ACS Chemical Neuroscience

Research Article

pubs.acs.org/chemneuro

Azaphilones Inhibit Tau Aggregation and Dissolve Tau Aggregates *in Vitro*

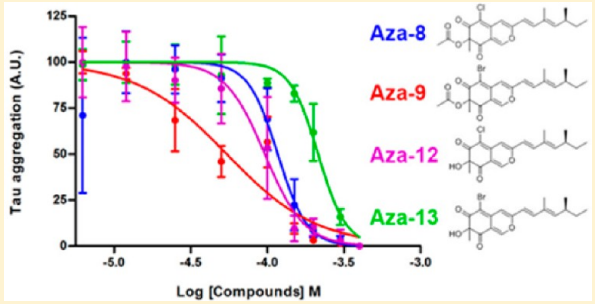
Smita R. Paranjape,*[,†] Andrew P. Riley,[‡] Amber D. Somoza,[§] C. Elizabeth Oakley,[†] Clay C. C. Wang,[§,∥] Thomas E. Prisinzano,[⊥] Berl R. Oakley,[†] and T. Chris Gamblin[†]

[†]Department of Molecular Biosciences, [‡]Department of Chemistry, [⊥]Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045, United States

[§]Department of Chemistry, [∥]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California 90089, United States

Supporting Information

ABSTRACT: The aggregation of the microtubule-associated protein tau is a seminal event in many neurodegenerative diseases, including Alzheimer's disease. The inhibition or reversal of tau aggregation is therefore a potential therapeutic strategy for these diseases. Fungal natural products have proven to be a rich source of useful compounds having wide varieties of biological activities. We have previously screened *Aspergillus nidulans* secondary metabolites for their ability to inhibit tau aggregation *in vitro* using an arachidonic acid polymerization protocol. One aggregation inhibitor identified was asperbenzaldehyde, an intermediate in azaphilone biosynthesis. We therefore tested 11 azaphilone derivatives to determine their tau assembly inhibition properties *in vitro*. All compounds tested inhibited tau filament assembly to some extent, and four of the 11 compounds had the advantageous property of disassembling preformed tau aggregates in a dose-dependent fashion. The addition of these compounds to the tau aggregates reduced both the total length and number of tau polymers. The most potent compounds were tested in *in vitro* reactions to determine whether they interfere with tau's normal function of stabilizing microtubules (MTs). We found that they did not completely inhibit MT assembly in the presence of tau. These derivatives are very promising lead compounds for tau aggregation inhibitors and, more excitingly, for compounds that can disassemble pre-existing tau filaments. They also represent a new class of anti-tau aggregation compounds with a novel structural scaffold.



KEYWORDS: *Tau, microtubule-associated protein, aggregation inhibitor, Alzheimer's disease, azaphilone, natural products, Aspergillus, Aspergillus nidulans*

Alzheimer's disease (AD) is the most common form of dementia. This devastating condition is made worse by the relative lack of therapies available for its treatment. Current therapeutics largely target cholinergic pathways and do little to slow or reverse the accumulation of aggregates of either beta amyloid or the microtubule-associated protein tau into senile plaques or neurofibrillary tangles, respectively. The location and amount of tau aggregation into neurofibrillary tangles directly correlates with the type and severity of the disease progression.[1] Therefore, there is great interest in identifying small molecules that may inhibit or reverse tau aggregation. Recently, a tau aggregation inhibitor, a stable, reduced form of methylthioninium chloride, has reached Phase III clinical trials,[2] validating the potential of tau aggregation as a target, but there is certainly a need for additional lead anti-tau aggregation compounds for further development into therapeutics.

An ideal tau aggregation inhibitor should inhibit the assembly of tau aggregates and disassemble preformed tau aggregates as well. Inhibitors of tau aggregation also should not impair the normal function of tau to bind and to stabilize microtubules. Previously identified tau aggregation inhibitors, including molecules belonging to the anthraquinone class, have had widely diverse structures, with fused ring structures being a commonly occurring structural motif.[3] Recent emphasis has been placed on identifying natural products with novel scaffolds that may have useful properties for the treatment of AD by inhibiting tau aggregation.[4,5]

Fungi have historically been a good source of secondary metabolites that have useful pharmacological properties such as antibiotics, immunosuppressants, and cholesterol-lowering drugs, among others.[6] We have identified numerous biosynthetic pathways in *Aspergillus nidulans* that lead to production of a wide range of secondary metabolites.[6−9] In a previous study, we tested several *A. nidulans* secondary metabolites for their ability to inhibit tau aggregation *in vitro* and

Received: January 9, 2015
Revised: March 26, 2015
Published: March 30, 2015

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

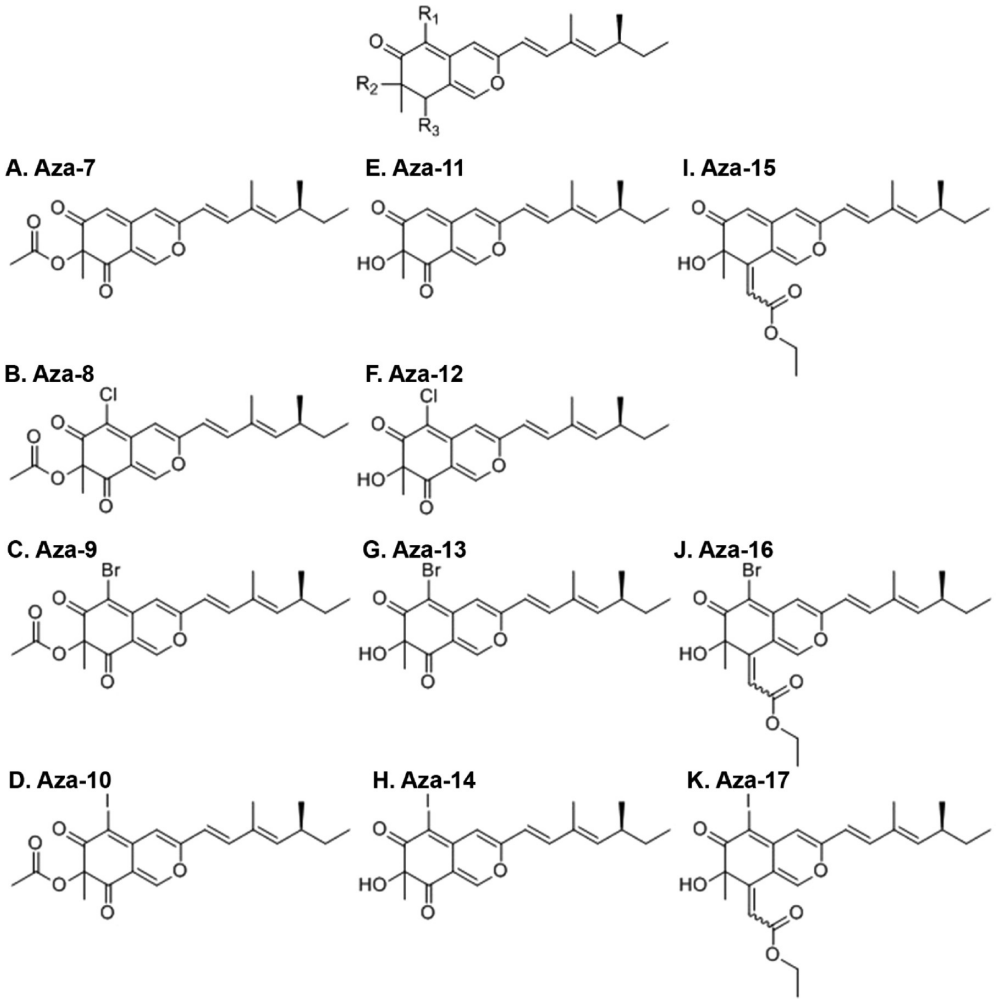


Figure 1. Compounds used in this study. The core structure of the azaphilone compounds is shown with the positions of modifications indicated by R_1, R_2, and R_3. (A–K) Structures of the 11 compounds used in this study: (A) aza-7, (B) aza-8, (C) aza-9, (D) aza-10, (E) aza-11, (F) aza-12, (G) aza-13, (H) aza-14, (I) aza-15, (J) aza-16, and (K) aza-17.

found that several were active inhibitors at micromolar concentrations, although they did not have tau disaggregation properties.[10] Among these, two, 2,ω-dihydroxyemodin and asperthecin, belong to the anthraquinone class of compounds, a class that includes compounds shown to inhibit tau aggregation. A third compound, asperbenzaldehyde, however, was structurally distinct from previously identified tau aggregation inhibitors. Asperbenzaldehyde is also interesting in that it is an intermediate in the biological synthesis of azaphilone compounds.[11]

Azaphilones are known to exhibit a great variety of biologically important activities, including inhibition of gp120–CD4 binding[12] and heat shock protein 90 (Hsp90),[13−15] among others. Several azaphilones have been shown to have lipoxygenase inhibitor activity.[11] Inhibition of lipoxygenases may help to reduce fatty acid metabolite levels that are elevated in AD.[16] Azaphilones, including lipoxygenase-inhibiting azaphilones, can be obtained from asperbenzaldehyde using a two to three step semisynthetic route.[11] We therefore sought to determine whether azaphilones derived from asperbenzaldehyde inhibit tau aggregation, hoping that they might be a useful step in finding compounds with two biological targets relevant to treating AD.

