003, 2004; Williams et al., 2005; Dolphin et al., 2008). Because compound 2 does not reduce monomer available for addition to preformed aggregates, this result provides additional evidence that compound 2 does not

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**Fig. 5.** Morphology of Aβ_{1–40} aggregates. SEC-isolated soluble Aβ_{1–40} (2 μM) aggregates in 40 mM Tris-HCl, pH 8.0, were incubated alone (A and D, negative control), elongated in the presence of 20 μM Aβ_{1–40} monomer (B, positive control), elongated in the presence of 20 μM Aβ_{1–40} monomer and 60 μM compound 2 (C), associated in the presence of 150 mM NaCl (E, positive control), or associated in the presence of 150 mM NaCl and 40 μM compound 2 (F). For samples containing compound 2, soluble aggregates and compound 2 were preincubated for 15 min before the initiation of aggregate growth. After 30 min of aggregate growth, samples were gridded and visualized by TEM as described under Materials and Methods. Results are representative of two independent experiments. Images are shown relative to a scale bar of 0.5 μm.
bind monomeric Aβ_{1-40}. Furthermore, the selectivity of compound 2 for the inhibition of soluble Aβ_{1-40} aggregate growth by lateral association demonstrates that binding of compound 2 to aggregated protein structures does not block sites of monomer addition. Instead, binding sites for this compound may be situated along the lateral surface of aggregates, positioning the bound inhibitor to selectively block association events. A similar selectivity was observed for the inhibition of soluble Aβ_{1-40} aggregate growth by nordihydroguaiaretic acid (Moss et al., 2004). As opposed to elongation in which monomers undergo a random coil to β-sheet transition upon incorporation into aggregate structures, association involves the coalescence of existing β-sheet structures and therefore does not create new amyloid material. Hence, selective inhibition of aggregate association might not be expected to affect aggregate formation from monomeric protein. Here, however, inhibition was observed for both aggregate association and monomer aggregation, suggesting that the ability of intermediate aggregates to associate contributes to the extent of aggregate formation. Association may create new sites for monomer addition or may stabilize aggregates to facilitate further aggregate growth. Still, complete blockage of fibril formation will require the inhibition of both mechanisms of aggregate growth such that effective therapeutic strategies should use multifunctional structures or multiple compounds. The inhibitory activity observed for compound 2 may be attributed to its aromatic structure and the reactive aldehyde group that can bind amino residues of the peptide. Several small molecules containing aromatic structures have been identified as inhibitors of Aβ self-assembly (Findeis, 2000; Hamaguchi et al., 2006; LeVine, 2007). Some of these compounds are proposed to recognize Aβ via interactions with aromatic residues (Porat et al., 2006; Barnham et al., 2008; Cellamare et al., 2008; Dolphin et al., 2008). In fact, aromatic residues are commonly found at critical positions within amyloidogenic proteins and may thermodynamically favor β-sheet formation (Gazit, 2002). Aβ contains four aromatic residues, including phenylalanine at residues 4, 19, and 20, and tyrosine at residue 10. Phe19 and Phe20 lie within the hydrophobic core of the protein, which is essential to amyloid formation (Hilbich et al., 1992). π-π-Stacking of aromatic residues is hypothesized to contribute to amyloid self-assembly (Gazit, 2002), and π-stacking of phenylalanine residues has been observed for other amyloid-forming proteins (Jack et al., 2006). π–π Interactions are also suggested to participate in the recognition of Aβ by small molecules containing aromatic structures (De Felice et al., 2004; Kanapathipillai et al., 2005; Porat et al., 2006; Barnham et al., 2008; Cellamare et al., 2008; Dolphin et al., 2008). Thus, π–π interactions between compound 2 and Aβ may disturb π-stacking between protein units within the β-structure, leading to disruption of Aβ self-assembly. The inference that compound 2 binds an on-pathway assembly intermediate suggests that Aβ monomers must reside in a β-sheet conformation for π–π interactions between the small molecule and the protein to occur. One proposed model for Aβ aggregate structure (Petkova et al., 2002) places Phe19 and Phe20 within the β-sheet structure of Aβ aggregates. Furthermore, these residues are positioned to participate in interactions involved in the lateral association of aggregates, which were found in the current study to be selectively disrupted by compound 2. Also positioned at this lateral association interface, the primary amine of Lys16 may interact with the aldehyde group to enhance binding. Alternatively, interaction between the aldehyde and Lys28 may disrupt its participation in a salt bridge with Asp23 that is suggested to stabilize a predicted β-turn. However, these reactions will be relatively slow at the experimental pH of 8.0.

The current study identifies a novel inhibitor of Aβ_{1-40} self-assembly. Using assays that isolate two distinct mechanisms of soluble Aβ_{1-40} aggregate growth, a julolidine aldehyde is shown to selectively inhibit the growth of soluble aggregates by lateral association but to have little effect on soluble aggregate elongation via monomer addition. Parallel inhibition of aggregate formation from monomeric protein implies a role for lateral aggregate association in the

![Figure 6](molpharm.aspetjournals.org)
overall extent of aggregate formation. Together, these results imply that this compound binds the lateral surface of a soluble on-pathway intermediate to prevent association with other aggregates required for continued assembly into mature fibrils. Furthermore, IC_{50} values suggest that a range of aggregate sizes may be recognized by this compound. These findings demonstrate that the quantitative evaluation of inhibition at different mechanistic steps within the Aβ self-assembly process can provide insight into the mechanism of inhibitor action to facilitate optimization of effective inhibitor structures.

Acknowledgments

We acknowledge the University of South Carolina Electron Microscopy Center for instrument use as well as scientific and technical assistance.

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