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Inhibition of Both Hsp70 Activity and Tau Aggregation *in Vitro* Best Predicts Tau Lowering Activity of Small Molecules

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Abstract

Three scaffolds with inhibitory activity against the heat shock protein 70 (Hsp70) family of chaperones have been found to enhance the degradation of the microtubule associated protein tau in cells, neurons, and brain tissue. This is important because tau accumulation is linked to neurodegenerative diseases including Alzheimer's disease (AD) and chronic traumatic encephalopathy (CTE). Here, we expanded upon this study to investigate the anti-tau efficacy of additional scaffolds with Hsp70 inhibitory activity. Five of the nine scaffolds tested lowered tau levels, with the rhodacyanine and phenothiazine scaffolds exhibiting the highest potency as previously described. Because phenothiazines also inhibit tau aggregation *in vitro*, we suspected that this activity might be a more accurate predictor of tau lowering. Interestingly, the rhodacyanines did inhibit *in vitro* tau aggregation to a similar degree as phenothiazines, correlating well with tau-lowering efficacy in cells and *ex vivo* slices. Moreover, other Hsp70 inhibitor scaffolds with weaker tau-lowering activity in cells inhibited tau aggregation *in vitro*, albeit at lower potencies. When we tested six well-characterized tau aggregation inhibitors, we determined that this mechanism of action was not a better predictor of tau-lowering than Hsp70 inhibition. Instead, we found that compounds possessing both activities were the most effective at promoting tau clearance. Moreover, cytotoxicity and PAINS activity are critical factors that can lead to false-

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ASSOCIATED CONTENT

Supporting Information

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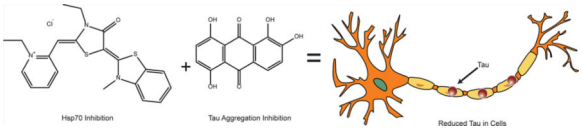
Supporting Figures 1–3 (PDF)

The authors declare no competing financial interest.

positive lead identification. Strategies designed around these principles will likely yield more efficacious tau-lowering compounds.

Graphical Abstract



Aggregation and accumulation of the microtubule associated protein tau is a common feature of several neurodegenerative diseases, referred to as tauopathies, which include chronic traumatic encephalopathy (CTE), Alzheimer's disease (AD), frontal temporal dementia linked to chromosome 17 (FTDP-17), Parkinson's disease, and Pick's disease.[1,2] A plethora of therapeutic strategies have been employed to attempt to prevent aberrant tau accumulation and toxicity, including kinase inhibitors, humanized antibodies, aggregation inhibitors, microtubule stabilizing compounds, and small molecules targeting degradation machinery.[3–11] Some of our original work sought to identify molecules that could simply facilitate tau clearance, regardless of any particular known mechanism of action.[12,13] While many of the compounds identified were largely cytotoxic, leading to a perceived reduction in tau, through these studies, we were able to identify chaperone modulators as one family of compounds capable of anti-tau activity independent of this toxicity.[12] In particular, inhibitors of the heat shock protein 70 kDa (Hsp70) chaperone family are highly effective at lowering tau.[8,14] When the activity of these molecular chaperones is pharmacologically or even genetically inhibited, tau can be degraded.[15–18] Recently, a number of other small molecules with anti-Hsp70 activity have been described. Some of these Hsp70 inhibitor scaffolds mimic ATP and act as a competitive inhibitor in the ATP pocket, while others are thought to act allosterically, blocking cofactor or substrate interactions.[19,20,8,9,21–28] But, the activity of these compounds against tau remains unknown.

In addition, several of the published molecules with Hsp70 inhibitory activity are known to have other mechanisms of action, perhaps as a result of their prevalence in existing chemical libraries and their possible pan-assay interference properties. In particular, the phenothiazine, methylene blue (MB), which has been identified as a potent Hsp70 inhibitor and an autophagy inducer, also possesses other activities due to its redox potential.[22,45] In fact, one such activity is inhibition of tau aggregation *in vitro*, an activity that was discovered two decades ago.[34] MB is thought to inhibit tau aggregation by covalent modification of tau *via* cysteine oxidation.[29,30] Because tau has two naturally occurring cysteine residues located in the microtubule binding domain, it can form intermolecular disulfide bonds with neighboring tau molecules leading to aggregate formation.[31–33] MB creates disulfide bonds within the same tau molecule disrupting fibrillization.[29] Since MB has been shown to reduce tau levels in multiple tauopathy models,[34–36] which has precipitated clinical trials of related derivatives for AD and FTD, it is difficult to know which activity, Hsp70 inhibition or aggregation inhibition, is most responsible for its ability to facilitate tau clearance.[34,37,38] In this regard, several other studies have identified tau aggregation inhibitors, but the ability of these compounds to promote tau clearance has not

been presented for most of these. For example, the olive oil phenols, aminothienopyridazine (ATPZ), rhodanines, and anthraquinones all prevent tau aggregation *in vitro*, yet it is not known if this activity is sufficient to facilitate tau reductions in living cells.[39,6,40–42]

On the basis of this information, we sought to evaluate if Hsp70 or aggregation inhibition was a better predictor of tau lowering in cells. With this in mind, we collected a number of known Hsp70 and aggregation inhibitors and assessed their activities against tau. Interestingly, neither *in vitro* activity alone was a strong predictor of tau-lowering in cells. Rather, only those molecules possessing potent activity against both Hsp70 ATPase function and tau aggregation *in vitro* facilitated tau clearance independent of toxicity. Here, we describe the implications of these findings for tau-based drug discovery efforts, and how this information could be used to improve the success rate for translation of leads identified from *in vitro* assays into preclinical and clinical studies.