Beginning with asperbenzaldehyde, which was purified from a fungal strain engineered to overproduce this compound, the azaphilones were prepared as previously described using two schemes.[11] The first employs *p*-toluenesulfonic acid to form the 2-benzopyrilium salt followed by oxidation by lead tetraacetate with or without halogenation. The second scheme employs the hypervalent-iodine-mediated phenol oxidative dearomatization of the 2-benzopyrilium salt with *o*-iodoxy-benzoic acid followed by halogenation and/or a Wittig olefination with carbethoxymethylenetriphenylphosphorane.

Using standard biochemical assays, we investigated the ability of these compounds to alter the aggregation of tau and its stabilization of microtubules. We found that while all compounds inhibited tau aggregation, a smaller subset had the added activity of disassembling preformed tau aggregates. The compounds most effective at inhibiting tau aggregation and disassembling preformed tau filaments also allowed tau to retain the majority of its microtubule-stabilizing functions.

RESULTS

Eleven compounds with the same azaphilone backbone differing at three points of diversity (R_1, R_2, and R_3) were used in this study (Figure 1). Tau polymerization was initiated *in vitro* using a standard arachidonic acid induction assay.[17] To determine whether the compounds could inhibit assembly of tau filaments, each of the compounds, at a final concentration of

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

200 μM, was preincubated with 2 μM tau for 20 min before the addition of 75 μM arachidonic acid. The degree of tau aggregation inhibition for each compound was determined using a membrane filter assay.[18] This assay has been used previously to screen *A. nidulans* secondary metabolites including anthraquinones, xanthones, polyketides, a benzophenone, and the asperbenzaldehyde compound that was the parent compound for the synthesis of the azaphilones used in this study.[10] A mixture of antibodies to the amino terminal, central, and carboxy terminal regions of tau (tau 12, tau 5, and tau 7, respectively) was used to detect tau aggregates. In this assay, only compound aza-11 significantly reduced the amount of tau aggregation detected (Figure 2A). Compounds aza-13 and aza-15 significantly increased the amount of tau aggregation, and the remaining compounds had no significant effect (Figure 2A). However, when antibodies against toxic species of tau were employed for detection, very different results were observed. All aza compounds completely abolished recognition by the TOC1 antibody, which recognizes toxic oligomers *in vitro* and in Alzheimer's disease tissue,[19] as compared to that for controls without compound (Figure 2B). Similarly, significant reductions in recognition by TNT1, an antibody that recognizes the phosphatase-activating domain of tau and is exposed in pathological forms of tau,[20] were observed for all aza compounds as compared to that for controls without compound (Figure 2C).

When the resulting tau aggregates from the inhibition reactions were visualized by electron microscopy, there were abundant numbers of long filaments in the absence of added compound (Figure 3). Surprisingly, all of the azaphilones inhibited the formation of long tau filaments that were observed in the absence of compounds. Instead, amorphous small aggregates were observed after treatment with the compounds (Figure 3). This degree of tau aggregation inhibition was similar to what was observed by electron microscopy for asperbenzaldehyde and asperthecin and was stronger than what was observed for 2,ω-dihydroxyemodin in a prior study.[10] Because tau aggregation inhibitors that inhibit filament formation have previously been shown to stabilize off-pathway soluble oligomers that are large enough to be trapped in the membrane filter assay,[21] we believe that the mixture of tau antibodies to normal tau was detecting these aggregates in the filter trap assay (Figure 2A). These aggregates do not seem to be toxic because of their lack of reactivity to TOC1 (Figure 2B) and TNT1 (Figure 2C).

To determine whether these compounds can disassemble preformed tau aggregates, tau aggregation was allowed to proceed for 6 h before the addition of compounds to a final concentration of 200 μM. After 12 h, the effect of compounds on the tau aggregation was examined by a filter trap assay using the mixture of antibodies against normal tau (Figure 4). All compounds reduced the amount of preformed tau filaments, with compounds aza-8, aza-9, aza-11, aza-12, and aza-13 having the greatest activity. Electron microscopy was used to validate and extend the results from the filter trap assay. Compounds aza-8, aza-9, aza-12, and aza-13 substantially reduced the preexisting filament mass, whereas the other compounds had less effect (Figure 5). To test whether compounds aza-8, aza-9, aza-12, and aza-13 were not simply blocking the adherence of tau filaments to the electron microscopy grids, compounds were added to preformed tau filaments and were immediately prepared for electron microscopy without allowing time for disassembly to occur. Under these conditions, none of the compounds blocked the binding of the filaments to the grid

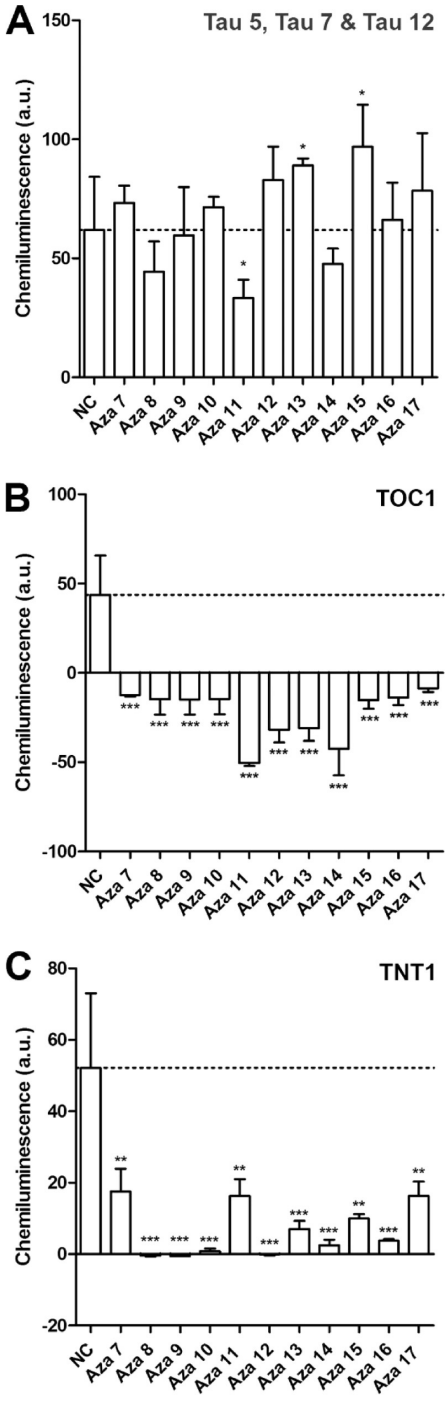


Figure 2. Filter trap assay of tau filament formation. Tau polymerization reactions in (A–C) were performed with 2 μM tau and 75 μM arachidonic acid either with or without 200 μM compound. The compounds used are listed on the *x* axis. The resulting amount of tau filament formation was determined using a filter trap assay. The values for tau filament formation were normalized to the amount of aggregation in the absence of compound (dashed line). Negative values indicate that there was less detectable tau on the filter after treatment with a compound than was observed with monomeric tau in the absence of arachidonic acid. The amount of tau on the filter was detected using (A) a mixture of antibodies tau 5, tau 7, and tau 12, (B) antibody TOC1, and (C) antibody TNT1. The observation that the values are lower in (B) and (C) suggests that the tau aggregates that are retained on the filters are not in the toxic oligomeric form recognized by the TOC1 antibody and that the phosphatase domain recognized by TNT1 antibody is not exposed. Values are the average of three trials ± SD. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751–760

