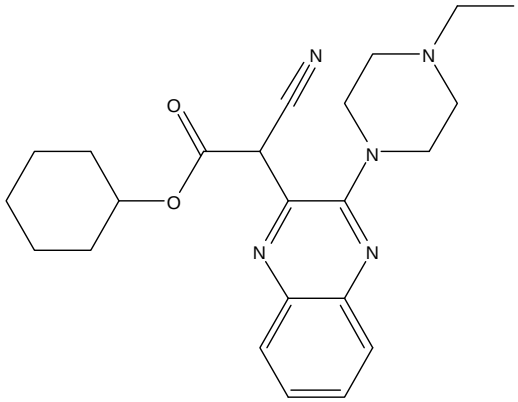
Project: Ligands for premortem diagnosis and treatment of Alzheimer's disease

Probe: cyclohexyl 2-cyano-2-[3-(4-ethylpiperazin-1-yl)quinoxalin-2-yl]acetate



SID: 862054
MLS000040166

PubChem Primary Bioassay Identifier (AID): 596

SID	IC50 (μM)	Antitarget	Selectivity*
862054	2.7	Human amyloid beta**	1 (1.4 uM IC50)
		Human alpha synuclein**	1 (1.8 uM IC50)
		Human caspase 1	≥21 (Inactive @57 μM)
		AmpC beta-lactamase	10 (Inactive @ 29 uM)
		Peroxiredoxin	≥21 (Inactive @57 μM)

*Selectivity = antitarget IC50/target IC50; fluorescent fibrillization assays

Assigned Assay Grant #: X01-MH077621-01

Screening Center Name: NIH Chemical Genomics Center
Principal Investigator of Screening Center: Christopher Austin

Assay Submitter & Institution: Jeff Kuret, Ohio State University

Assay or Pathway Target: Human tau

Assay provider information

Specific Aim: Identify ligands capable of binding amyloidgenic tau conformations with high affinity.

Significance: Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by selective neuronal death in specific brain regions. Pathological hallmarks that accompany and/or precede neuronal death include neurofibrillary lesions and amyloid

plaques. This project focuses on the neurofibrillary pathology, which develops as the disease progresses, and whose spatial and temporal deposition correlates well with disease severity. Its principal components are straight and paired-helical filaments composed of the microtubule-associated protein tau. Because of its utility as a surrogate marker for neurodegeneration in AD and other "tauopathic" neurofibrillary degenerative diseases, detection of tau conformation is of practical importance for disease diagnosis and staging. Abnormalities in tau biology also may contribute directly to neuronal death. If so, then pharmacological modulation of the aggregation pathway could have therapeutic utility. For these reasons, characterization of neuritic lesion pharmacology is of critical importance for the AD field.

Rationale: This *in vitro* tau fibrillization assay screens for small molecules that displace binding of Thioflavin S to tau filaments. Such molecules may be useful detection agents for neurofibrillary lesions, particularly those that show selectivity for tau over beta amyloid. In addition, actives identified via this assay may disrupt tau filament formation. These inhibitors may have additional utility as therapeutic agents for AD.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: qHTS Assay for Tau Filament Binding

List of PubChem bioassay identifiers generated for this screening project (AIDs):

AID	Target	Concentration	Bioassay type
596	Human tau	57μM – 3.7 nM	Primary qHTS
911	Human tau	10μM – 1.5 nM	Confirmation
937	Human alpha-synuclein	10μM – 1.5 nM	Secondary
956	Human beta-amyloid	10μM – 1.5 nM	Secondary
991	Human tau	8μM – 0.2μM	Secondary

Primary Assay Description as defined in PubChem:

Overview:

Tau monomers form filaments *in vitro* in the presence of anionic detergents or fatty acids. The dye Thioflavine S (ThS) binds to tau filaments and upon binding, increases in fluorescence several fold. Small molecules that displace ThS binding or prevent filament binding are identified by a reduction in ThS fluorescence. This assay used histidine-tagged, recombinant human tau protein containing the products of exons 2, 3, and 10 that was provided by Jeff Kuret, Ohio State University. To form filaments, 1 uM tau was incubated with 30 uM sodium octadecylsulfate (ODS) in reagent buffer (10 uM thioflavine S, 5 mM 1,4-dithioerythreitol (DTT), 100 mM sodium chloride, 10 mM HEPES pH 7.4) for 15 hrs at 37 C.