RESULT AND DISCUSSION

On the basis of our previous reports that methylene blue and the compound YM-01, a derivative of MKT-077 from the rhodacyanine scaffold, both inhibited Hsp70 activity and lowered tau levels in a cell tauopathy model,[8,14] we hypothesized that it was in fact the Hsp70 ATPase inhibition that was the best predictor of tau-lowering activity in cells. To investigate this, we examined the tau-lowering capability of several other published Hsp70 inhibitors, listed in Table 1. Compounds for each scaffold were assessed for tau-lowering efficacy. Human embryonic kidney (HEK293T) cells, transiently overexpressing WT4R0N tau were treated with increasing concentrations of each compound for 24 h. Interestingly, vast differences in tau-lowering activity were found among the molecules. Compounds from the piperidine-3-carboxamide and the adenosine analog scaffolds surprisingly increased tau levels. In contrast, the rhodacyanine and phenothiazine compounds still potently reduced tau levels at all concentrations. However, the dihydropyrimidine, phenoxy-N-arylacetamide, sulfonamide, and flavonol scaffolds only lowered tau levels at the highest concentration tested, 30 μM (Figure 1A,B). Similar trends were observed for these compounds in a stably transfected HEK P301L tau cell line (Supporting Information Figure 1). These data indicate that allosteric Hsp70 inhibitors might be more likely to possess tau-lowering activity than those that directly target the ATP binding site. Perhaps more importantly, because all of these compounds target the same mechanism of action, we concluded that Hsp70 inhibition alone was insufficient to predict tau lowering activity by greater than ~60%.

Because methylene blue also inhibits *in vitro* tau aggregation, we then speculated that tau lowering efficacy could be better predicted by *in vitro* anti-tau aggregation activity. To test this, we evaluated the tau lowering activity of several commercially available tau aggregation inhibitor scaffolds including carbocyanine, aminothienopyridazine (ATPZ), polyphenols, anthraquinone, and rhodanine (Table 2). Surprisingly, only two of these compounds lowered tau levels in our HEK293T tauopathy cell model: carbocyanine and anthraquinone (Figure 2A,B). However, the activity of carbocyanine coincided with very high cytotoxicity as suggested by the reduced actin levels. Thus, only the anthraquinone at 30 μM effectively lowered tau levels, possibly suggesting another unknown mechanism for

this particular molecule. In this way, tau aggregation inhibition alone is an even worse predictor of tau lowering activity than Hsp70 inhibition.

Since neither of these mechanisms alone was sufficient to predict tau-lowering activity, we speculated that perhaps compounds with dual activities might be more likely to facilitate tau clearance in cells. This was largely based on the known pleiotropy of methylene blue. Therefore, we investigated whether those Hsp70 inhibitors capable of lowering tau might also have activity against its aggregation *in vitro*. To test this, 10 μM 4R0N human recombinant tau was incubated with each compound at increasing concentrations for 1 h at 37 °C. After 1 h of incubation, 10 μM heparin was added to the tau compound mixture and incubated for a further 24 h at 37 °C prior to reading the fluorescent intensity of each mixture. Increases in fluorescence intensity indicate enhanced amyloid formation. Interestingly, we found that compounds from the rhodacyanine and phenothiazine scaffolds had high anti-tau aggregation potency with IC_{50}'s of 0.813 μM and 1.35 μM, respectively. Less potent tau-lowering scaffolds such as dihydropyrimidine, phenoxy-N-arylacetamide, and flavonols had higher IC_{50}'s (22.7, 6.93, and 9.74 μM, respectively). In contrast, the compounds that increased tau levels in our cell model, piperidine-3-carboxamide and an adenosine analog, also increased tau aggregation levels *in vitro* (Figure 3A). To confirm *in vitro* anti-tau aggregation activity of the tau-lowering compounds, nondenaturing gel electrophoresis was performed. The most potent tau-lowering scaffold compounds, rhodacyanine and phenothiazine, also most potently reduced high molecular weight tau compared to other compounds and the tau alone control; however all compounds with anti-tau activity also reduced high molecular weight tau aggregation (Figure 3B,C). Since MB is known to alter cysteine oxidation in tau, a mechanism that can contribute to its antiaggregation activity,[29] we suspected that the rhodacyanines may be acting through a similar mechanism. However, when we tested for the production of hydrogen peroxide following incubation of recombinant tau with 30 μM rhodacyanine, no significant differences were observed (Supporting Information Figure 2). Thus, those compounds capable of inhibiting both Hsp70 activity and tau aggregation *in vitro* predicted tau lowering activity by ~80%.

One interesting outcome from these analyses was the discovery that the antiaggregant compound ATPZ did not lead to tau lowering, despite it having the same exact antiaggregation mechanism as MB: alteration of cysteine oxidation.[29] We speculated that the lack of tau-lowering activity may be due to ATPZ's inability to inhibit Hsp70 ATPase activity. Therefore, we examined the Hsp70 inhibitory activity of not only ATPZ but of each aggregation inhibitor. Recombinant human Hsp70 and DnaJA2 were incubated with increasing concentrations of each compound for 30 min, followed by the addition of ATP and subsequent 3 h incubation. We measured absorbance by malachite green assay as previously described.[21] None of the aggregation inhibitor scaffolds that failed to lower tau inhibited Hsp70 ATPase activity, with the exception of the rhodanine scaffold (Figure 4), a known PAINS molecule that can be effective in any assay due to Michael reactions.[43,44]

One final variable that we needed to consider for these results was the role of cytotoxicity in perceived tau reductions. We previously found that compounds with high cytotoxicity caused tau lowering, perhaps due to microtubule breakdown.[12,45,46] We speculated this to be

the case for the carbocyanine and possibly some of the other outliers in these studies. Therefore, we tested the cytotoxicity of all compounds at increasing concentrations using a lactate dehydrogenase (LDH) assay. The data revealed that both the sulfonamide compound and the carbocyanine had high toxicity relative to the other compounds, explaining why they were leading to perceived tau reductions (Figure 5). Thus, compounds with activity against both Hsp70 activity and tau aggregation *in vitro* can predict tau-lowering activity at greater than 90% when both cytotoxicity and PAINS classification are accounted for.