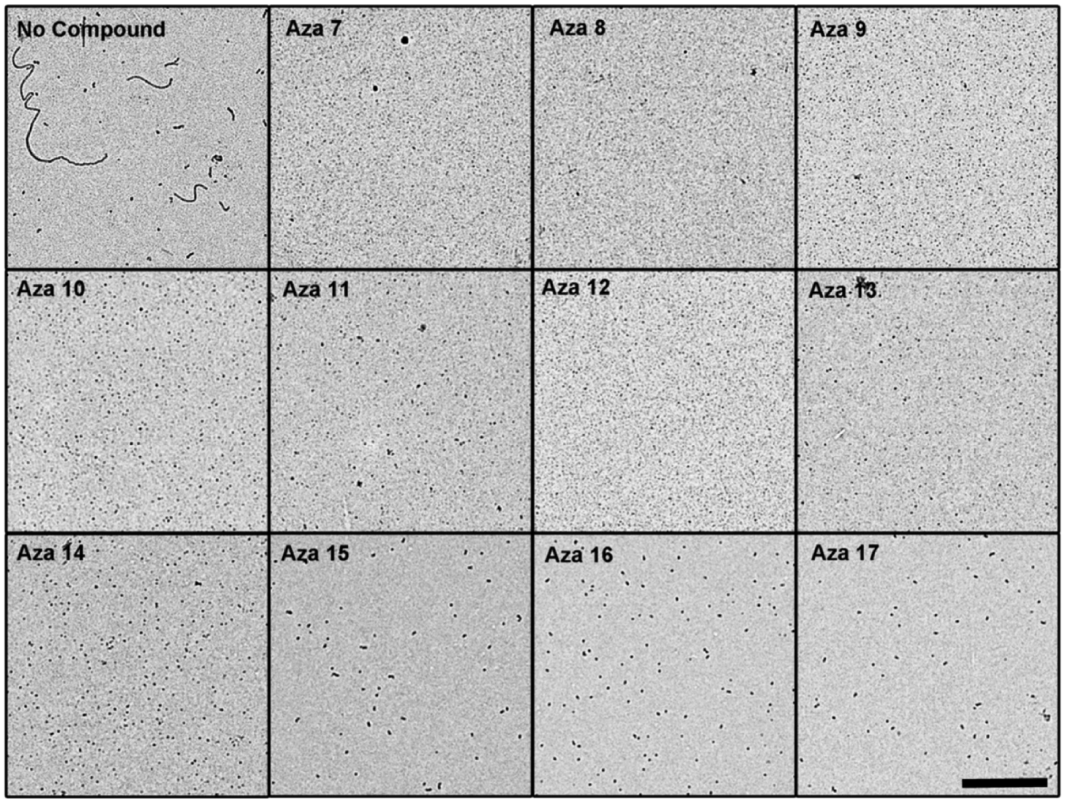


Figure 3. Electron microscopy of tau filament formation in the presence of azaphilone derivatives. Tau polymerization reactions were performed with 2 μM tau and 75 μM arachidonic acid either with or without 200 μM compound. Aliquots of the reactions were prepared for negative stain electron microscopy. Representative images are shown for no compound control, aza-7, aza-8, aza-9, aza-10, aza-11, aza-12, aza-13, aza-14, aza-15, aza-16, and aza-17. The scale bar in the lower right panel represents 1 μm and is applicable to all images.

(Supporting Information Figure S2). Quantitative analysis of the filament lengths in the presence and absence of compounds confirmed that compounds aza-8, aza-9, aza-12, and aza-13 had fewer aggregates overall compared to that in reactions without compound and virtually no filaments remaining that were greater than 200 nm in length (Figure 6).

The IC_{50} of the four most potent compounds was determined using the filter trap assay. The amount of preformed filaments remaining following treatment with compounds for 12 h was reduced in a concentration-dependent manner for all four compounds tested (Figure 7). Compound aza-9 had an IC_{50} of 56 $\pm$ 14 μM, compared to 118 $\pm$ 19, 98 $\pm$ 16, and 216 $\pm$ 18 μM for compounds aza-8, aza-12, and aza-13 respectively, indicating that aza-9 has the most activity for dissolving preformed tau filaments *in vitro* (Figure 7).

In a number of studies, heparin has been used to induce tau aggregation. Because heparin-induced tau filaments might be different from arachidonic acid induced filaments, we wished to determine if aza-9 inhibited heparin-induced tau aggregation or disassembled heparin-assembled tau aggregates. We chose aza-9 because it was the most potent compound among the 11 azaphilones. Filter trap assays were performed using the mixture of antibodies against normal tau, the TOC1 antibody, and the TNT1 antibody. Aza-9 significantly reduced the assembly of TOC1- and TNT1-positive aggregates (Figure 8A). The addition of aza-9 also resulted in the significant disassembly of preformed filaments recognized by the TOC1 and TNT1 antibodies (Figure 8B).

We chose the most potent azaphilone derivatives, aza-8, aza-9, aza-12, and aza-13, to determine their effects on the normal function of tau to stabilize microtubules. Tubulin was mixed with tau in the presence or absence of 90 μM compound, and the resulting microtubule formation was monitored by turbidity. All polymerization curves were fit using a Gompertz growth equation (Figure 9). While all four compounds affected the apparent rate and maximum amount of microtubule formation at the concentration tested (Table 1), tau still retained a significant ability to stabilize microtubule formation.

DISCUSSION

Tau-based therapeutic strategies have recently been gaining additional attention largely due to the major role that tau pathology plays in many neurological disorders including Alzheimer's disease. Several tau-directed therapeutic strategies with disease-modifying potential have been identified, including modulating tau phosphorylation, microtubule stabilization, tau aggregation inhibitors, and tau clearance using antibodies.[22−30] Conversion of soluble monomeric tau into insoluble tau aggregates could, potentially, result in both loss-of-function and gain-of-function toxicities.[31] Therefore, inhibiting aggregation of tau might prevent formation of the toxic oligomers or tangles. Inhibiting aggregation could also increase the levels of monomeric tau, thereby increasing the chances for its clearance through chaperone-mediated processes.[32] Previous studies have identified several tau aggregation inhibitor (TAI) molecules, including those belonging to the class of anthraquinones, phenothiazines, and a benzothiazolidine derivative, among others.[3,33,34] One TAI, a stable, reduced form of methylthioninium chloride, is currently in Phase III clinical trials,[2]

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

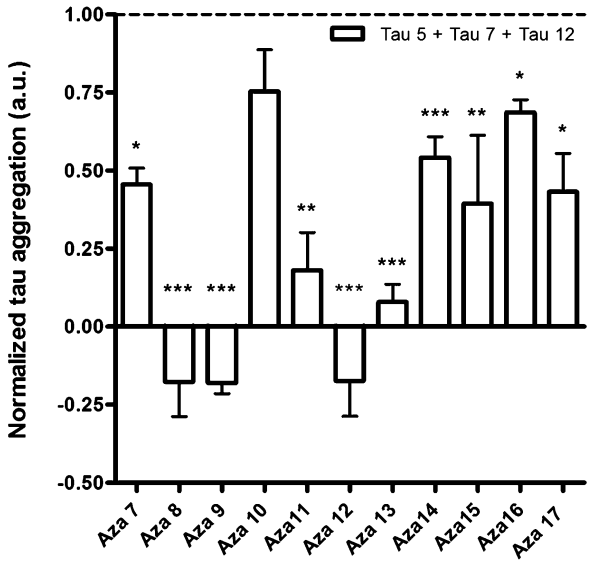


Figure 4. Filter trap assay of tau filament disassembly. Tau polymerization reactions were performed with 2 μM tau and 75 μM arachidonic acid at room temperature. After 6 h, 200 μM compound or an equal volume of DMSO was added to the reactions. The compounds used are listed on the *x* axis. The resulting amount of tau filament formation was determined using a filter trap assay. The values for tau filament formation were normalized to the amount of aggregates detected in the absence of compound (dashed line). Negative values indicate that there was less detectable tau on the filter after treatment with a compound than was observed with monomeric tau in the absence of arachidonic acid. The amount of tau on the filter was detected using a mixture of antibodies tau 5, tau 7, and tau 12. Values are the average of three trials ± SD. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

indicating that this approach has promise and that it is, consequently, worthwhile to identify additional structural backbones with this activity. Many of the previously identified TAIs are composed of fused ring structures believed to be capable of interacting with the β-sheet structures formed in tau aggregates, thereby inhibiting formation of tau filaments.[10,21]

Fungal extracts are known to include pharmaceutically important secondary metabolites.[35] We therefore previously screened 17 secondary metabolites obtained from the fungus *A. nidulans* for TAIs due to their structural similarity to previously identified TAIs.[10] From this screen, we identified three compounds that inhibited tau aggregation at micromolar concentrations. Two of these compounds belong to the anthraquinone class of compounds, and one was structurally unique from all previously identified TAIs. We were particularly interested in this compound, asperbenzaldehyde. Asperbenzaldehyde is a precursor to an important class of natural products called azaphilones. Azaphilones are a structurally diverse group of polyketides that share a highly oxygenated bicyclic core and chiral quaternary center.[36] The azaphilones used in this study were obtained by semisynthetic diversification of asperbenzaldehyde.

All 11 azaphilones inhibited the formation of tau filaments, but some of them produced small amorphous tau aggregates, which can be seen in the electron micrographs in Figure 3. These aggregates were not recognized by TOC1[19] and TNT1[20] antibodies, which bind to toxic forms of tau; therefore, we believe that these compounds promote the formation of small off-pathway aggregates of tau that are not toxic and do not act as seeds for further tau filament assembly. The induction of these aggregates could be similar to soluble aggregates of tau

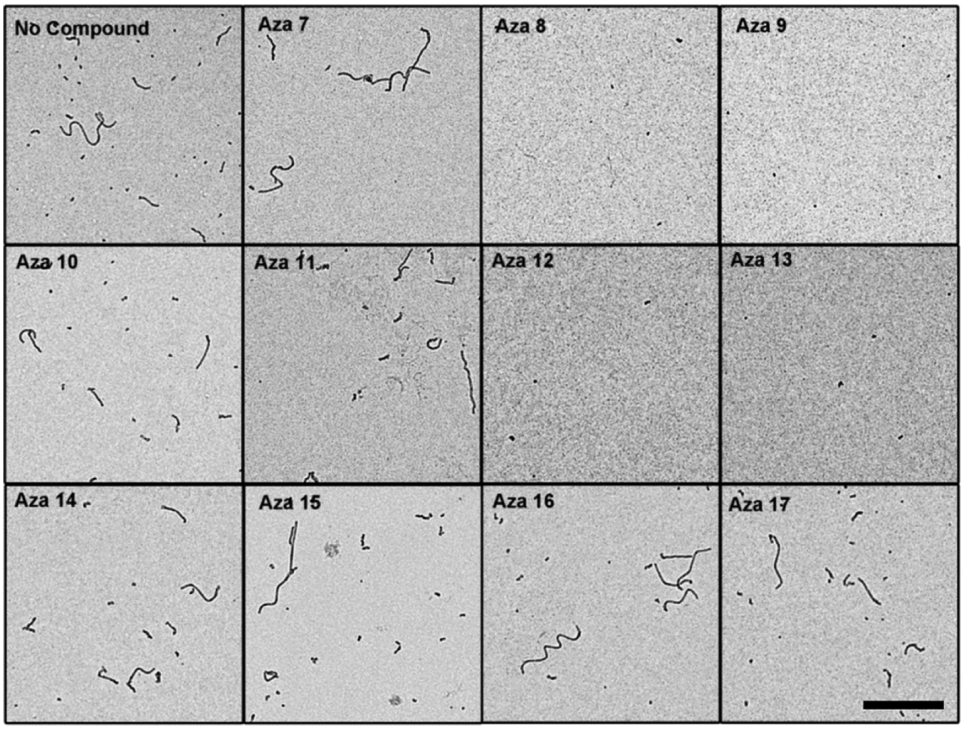


Figure 5. Electron microscopy of tau filament disassembly in the presence of azaphilone derivatives. Tau polymerization reactions were performed with 2 μM tau and 75 μM arachidonic acid at room temperature. After 6 h, 200 μM compound or equal volume of DMSO was added to the reactions. Aliquots of the reactions were prepared for negative stain electron microscopy. Representative images are shown for no compound control, aza-7, aza-8, aza-9, aza-10, aza-11, aza-12, aza-13, aza-14, aza-15, aza-16, and aza-17. The scale bar in the lower right panel represents 1 μm and is applicable to all images.

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

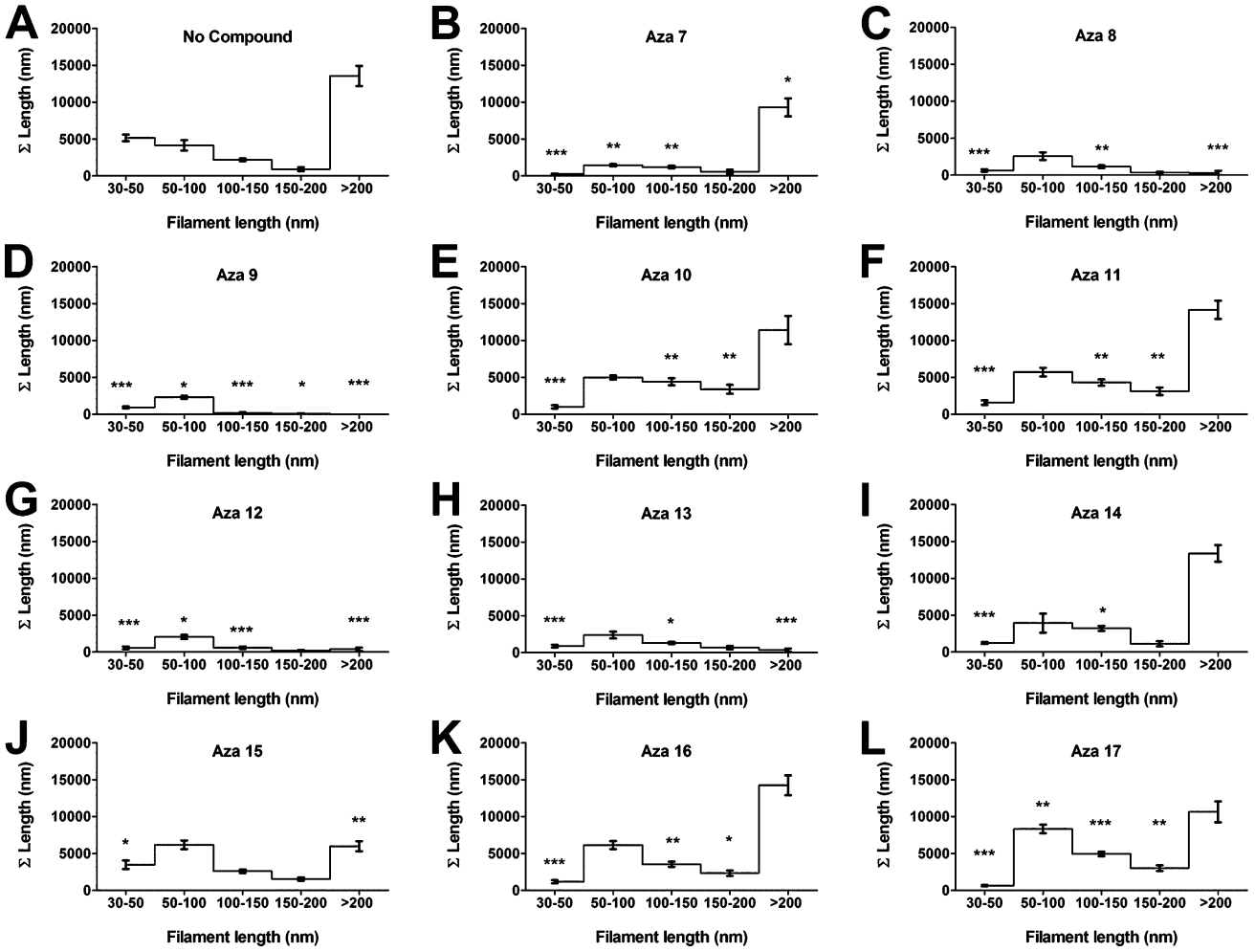


Figure 6. Filament length distributions. Tau disassembly reactions were performed and viewed by electron microscopy. The filaments remaining following incubation with or without compound were measured and placed into bins according to their length (30−50, 50−100, 100−150, 150−200, and >200 nm). The lengths within a bin were summed to determine the total amount of filament length in each bin. (A) Length distributions for filaments in the control reaction without compound. The length distributions are also shown for filaments remaining following incubation in the presence of (B) aza-7, (C) aza-8, (D) aza-9, (E) aza-10, (F) aza-11, (G) aza-12, (H) aza-13, (I) aza-14, (J) aza-15, (K) aza-16, and (L) aza-17. Each point is the average distribution for images of five different fields ± SD. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

induced by porphyrin phthalocyanine tetrasulfonate that have a different conformation from that of insoluble toxic tau oligomers.[21]