Overall, neither Hsp70 inhibition nor anti-tau aggregation activity alone is sufficient to predict tau lowering in an *in vitro* model by more than 50%, although Hsp70 inhibition appears to be a better predictor than aggregation inhibition. But compounds that possess both activities, exemplified by the phenothiazines and rhodacyanines, are highly likely to promote tau clearance in cells and neurons (Supporting Information Figure 3). Importantly, the higher the potency of Hsp70 inhibitors against tau aggregation, the more potent the tau lowering, suggesting a synergy of these two mechanisms. Perhaps compounds with both activities can keep tau in a unique conformation that better facilitates its clearance. While there are certainly other mechanisms of tau clearance and molecules with distinct activities that could lead to tau degradation, these findings suggest that a drug screening campaign based first on Hsp70 inhibition with follow up assays examining tau aggregation, cytotoxicity, and pan-assay interference will likely yield a high number of low false-positive leads with regard to cellular activity against tau.[47] This concept of polypharmacy has been gaining ground over the past few years. While target specificity is still a major goal of modern drug discovery, it is clear that compounds with multiple targets can have a clinical benefit despite their pleiotropy.[48–50] In particular, compounds that target multiple relevant mechanisms for single diseases may be more effective than compounds with single mechanisms of action.[48,49]

METHODS

Cell Culture

HEK293T cells were maintained in DMEM plus 10% FBS (Life Technologies), 1% penicilin streptomycin (Invitrogen), and 1% Glutamax (complete media; Thermo Scientific). Transfections were performed following the Invitrogen Plasmid DNA Transfection protocol using Opti-MEM. All cells were treated as indicated and were harvested as previously described.[9] Cells were washed twice in ice-cold PBS, then scraped in cold mammalian protein extraction reagent (M-PER; Fisher Scientific) containing PMSF, protease and phosphatase inhibitors. Samples were incubated on ice, vortexed, then centrifuged at 10 000 rpm for 5 min at 4 °C to clear debris. Assays were run in triplicate, and statistical significance was assessed by *t* test.

Aggregation Kinetics (Thioflavin T) Assay and immunoblot

Samples consisting of 10 μM purified human recombinant Tau P301L, 2 mM diothiothreitol, the indicated drug concentration, 10 μM heparin, and 10 μM thioflavin T in 100 mM sodium acetate buffer at a pH of 7.4 were prepared. A total of 200 μL of indicated sample was added to a black, sealed, clear-bottom 96 well plate (Corning costar 3603). Plates were incubated at

37 °C, and fluorescence (440 emission, 490 excitation) was observed over a 24 h time course, with periodic readings using a BioTek Synergy H1 microplate reader. Following ThT analyses, recombinant fibrils were analyzed by Native-PAGE (nondenaturing conditions), followed by immunoblotting for tau. Specifically, samples incubated with 30 μM of each compound were analyzed in this way.

Hydrogen Peroxide Assay

Hydrogen peroxide production was measured as previously described.[29] Briefly, recombinant human P301L tau (10 μM) was incubated with 30 μM rhodacyanine or equivalent vehicle (DMSO). Hydrogen peroxide production was measured using the PeroXQuant assay (Pierce 23280) according to manufacturer instructions.

Hsp70 ATPase Assay

Recombinant human Hsp70 and DnaJA2 were used in a malachite green assay. A master mix of Hsp70:DnaJA2 at 1 μM was prepared in assay buffer (0.017% Triton X-100, 100 mM Tris–HCl, 20 mM KCl, and 6 mM MgCl2, pH 7.4). An aliquot (14 μL) of this mixture was added into each well of a 96-well plate. To this solution, 1 μL of either compound (3, 10, 30, 100, or 200 μM) or DMSO was added, and the plate was incubated for 30 min at 37 °C before adding 10 μL of 2.5 mM ATP to start the reaction. Thus, the final reaction volume was 25 μL, and the conditions were 1 μM Hsp70, 1 μM DnaJA2, 4% DMSO, 0.01% Triton X-100, and 1 mM ATP. After 3 h of incubation at 37 °C, 80 μL of malachite green reagent was added into each well. Immediately after this step, 10 μL 34% sodium citrate was used to stop the nonenzymatic hydrolysis of ATP. The samples were mixed thoroughly and incubated at 37 °C for 15 min before measuring OD620 on a BioTek Synergy H1 microplate reader as previously described.[21]

Lactate Dehydrogenase (LDH) Assay

LDH cytotoxicity was measured using the Pierce LDH Cytotoxicity Assay Kit. Values are presented as the percent of LDH release compared to DMSO (Vehicle) treated cells.