From a therapeutic point of view, TAIs would be more useful if they could also dissolve preformed tau filaments because they could theoretically be beneficial to patients that already demonstrate cognitive impairments. We found that a subset of the compounds, aza-8, aza-9, aza-12, and aza-13, showed this property. These four compounds have Br or Cl at position R1, whereas the other compounds have either I or H at R1 (Figure 1). Therefore, halogenation at position R1 may not necessarily be important for inhibition of tau filament formation, but electron-withdrawing groups at R1 specifically seem to enhance disassembly of tau filaments. Cl and Br are more electronegative (3.0 and 2.8, respectively) than I (2.5) and H (2.1), indicating that increased electronegativity at position R1 could have a significant impact on the activity of tau aggregation inhibitors with this scaffold. The four disassembly causing compounds have a ketone at position R3, whereas the presence of the $CHCO_2Et$ moiety at the same position seems to virtually eliminate disassembly, even with halogenation. The impact of the chemical groups at the R2 position seems to be dependent upon the substitution at R1. Compounds aza-8 and aza-12 both have Cl at R1, but they possess acetate and hydroxyl groups at R2, respectively. Despite this structural difference, there is no significant difference in their activity levels. However, compounds aza-9 and aza-13, both containing Br at R1 and acetate and hydroxyl groups at R2, respectively, differ in their levels of activity. Aza-9 is more potent than aza-13; therefore, positioning of the acetate group at R2 in the presence of a Br at R1 might be important for compound activity.

Additionally, all four disassembly causing compounds have lipoxygenase-1 inhibitory activity in the low micromolar range (IC_{50} of 2−8 μm).[11] Inhibition of LOX-1 may help to reduce fatty acid metabolites of arachidonic acid and docosahexaenoic acid that are elevated in Alzheimer's disease.[16] These compounds could therefore have two positive therapeutic activities in tau dementias. The relatively high IC_{50} values of our compounds indicate that they are unlikely to be of therapeutic value, and we do not know if they have suitable bioavailability or pharmacokinetic properties. It is important, however, to identify new scaffolds with the appropriate biological activity for further development. We believe that these compounds provide

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

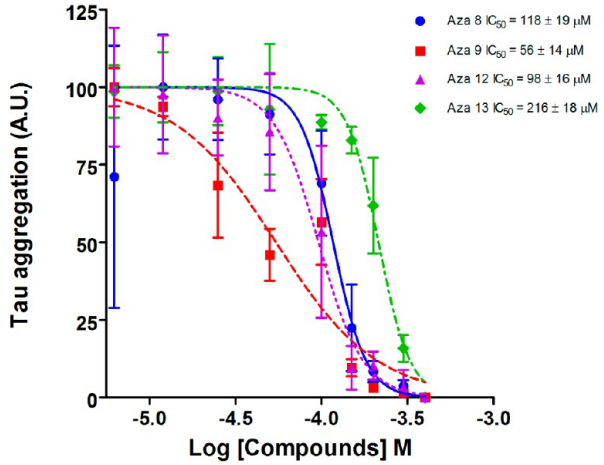


Figure 7. IC_{50} determination tau filament disassembly. Polymerization reactions at 2 μM tau and 75 μM arachidonic acid were performed at room temperature. After 6 h, compounds were added to these reactions at several different concentrations, and the mixtures were incubated for an additional 12 h. The resulting amount of tau filaments in the reaction was determined by a filter trap assay detected by a mixture of antibodies to normal tau (tau 5, tau 7, and tau 12). The amount of polymerization was normalized to controls in the absence of compound (100%). The normalized data was plotted against the log of the inhibition concentration and fit to a dose−response curve to determine the IC_{50} for Aza-8 (blue circles), Aza-9 (red squares), Aza-12 (purple triangles) and Aza-13 (green diamonds). Data points are the average of three trials ± SD.

a novel TAI scaffold with the added features that they inhibit LOX-1 and some of them disassemble preformed tau aggregates.

In conclusion, this study shows that these compounds inhibit assembly of tau aggregates, disassemble preformed tau aggregates, and partially preserve tau's ability to bind to tubulin and promote microtubule assembly. These compounds provide a promising novel scaffold for TAI molecules. The structure−activity relationship studies give us several leads for the probable important chemical groups required in this scaffold structure required for the anti-tau aggregation activity of the compounds. Further studies on the interaction between the compounds and tau will help to determine the precise mechanism of action of these compounds.

METHODS

Chemicals and Reagents. Full-length 2N4R tau (441 amino acids) was expressed in *Escherichia coli* and purified as described previously.[37] Arachidonic acid (ARA) was purchased from Cayman Chemicals (Ann Arbor, MI). Heparin sodium salt was purchased from Sigma (St. Louis, MO). Asperbenzaldehyde was purified from *A. nidulans* and converted to the compounds aza-7−aza-17 (Figure 1) as described previously[11] with the following minor modifications. We constructed a number of strains with various promoter combinations and used a variety of induction conditions to maximize asperbenzaldehyde production. Our best yields were obtained with strain LO8355 (*pyrG*89, *pyroA*4, *riboB*2, *nkuA*::*argB*, *stcJ*::*AfriboB*, AN1029(p)::*AfpyrG-alcA*(p), AN1033::*AfpyroA*, *alcR*(p)::*ptrA-gpdA*(p)). In this strain, the promoter of asperfuranone biosynthesis transcription factor AN1029 (using the *AspGD* nomenclature; http://aspergillusgenomes.org/) is replaced with the highly inducible *alcA* promoter, and the *alcR* promoter is replaced with the strong, constitutive *gpdA* promoter (−1241 to −1). AN1033 is replaced with the *AfpyroA* gene to interrupt the asperfuranone biosynthesis pathway, causing asperbenzaldehyde to accumulate. Growth was in lactose minimal medium (20 g/L lactose, 6 g/L $NaNO_3$, 0.52 g/L KCl, O.52 g/L $MgSO_4{\cdot}7H_2O$, 1.52 g/L KH_2PO_4, 1 mL/L trace elements solution).[38] Spores were inoculated at 10^6/mL into 500 mL of medium in a 2 L flask. Incubation was at 37 °C on a gyratory shaker, and induction was with 30 mM methyl-ethyl-ketone, added 55 h after inoculation. Cultures were harvested 6 days after inoculation. Yields of purified asperbenzaldehyde