Western Blotting

After normalization of protein concentration by bicinchoninic acid (BCA; ThermoFisher), samples were run on 10% SDS-PAGE gels (for nondenaturing gel electrophoresis, samples were run without β-mercaptoethanol on a 4–15% gradient PAGE gel) and transferred to PVDF membranes (Immobilon, EMD Millipore). Membranes were blocked for 1 h in 7% nonfat dry milk in TBS-T before being probed with antibodies directed against total tau (H150; 1:1000; Santa Cruz Biotechnology), tau phosphorylated at S396/404 (PHF1; 1:500; provided by P. Davies), β-actin (1:1000; Sigma-Aldrich), or GAPDH (1:1000; Biodesign). Primary antibodies were detected by species appropriate secondary antibodies (Southern Biotech), and chemiluminescence was detected by ECL (Thermo-Fisher). Scion Image software was used to calculate densitometry of all Western blots. All values are shown as percent vehicle following actin normalization ± standard error of the mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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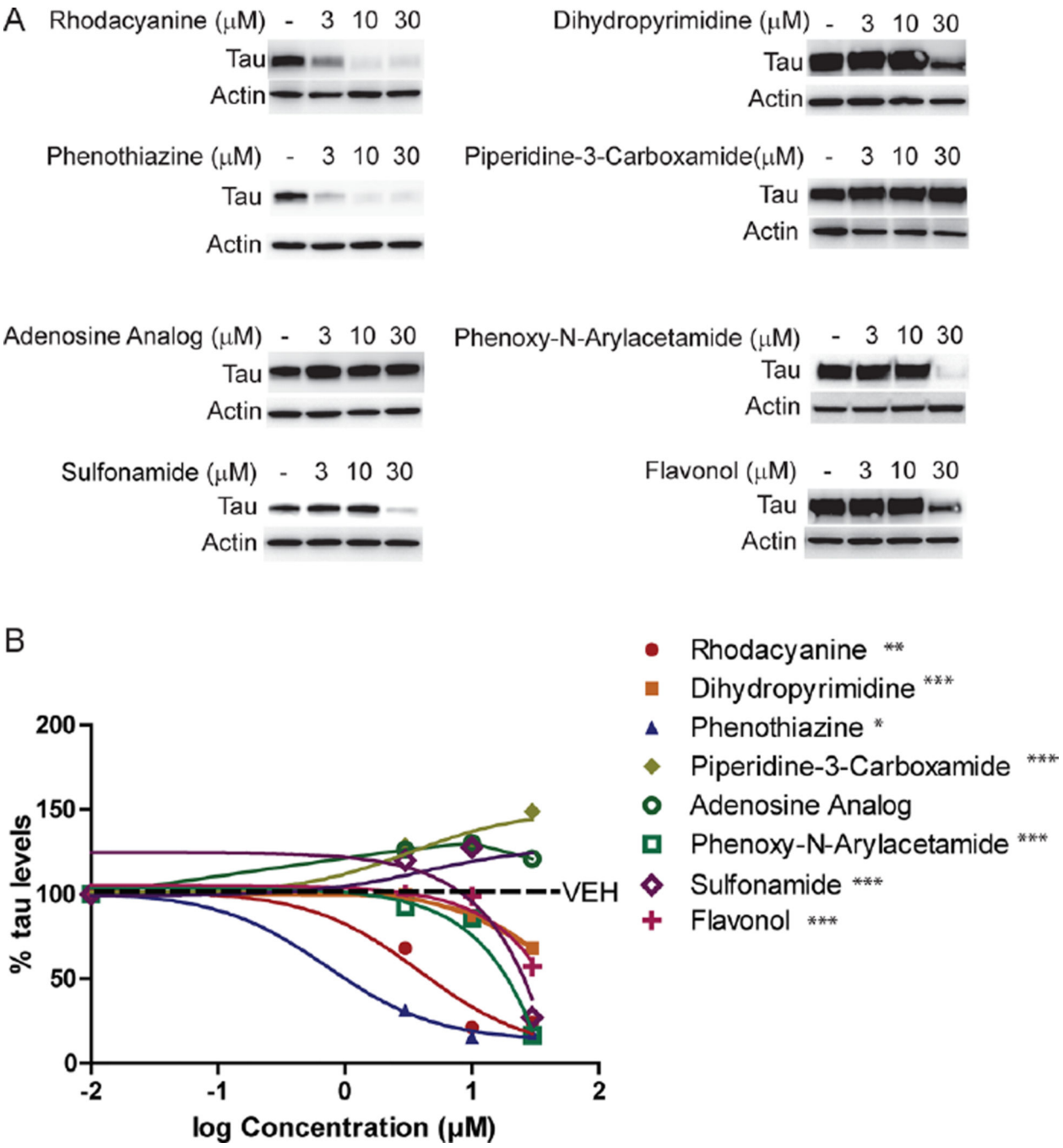


Figure 1.

Diverse Hsp70 inhibitor scaffolds having differing effects on tau levels. (A) Representative Western blot analysis of HEK293T cells transiently transfected WT4R0N tau and treated with each Hsp70 inhibitor at indicated concentrations for 24 h. (B) Quantification of tau levels in panel A as a percentage of vehicle treated ± standard error of the mean (SEM), $n = 3$. By linear regression analyses, *** indicates $p < 0.001$, and ** indicates $p < 0.01$.