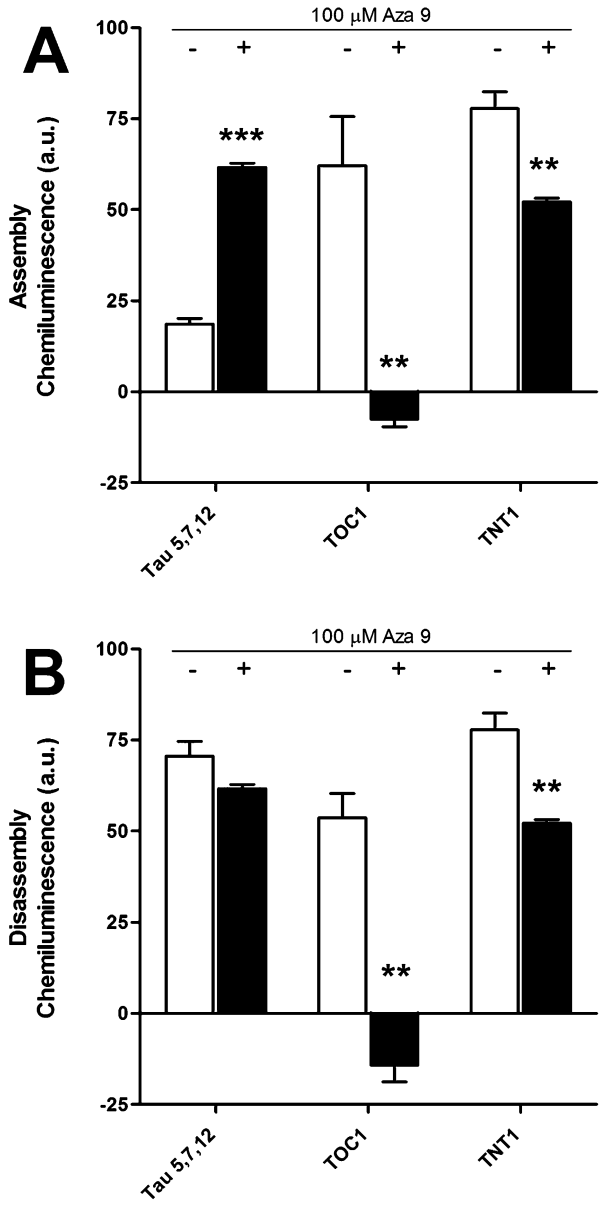


Figure 8. Filter trap assay for filament assembly and disassembly of heparin-induced tau filaments. (A) For assembly inhibition, 100 μM aza-9 was incubated with 2 μM tau for 20 min before the addition 0.6 μM heparin. Sixteen hours after induction, the degree of aggregation was determined using the filter trap assay detected by a mixture of antibodies to normal tau (tau 5, tau 7, and tau 12), an antibody to oligomeric tau (TOC1), and an antibody to a toxic conformation of tau (TNT1). The average of three independent trials ± SD is shown for no compound controls (white bars) and 100 μM aza-9 (black bars). (B) 2 μM tau and 0.6 μM heparin were incubated together for 12 h prior to the addition of 100 μM aza-9 or an equal volume of DMSO. Disassembly reactions proceeded for an additional 24 h, and the degree of aggregation was determined using the filter trap assay detected by a mixture of antibodies (tau 5, tau 7, and tau 12), TOC1, and TNT1. The average of three independent trials ± SD is shown for no compound controls (white bars) and 100 μM aza-9 (black bars). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

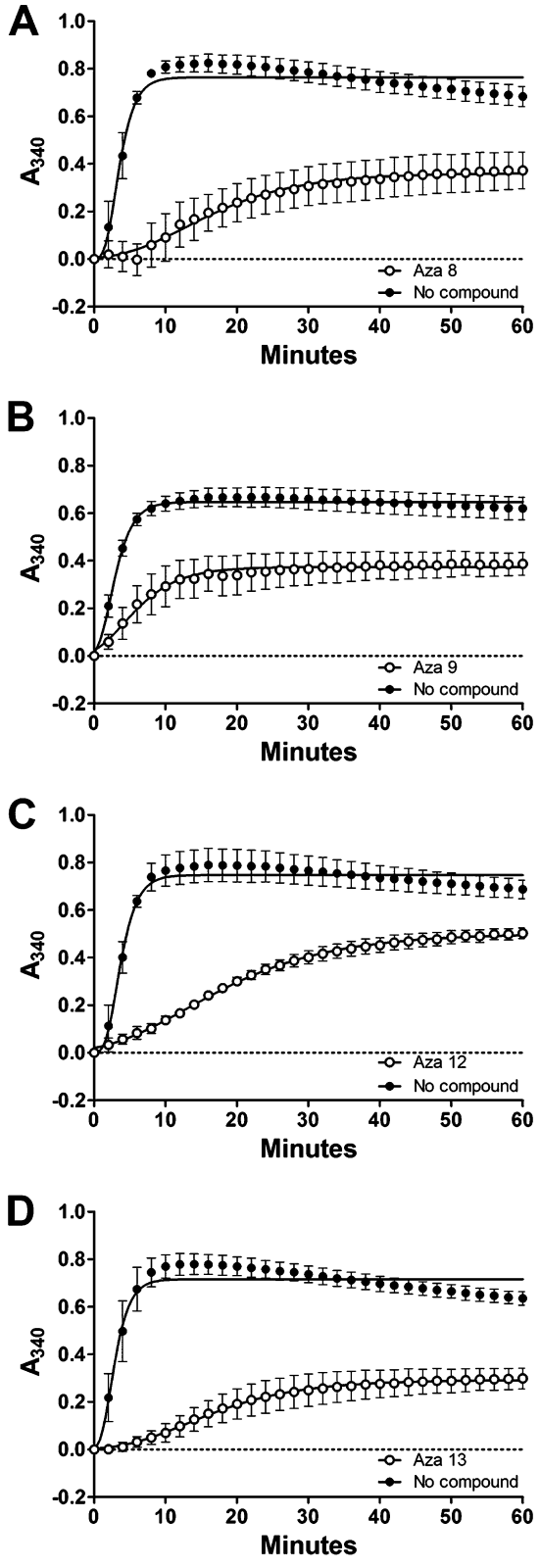


Figure 9. Microtubule assembly in the presence of the most potent azaphilone tau aggregation inhibitors. Tubulin was incubated with either tau protein alone or tau in the presence of (A) aza-8, (B) aza-9, (C) aza-12, or (D) aza-13 at a concentration of 90 μM. Microtubule assembly was monitored by absorbance at 340 nm (y axis) over time (x axis). Each point is the average of three independent trails ± SD. All data were fit to Gompertz growth curve (dashed and solid lines for no compound and 90 μM azaphilones, respectively). For clarity, only every other time point is shown on the graph. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Table 1. Statistical Analysis of the Effects of Compounds Aza-8, Aza-9, Aza-12, and Aza-13 on the Stabilization of Microtubules[a]

		t_i (min)	k_{app} (min^{-1})	max (A_{340})
Aza-8	−	3.08 ± 1.28	0.87 ± 0.14	0.76 ± 0.36
	+	13.42 ± 6.59	0.12 ± 0.02***	0.36 ± 0.14*
Aza-9	−	2.26 ± 0.65	0.62 ± 0.08	0.65 ± 0.38
	+	5.03 ± 1.93	0.26 ± 0.19*	0.38 ± 0.10*
Aza-12	−	3.11 ± 1.22	0.78 ± 0.09	0.75 ± 0.10
	+	12.83 ± 1.35***	0.09 ± 0.02***	0.50 ± 0.04*
Aza-13	−	2.58 ± 1.29	0.86 ± 0.17	0.72 ± 0.06
	+	15.65 ± 7.72*	0.12 ± 0.05**	0.30 ± 0.07**

[a]Max (A_{340}) is the maximum amount of microtubule polymerization, t_i is the point of inflection of the curve at the time of maximum growth rate in minutes, and b is inversely proportional to the apparent rate of polymerization (k_{app}, min^{-1}), as determined from the fit of three individual microtubule polymerization curves for each condition to a nonlinear Gompertz growth function (see Methods). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

were greater than 2 g/L, representing an approximate conversion of 10% of the carbon source to final product. Additional information on the purity of the compounds can be found in Supporting Information.

Inhibition of Tau Aggregation. 75 μM arachidonic acid was used to initiate the aggregation of 2 μM tau in polymerization buffer (PB, 10 mM HEPES (pH 7.64), 5 mM DTT, 100 mM NaCl, 0.1 mM EDTA, and 3.75% ethanol) *in vitro* as previously described.[17] Compounds dissolved in DMSO were added to a final concentration of 200 μM and incubated with tau protein in PB 20 min prior to the addition of arachidonic acid. The reactions were allowed to proceed for 16 h at room temperature before analysis.[10] For heparin induced tau assembly inhibition reactions, 0.6 μM heparin was used to initiate aggregation on 2 μM tau in polymerization buffer for heparin (PBh, 10 mM HEPES (pH 7.64), 5 mM DTT, 17.7 mM NaCl) *in vitro*. Compounds dissolved in DMSO were added to a final concentration of 100 μM and incubated with tau protein in PB 20 min prior to the addition of heparin. The reactions were allowed to proceed for 16 h at 37 °C before analysis.