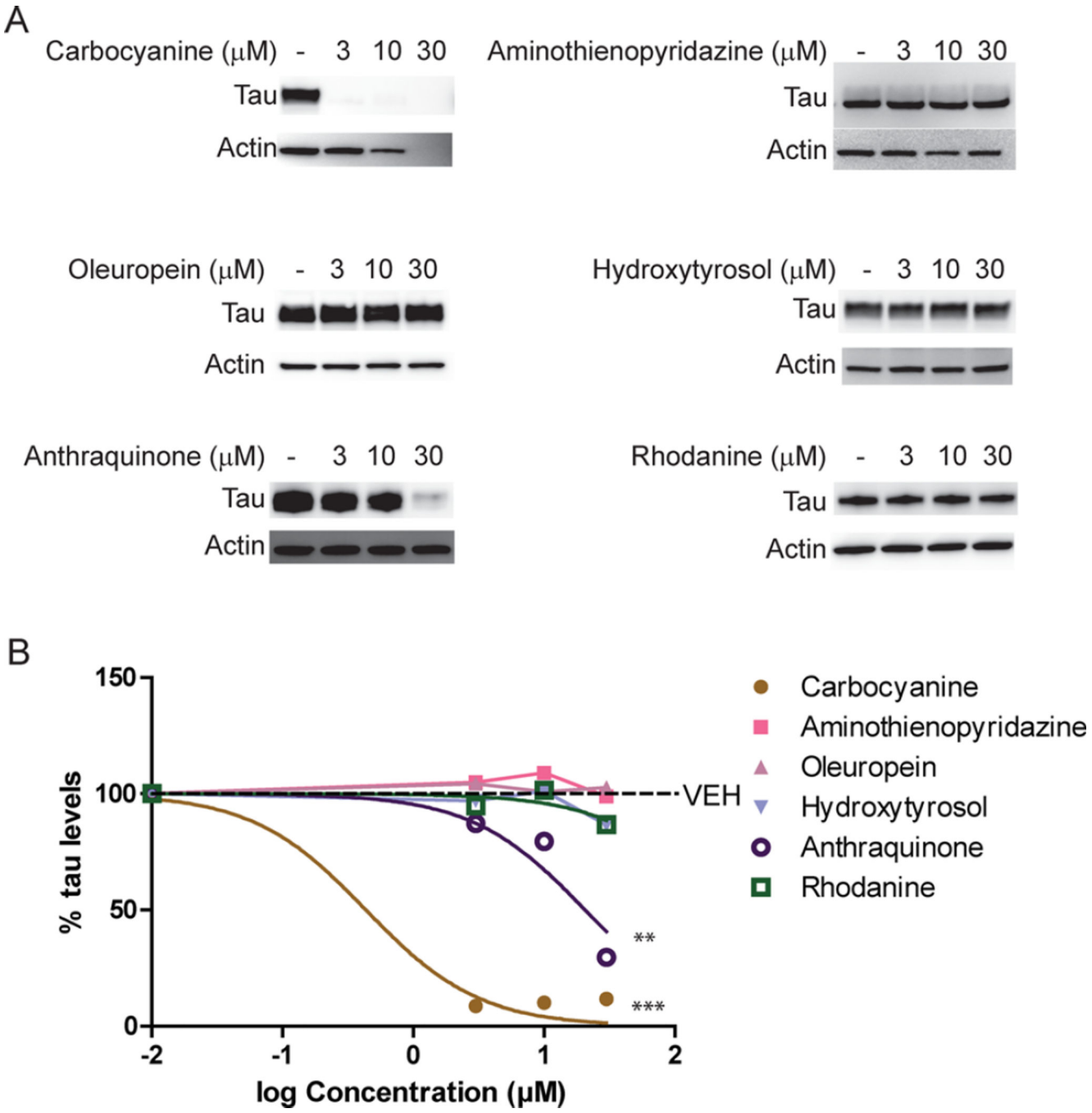


Figure 2.

Diverse tau aggregation inhibitor scaffolds having differing effects on tau levels. (A) Representative Western blot analysis of HEK293T cells transiently transfected WT4R0N tau and treated with each tau aggregation inhibitor at indicated concentrations for 24 h. (B) Quantification of tau levels in panel A as a percentage of vehicle treated ± standard error of the mean (SEM), $n = 3$. By linear regression analyses, *** indicates $p < 0.001$, ** indicates $p < 0.01$, and * indicates $p < 0.05$.

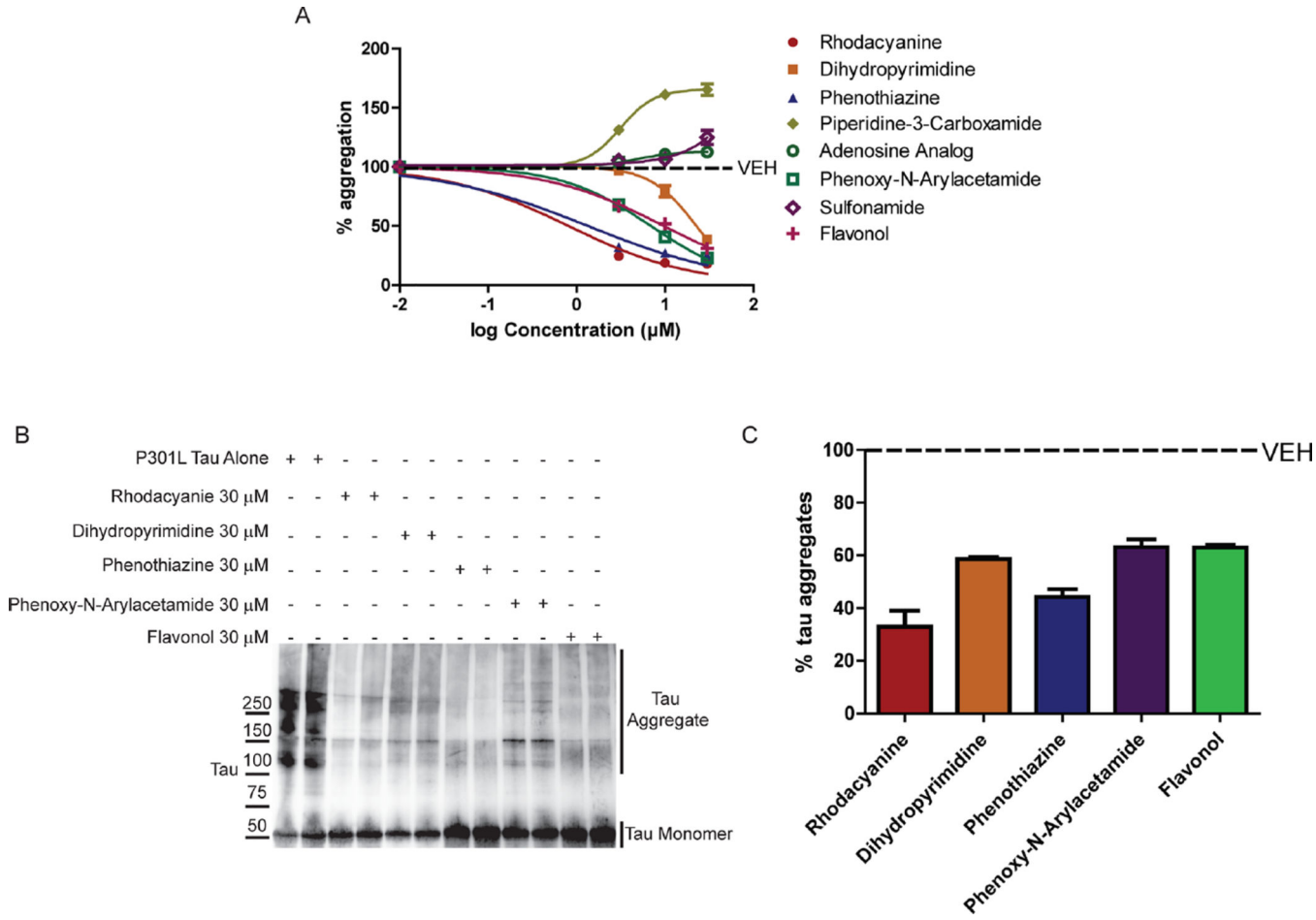


Figure 3.

Diverse Hsp70 inhibitor scaffolds having differing effects on tau aggregation *in vitro*. (A) anti-Hsp70 compounds; quantification of *in vitro* ThT assay using recombinant P301L tau incubated with the drug at increasing concentrations for 1 h prior to addition of 20 μM heparin which was then incubated for 24 h. Data are average ± standard error of the mean (SEM), $n = 3$. (B) Nondenaturing gel electrophoresis of antiaggregation ThT samples from A. (C) Quantification of tau aggregate levels from B. Data are average ± standard error of the mean (SEM), $n = 3$.

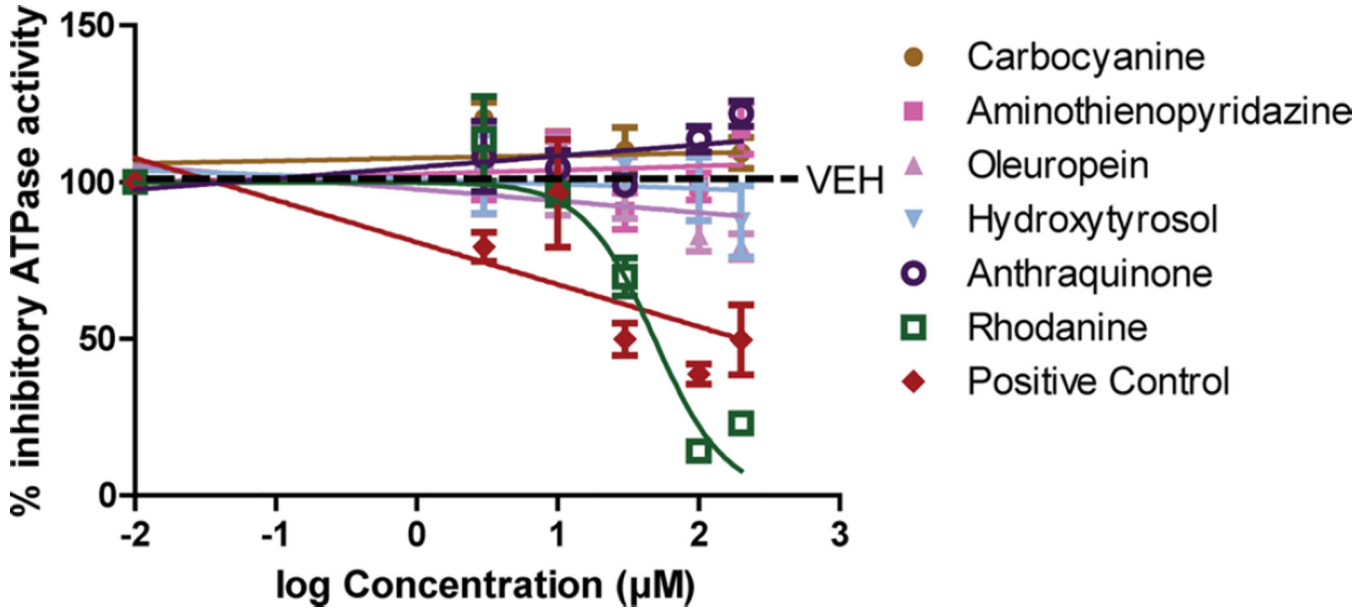


Figure 4.

Tau aggregation inhibitor scaffolds not inhibiting Hsp70 ATPase activity. Hsp70 quantification of ATPase activity using recombinant human Hsp70 and DnaJA2 protein. Activities are a percentage of vehicle treated ± standard error of the mean (SEM), $n = 3$.