Disassembly of Preformed Filaments. Preformed tau filaments were generated with 2 μM tau and 75 μM ARA in PB as described above for 6 h at room temperature. Compounds dissolved in DMSO were added to the tau solution at final concentrations indicated in the Results section and figure legends. The reactions were allowed to proceed at room temperature for 12 h before analysis. For heparin-induced tau aggregation assays, preformed tau filaments were generated with 2 μM tau and 0.6 μM heparin in PBh as described above for 12 h at 37 °C. Compounds dissolved in DMSO were added to the tau solution at final concentrations indicated in the Results section and figure legends. The reactions were allowed to proceed at 37 °C for 24 h before analysis.

Filter Trap Assay. The amount of tau aggregates following assembly or disassembly reactions was determined by filter trap assay as described previously.[10] Reactions were diluted into TBS such that they contained 20 ng of protein in 300 μL. For heparin-induced tau aggregation reactions, the reactions were diluted into TBS such that they contained 60 ng of protein in 300 μL. Solutions were passed through a nitrocellulose membrane using house vacuum in a dot-blot apparatus. The aggregates trapped on the membrane were detected by either general antibodies (a mixture of tau 5[39] at 1:50 000 dilution, tau 12[40] at 1:250 000 dilution, and tau 7[41] at 1:250 000 dilution) or antibodies to toxic conformations (TNT1[19] at 1:200 000 or TOC1[20] at 1:7000). All antibodies were a kind gift from Drs. Nick Kanaan and Lester I. Binder. HRP-linked goat anti-mouse IgG (general antibodies and TNT1) or HRP-linked goat anti-mouse IgM (TOC1) (Thermo Scientific, Rockford, IL) were used as the secondary antibodies, and blots were developed using ECL (enhanced chemiluminescence) western blotting analysis system (GE Healthcare, Buckinghamshire, UK).

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

Images were captured with a Kodak Image Station 4000R or ChemiDoc-It^2 imager and were quantified using the histogram function of Adobe Photoshop 7.0. Statistical analyses were performed using unpaired *t*-tests to compare the triplicate values to control values.

Transmission Electron Microscopy. Polymerization reaction samples were diluted 1:10 in PB and fixed with 2% glutaraldehyde for 5 min. Fixed samples were placed on Formvar carbon-coated grids and stained with uranyl acetate as previously described.[10] Images were captured with a Technai F20 XT field emission transmission electron microscope (FEI Co., Hillsboro, OR) and Gatan Digital Micrograph imaging system (Gatan, Inc., Pleasanton, CA). The filaments were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD) as previously described.[10] For quantitative analysis, filament lengths were placed into bins as described in Results. Statistical analyses were performed using unpaired *t*-tests to compare four or five replicates for each bin size with the no-compound data serving as reference values.

Tubulin Polymerization Assay. Polymerization of tubulin was measured using a tubulin polymerization assay kit (BK006P, Cytoskeleton, Inc., Denver, CO) following the manufacturer's protocol. Briefly, 2 mg/mL porcine tubulin was incubated with 1.5 μM tau and 90 μM aza-8, aza-9, aza-12, or aza-13 compound. Tubulin polymerization was monitored by turbidity at 340 nm in a Varian 50 MPR microplate reader at 37 °C for 1 h. Experiments were performed in triplicate, averaged, and fit to a Gompertz growth equation as previously described[42] using the equation

$$y = a\, e^{-e^{-\left(\frac{t-t_i}{b}\right)}}$$

where y is the amount of microtubule polymerization measured at time t, a is the maximum amount of microtubule polymerization observed at an absorbance of 340 nm (max), t_i is the point of inflection of the curve at the time of maximum growth rate in minutes, and b is inversely proportional to the apparent rate of polymerization (k_{app}, min^{-1}). The average values for parameters a, b, and t_i were determined and compared to the no-compound control using a paired *t*-test to determine statistical significance. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: Representative example ^{1}H NMR spectrum used to determine purity and diastereomeric ratio. Figure S2: Preformed tau filaments binding to electron microscopy grids in the presence of azaphilone compounds. Table S1: Diastereomeric ratios of aza-7−aza-17. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: smita_paranjape@hotmail.com.

Author Contributions

S.R.P. carried out the tau assembly and disassembly experiments and drafted the manuscript. A.P.R. purified asperbenzaldehyde, generated and purified azaphilone derivatives, and assisted with analysis of structure/activity relationships. A.D.S. purified asperbenzaldehyde and generated and purified derivatives of azaphilones. C.E.O. constructed the *A. nidulans* strains for asperbenzaldehyde overproduction and cultured the strains for overproduction. C.C.C.W. and B.R.O. conceived of the generation and purification of derivatives of asperbenzaldehyde and azaphilones. T.E.P. assisted with analysis of structure/activity relationships. B.R.O. and T.C.G. conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Funding

Funding was provided in part by P01-GM084077 from the National Institute of General Medical Sciences (C.C.C.W. and B.R.O.), T32 GM008545 from the National Institute of General Medical Sciences (A.P.R.), R01-NS083391 from the National Institute of Neurological Disorders and Stroke (T.C.G.), and by the H.L. Snyder Medical Foundation (B.R.O. and T.C.G.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Adam Miltner for assistance with protein expression and purification. We thank Drs. Nick Kanaan and Lester I. Binder for their kind gift of the antibodies used in this study.

■ REFERENCES

(1) Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T. (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology 42*, 631−639.

(2) Wischik, C. M., Harrington, C. R., and Storey, J. M. (2014) Tau-aggregation inhibitor therapy for Alzheimer's disease. *Biochem. Pharmacol. 88*, 529−539.

(3) Pickhardt, M., Gazova, Z., von Bergen, M., Khlistunova, I., Wang, Y., Hascher, A., Mandelkow, E. M., Biernat, J., and Mandelkow, E. (2005) Anthraquinones inhibit tau aggregation and dissolve Alzheimer's paired helical filaments in vitro and in cells. *J. Biol. Chem. 280*, 3628−3635.

(4) Calcul, L., Zhang, B., Jinwal, U. K., Dickey, C. A., and Baker, B. J. (2012) Natural products as a rich source of tau-targeting drugs for Alzheimer's disease. *Future Med. Chem. 4*, 1751−1761.

(5) Fang, L., Gou, S., Fang, X., Cheng, L., and Fleck, C. (2013) Current progresses of novel natural products and their derivatives/analogs as anti-Alzheimer candidates: an update. *Mini-Rev. Med. Chem. 13*, 870−887.

(6) Yaegashi, J., Oakley, B. R., and Wang, C. C. (2014) Recent advances in genome mining of secondary metabolite biosynthetic gene clusters and the development of heterologous expression systems in Aspergillus nidulans. *J. Ind. Microbiol. Biotechnol. 41*, 433−442.

(7) Nayak, T., Szewczyk, E., Oakley, C. E., Osmani, A., Ukil, L., Murray, S. L., Hynes, M. J., Osmani, S. A., and Oakley, B. R. (2006) A versatile and efficient gene-targeting system for Aspergillus nidulans. *Genetics 172*, 1557−1566.

(8) Oakley, C. E., Edgerton-Morgan, H., and Oakley, B. R. (2012) Tools for manipulation of secondary metabolism pathways: rapid promoter replacements and gene deletions in *Aspergillus nidulans*. *Methods Mol. Biol. 944*, 143−161.

(9) Szewczyk, E., Nayak, T., Oakley, C. E., Edgerton, H., Xiong, Y., Taheri-Talesh, N., Osmani, S. A., and Oakley, B. R. (2006) Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat. Protoc. 1*, 3111−3120.

(10) Paranjape, S. R., Chiang, Y. M., Sanchez, J. F., Entwistle, R., Wang, C. C., Oakley, B. R., and Gamblin, T. C. (2014) Inhibition of tau aggregation by three *Aspergillus nidulans* secondary metabolites: 2,omega-dihydroxyemodin, asperthecin, and asperbenzaldehyde. *Planta Med. 80*, 77−85.

(11) Somoza, A. D., Lee, K. H., Chiang, Y. M., Oakley, B. R., and Wang, C. C. (2012) Reengineering an azaphilone biosynthesis pathway in *Aspergillus nidulans* to create lipoxygenase inhibitors. *Org. Lett. 14*, 972−975.

(12) Matsuzaki, K., Tahara, H., Inokoshi, J., Tanaka, H., Masuma, R., and Omura, S. (1998) New brominated and halogen-less derivatives and structure−activity relationship of azaphilones inhibiting gp120−CD4 binding. *J. Antibiot. 51*, 1004−1011.

(13) Clark, R. C., Lee, S. Y., Searcey, M., and Boger, D. L. (2009) The isolation, total synthesis and structure elucidation of chlorofusin, a

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760

natural product inhibitor of the p53−mDM2 protein-protein interaction. *Nat. Prod. Rep. 26*, 465−477.
(14) Musso, L., Dallavalle, S., Merlini, L., Bava, A., Nasini, G., Penco, S., Giannini, G., Giommarelli, C., De Cesare, A., Zuco, V., Vesci, L., Pisano, C., Castorina, M., Milazzo, F., Cervoni, M. L., Dal Piaz, F., De Tommasi, N., and Zunino, F. (2010) Natural and semisynthetic azaphilones as a new scaffold for Hsp90 inhibitors. *Bioorg. Med. Chem. 18*, 6031−6043.
(15) Nam, J. Y., Kim, H. K., Kwon, J. Y., Han, M. Y., Son, K. H., Lee, U. C., Choi, J. D., and Kwon, B. M. (2000) 8-O-Methylsclerotiorinamine, antagonist of the Grb2-SH2 domain, isolated from *Penicillium multicolor*. *J. Nat. Prod. 63*, 1303−1305.
(16) Chu, J., and Pratico, D. (2011) Pharmacologic blockade of 5-lipoxygenase improves the amyloidotic phenotype of an Alzheimer's disease transgenic mouse model involvement of gamma-secretase. *Am. J. Pathol. 178*, 1762−1769.
(17) Carlson, S. W., Branden, M., Voss, K., Sun, Q., Rankin, C. A., and Gamblin, T. C. (2007) A complex mechanism for inducer mediated tau polymerization. *Biochemistry 46*, 8838−8849.
(18) Chang, E., and Kuret, J. (2008) Detection and quantification of tau aggregation using a membrane filter assay. *Anal. Biochem. 373*, 330−336.
(19) Ward, S. M., Himmelstein, D. S., Ren, Y., Fu, Y., Yu, X. W., Roberts, K., Binder, L. I., and Sahara, N. (2014) TOC1: a valuable tool in assessing disease progression in the rTg4510 mouse model of tauopathy. *Neurobiol. Dis. 67*, 37−48.
(20) Kanaan, N. M., Morfini, G. A., LaPointe, N. E., Pigino, G. F., Patterson, K. R., Song, Y., Andreadis, A., Fu, Y., Brady, S. T., and Binder, L. I. (2011) Pathogenic forms of tau inhibit kinesin-dependent axonal transport through a mechanism involving activation of axonal phosphotransferases. *J. Neurosci. 31*, 9858−9868.
(21) Akoury, E., Gajda, M., Pickhardt, M., Biernat, J., Soraya, P., Griesinger, C., Mandelkow, E., and Zweckstetter, M. (2013) Inhibition of tau filament formation by conformational modulation. *J. Am. Chem. Soc. 135*, 2853−2862.
(22) Bhat, R. V., Budd Haeberlein, S. L., and Avila, J. (2004) Glycogen synthase kinase 3: a drug target for CNS therapies. *J. Neurochem. 89*, 1313−1317.
(23) Lagoja, I., Pannecouque, C., Griffioen, G., Wera, S., Rojasdelaparra, V. M., and Van Aerschot, A. (2011) Substituted 2-aminothiazoles are exceptional inhibitors of neuronal degeneration in tau-driven models of Alzheimer's disease. *Eur. J. Pharm. Sci. 43*, 386−392.
(24) Brunden, K. R., Yao, Y., Potuzak, J. S., Ferrer, N. I., Ballatore, C., James, M. J., Hogan, A. M., Trojanowski, J. Q., Smith, A. B., III, and Lee, V. M. (2011) The characterization of microtubule-stabilizing drugs as possible therapeutic agents for Alzheimer's disease and related tauopathies. *Pharmacol. Res. 63*, 341−351.
(25) Zhang, B., Carroll, J., Trojanowski, J. Q., Yao, Y., Iba, M., Potuzak, J. S., Hogan, A. M., Xie, S. X., Ballatore, C., Smith, A. B., III, Lee, V. M., and Brunden, K. R. (2012) The microtubule-stabilizing agent, epothilone D, reduces axonal dysfunction, neurotoxicity, cognitive deficits, and Alzheimer-like pathology in an interventional study with aged tau transgenic mice. *J. Neurosci. 32*, 3601−3611.
(26) Chang, E., Congdon, E. E., Honson, N. S., Duff, K. E., and Kuret, J. (2009) Structure−activity relationship of cyanine tau aggregation inhibitors. *J. Med. Chem. 52*, 3539−3547.
(27) Honson, N. S., Jensen, J. R., Darby, M. V., and Kuret, J. (2007) Potent inhibition of tau fibrillization with a multivalent ligand. *Biochem. Biophys. Res. Commun. 363*, 229−234.
(28) Pickhardt, M., Larbig, G., Khlistunova, I., Coksezen, A., Meyer, B., Mandelkow, E. M., Schmidt, B., and Mandelkow, E. (2007) Phenylthiazolyl-hydrazide and its derivatives are potent inhibitors of tau aggregation and toxicity in vitro and in cells. *Biochemistry 46*, 10016−10023.
(29) Asuni, A. A., Boutajangout, A., Quartermain, D., and Sigurdsson, E. M. (2007) Immunotherapy targeting pathological tau conformers in a tangle mouse model reduces brain pathology with associated functional improvements. *J. Neurosci. 27*, 9115−9129.
(30) Krishnamurthy, P. K., Deng, Y., and Sigurdsson, E. M. (2011) Mechanistic studies of antibody-mediated clearance of tau aggregates using an ex vivo brain slice model. *Front. Psychiatry 2*, 59.
(31) Sahara, N., Maeda, S., and Takashima, A. (2008) Tau oligomerization: a role for tau aggregation intermediates linked to neurodegeneration. *Curr. Alzheimer Res. 5*, 591−598.
(32) Blair, L. J., Zhang, B., and Dickey, C. A. (2013) Potential synergy between tau aggregation inhibitors and tau chaperone modulators. *Alzheimer's Res. Ther. 5*, 41.
(33) Chirita, C., Necula, M., and Kuret, J. (2004) Ligand-dependent inhibition and reversal of tau filament formation. *Biochemistry 43*, 2879−2887.
(34) Wischik, C. M., Edwards, P. C., Lai, R. Y., Roth, M., and Harrington, C. R. (1996) Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines. *Proc. Natl. Acad. Sci. U.S.A. 93*, 11213−11218.
(35) Keller, N. P., Turner, G., and Bennett, J. W. (2005) Fungal secondary metabolism—from biochemistry to genomics. *Nat. Rev. Microbiol. 3*, 937−947.
(36) Gao, J. M., Yang, S. X., and Qin, J. C. (2013) Azaphilones: chemistry and biology. *Chem. Rev. 113*, 4755−4811.
(37) Rankin, C. A., Sun, Q., and Gamblin, T. C. (2005) Pseudo-phosphorylation of tau at Ser202 and Thr205 affects tau filament formation. *Brain Res. 138*, 84−93.
(38) Vishniac, W., and Santer, M. (1957) The thiobacilli. *Bacteriol. Rev. 21*, 195−213.
(39) LoPresti, P., Szuchet, S., Papasozomenos, S. C., Zinkowski, R. P., and Binder, L. I. (1995) Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes. *Proc. Natl. Acad. Sci. U.S.A. 92*, 10369−10373.
(40) Horowitz, P. M., Patterson, K. R., Guillozet-Bongaarts, A. L., Reynolds, M. R., Carroll, C. A., Weintraub, S. T., Bennett, D. A., Cryns, V. L., Berry, R. W., and Binder, L. I. (2004) Early N-terminal changes and caspase-6 cleavage of tau in Alzheimer's disease. *J. Neurosci. 24*, 7895−7902.
(41) Horowitz, P. M., LaPointe, N., Guillozet-Bongaarts, A. L., Berry, R. W., and Binder, L. I. (2006) N-Terminal fragments of tau inhibit full-length tau polymerization in vitro. *Biochemistry 45*, 12859−12866.
(42) Combs, B., Voss, K., and Gamblin, T. C. (2011) Pseudohyperphosphorylation has differential effects on polymerization and function of tau isoforms. *Biochemistry 50*, 9446−9456.

DOI: 10.1021/acschemneuro.5b00013
ACS Chem. Neurosci. 2015, 6, 751−760