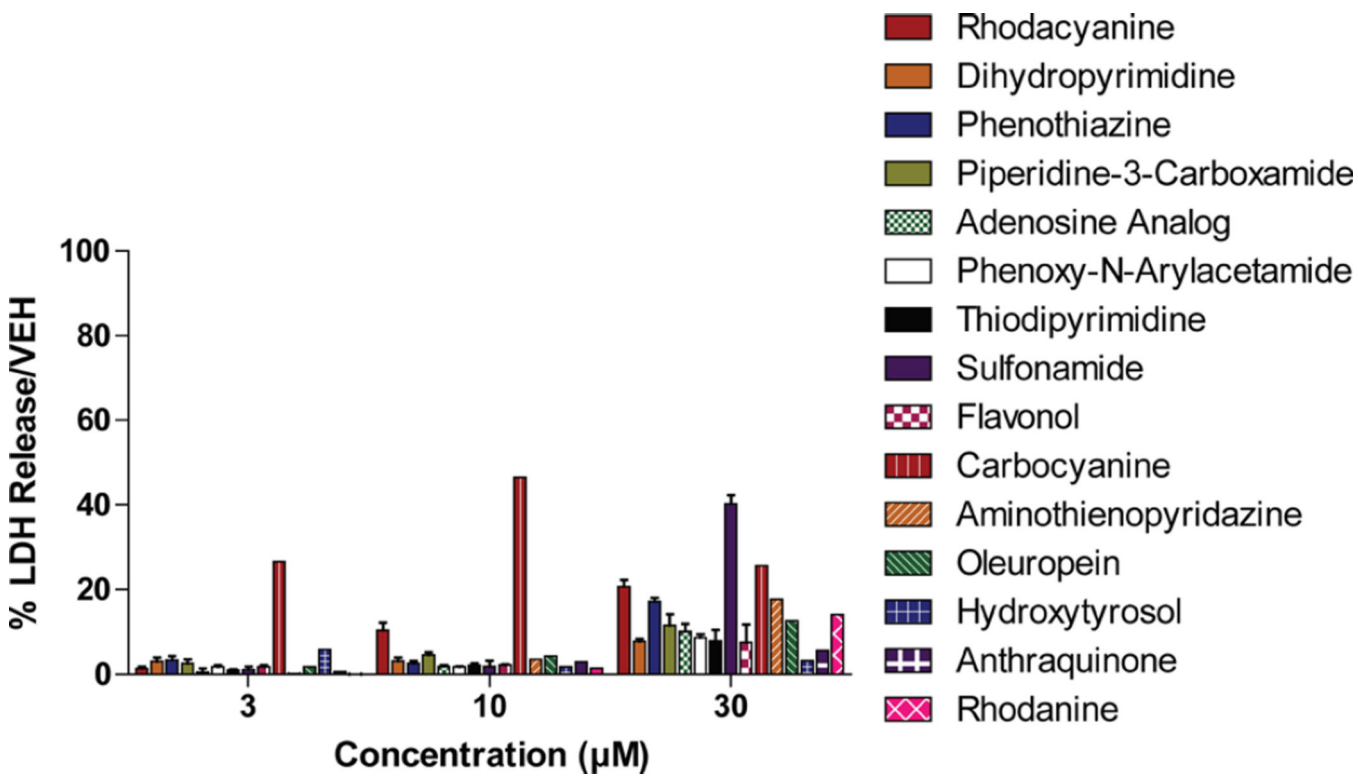
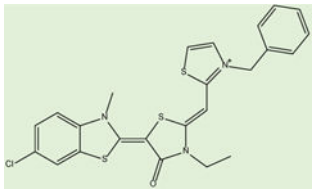
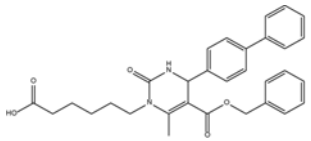
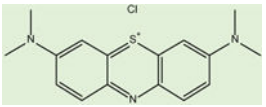
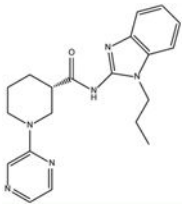


Figure 5.

Determination of LDH cytotoxicity of Hsp70 and Tau aggregation inhibitors in HEK293T cells. HEK293T cells (10 000 cells per well) were plated in a 96-well plate and incubated overnight at 37 °C. After 24 h, increasing concentrations of inhibitors were added to the culture media and incubated for 24 h at 37 °C. LDH cytotoxicity was measured using the Pierce LDH Cytotoxicity Assay Kit. Values are relative to DMSO control.

Table 1

Summary of Published Hsp70 Inhibitor Scaffolds

Scaffold	Nomenclature (Compound Name)	Compound Structure	Hsp70/DnaJ Inhibitory Activity Reported	Hsp70 Family member targeted	Anti-Tau Activity Reported
Rhodacyanine	3-benzyl-2-((Z)-((E)-5-(6-chloro-3-methylbenzo[d]thiazol-2(3H)-ylidene)-3-ethyl-4-oxothiazolidin-2-ylidene)methyl)thiazol-3-ium chloride **(JG-98)**		(Li et al., 2013; Rousaki et al., 2011)	HSPA8 (Hsc70); HSPA9 (Mortalin); HSPA1 (Hsp72); SSA1 (Hsp70 yeast) DnaK (Hsp70 Bacterial)	(Fontaine et al., 2015; Rousaki et al., 2011)
Dihydropyrimidine	4-[1,1'-Biphenyl]-4-yl-3,4-dihydro-6-methyl-2-oxo-5-[(phenylmethoxy)carbonyl]-1(2H)-pyrimidinehexanoic acid **(116-9E)**		(Chang et al., 2008; Wisen et al., 2010)	DnaK (Hsp70 Bacterial)	Unknown
Phenothiazine	3,7-bis(Dimethylamino)phenazathionium chloride **(Methylene Blue)**		(Chang et al., 2008; Jinwal et al., 2009; Miyata et al., 2012)	HSPA1 (Hsp72)	(Jinwal et al., 2009; Wischik, Edwards, Lai, Roth, & Harrington, 1996)
Piperidine-3-Carboxamide	(S)-N-(1-propyl-1H-benzimidazol-2-yl)-1-(2-pyrazinyl)piperidine-3-carboxamide **(HS72)**		(Howe et al., 2014)	HSPA1 (Hsp72)	Unknown

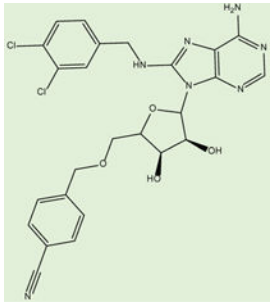
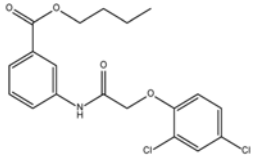
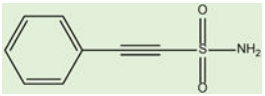
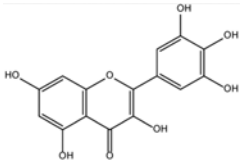
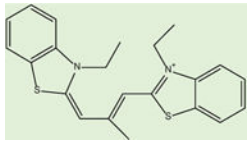
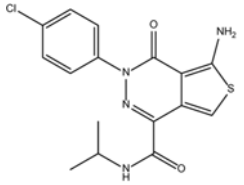
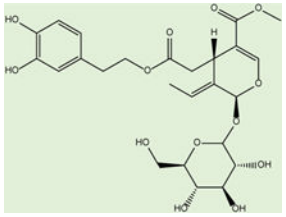
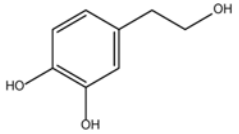
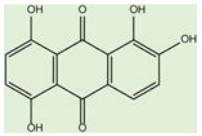
Scaffold	Nomenclature (Compound Name)	Compound Structure	Hsp70/DnaJ Inhibitory Activity Reported	Hsp70 Family member targeted	Anti-Tau Activity Reported
Adenosine Analog	5'-O-[(4-Cyanophenyl)methyl]-8-[[(3,4-dichlorophenyl)methyl]amino]-adenosine **(VER-155008)**		(Williamson et al., 2009)	HSPA8 (Hsc70); HSPA1 (Hsp72)	Unknown
Phenoxy-N-Arylacetamide	butyl 3-[2-(2,4-dichlorophenoxy)acetamido] benzoate **(ALSB-2970)**		(Cassel, llyin, McDonnell, & Reitz, 2012)	targets and binds to DNAJ	Unknown
Sulfonamide	2-Phenylethynesulfonamide **(PES)**		(Leu, Pimkina, Frank, Murphy, & George, 2009)	HSPA1 (Hsp72) DnaK (Hsp70 Bacterial)	Unknown
Flavonol	3,3',4',5,5',7-Hexahydroxyflavone **(Myricetin)**		(Chang et al., 2008; Chang et al., 2011; Jinwal et al., 2009)	HSPA8 (Hsc70) DnaK (Hsp70 Bacterial)	(Jinwal et al., 2009)

Table 2

Summary of Published Tau Aggregation Inhibitor Scaffolds

Scaffold	Nomenclature (Compound Name)	Compound Structure	Anti-Tau Aggregation Inhibitory Activity Reported	Tau Mutant Targeted	Intracellular Anti-Tau Activity Reported
Carbocyanine	3,3'-Diethyl-9-methylthiacarbocyanine iodide **(C11)**		(Congdon et al., 2009; Duff, Kuret, & Congdon, 2010)	Wild Type	(Congdon et al., 2009)
Aminothienopyridazine	5-amino-3-(4-chlorophenyl)-N-cyclopropyl-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide **(ATPZ)**		(Ballatore et al., 2010; Crowe et al., 2009)	Wild Type; P301L; K311D	Unknown
Polyphenol	(2S,3E,4S)-3-Ethylidene-2-(β-D-glucopyranosyloxy)-3,4-dihydro-5-(methoxycarbonyl)-2H-pyran-4-acetic acid 2-(3,4-dihydroxyphenyl)ethyl ester **(Oleuropein)**		(Daccache et al., 2011)	P301L	Unknown
Polyphenol	2-(3,4-Dihydroxyphenyl)ethanol, 3,4-Dihydroxyphenethyl alcohol **(Hydroxytyrosol)**		(Daccache et al., 2011)	P301L	Unknown
Anthraquinone	1,2,5,8-Tetrahydroxy-9,10-anthraquinone, 1,2,5,8-Tetrahydroxyanthraquinone **(Quinalizarin)**		(Duff et al., 2010; Pickhardt et al., 2005)	Wild Type; ΔK280	Unknown

Scaffold	Nomenclature (Compound Name)	Compound Structure	Anti-Tau Aggregation Inhibitory Activity Reported	Tau Mutant Targeted	Intracellular Anti-Tau Activity Reported
Rhodanine	2-((Z)-5-((5-(3-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)ethanoic acid **(Compound 1)**		(Bulic et al., 2007; Bulic, Pickhardt, Mandelkow, & Mandelkow, 2010)	Wild Type; ΔK280	Unknown