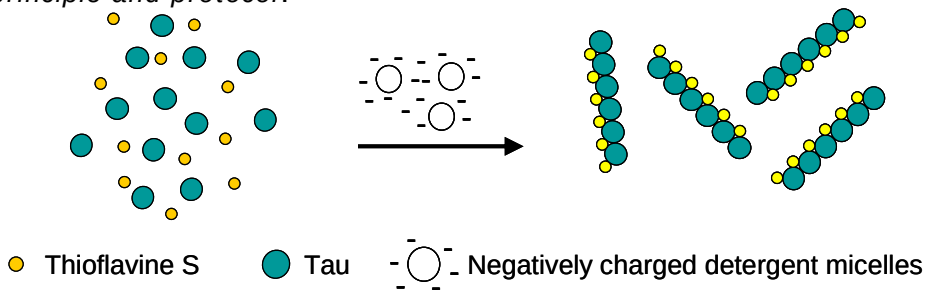
Protocol:

Two uL/well of 2 uM tau in reagent buffer (10 uM thioflavine S, 5 mM DTT, 100 mM sodium chloride, 10 mM HEPES pH 7.4) was dispensed into solid black 1536-well plates. Twenty-

three nL compound was transferred to the assay plate after which 2 uL/well of 60 uM ODS in reagent buffer was dispensed for a final assay volume of 4 uL /well. Following 15 hours of incubation at 37 C, fluorescence was detected by an Envision (Perkin Elmer) using 450/8 nm excitation and 510/23 nm emission.

Center Summary of the Primary Screen:

Assay principle and protocol:



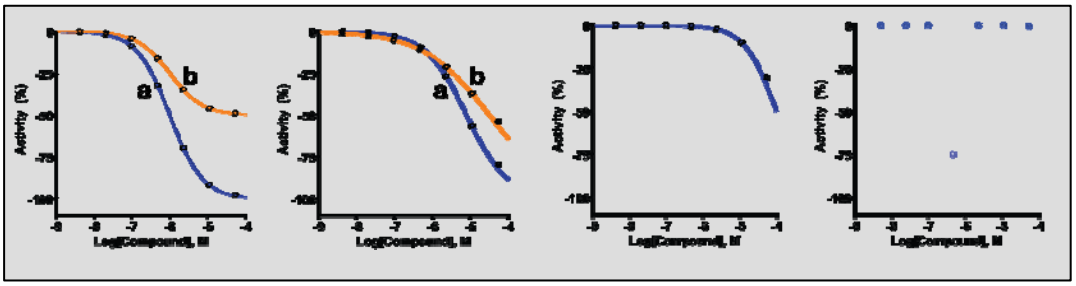
Tau Filament Binding Assay 1536-well qHTS Protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	2 uL	1 uM Tau
2	Compound	23 nL	57 uM – 3.7 nM
3	Reagent	2 uL	30 uM octadecyl sodium sulfate
4	Time	15 hr	37 C incubation
5	Detector	Fluorescence	Envision

Step	Notes
1	1536-well black solid plate. Tau (1 μM final concentration) or mixed histones (50 nM final concentration) in Reagent Buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol, 10 μM ThS).
2	
3	30 uM octadecyl sodium sulfate (final) in Reagent Buffer
4	
5	Envision fluorescence settings- 450/8 nm excitation and 510/23 nm emission

Figure 1: Tau fibrillization assay and protocol. *Top*, Tau monomers polymerize in the presence of negatively-charged detergent micelles, such as octadecyl sodium sulfate. Thioflavine S (ThS) binding to tau filaments results in increased ThS fluorescence. *Bottom*, the protocol used to screen the Tau assay is shown.

Identification of Active Clusters: Following the quantitative high throughput screen (qHTS), the concentration-response curve (CRC) data was classified to rank the curve quality as described (1). Briefly, CRCs are placed into four classes. Class 1 contains complete CRCs showing both upper and lower asymptotes and r^2 values > 0.9. Class 2 contains incomplete CRCs lacking the lower asymptote and shows r^2 values greater than 0.9. Class 3 curves are of the lowest confidence because they are defined by a single concentration point where the

minimal acceptable activity is set at 3 SD of the mean activity calculated as described above. Finally, class 4 contains compounds that do not show any CRCs and are therefore classified as inactive. Both activators and inhibitors were recovered from the qHTS; however, activators were not considered further as their activity likely arose from compound fluorescence.



Scheme 1: Example classification scheme for assignment of resulting curve-fit data into classes. Curves fitting the following classification criteria are then used to establish SAR: Class 1 curves display two asymptotes, an inflection point, and r2 ≥0.9; subclasses 1a vs. 1b are differentiated by full (>80%) vs. partial (≤ 80%) response. Class 2 curves display a single left-hand asymptote and inflection point; subclasses 2a and 2b are differentiated by a max response and r2, >80% and >0.9 or <80% and <0.9, respectively. Class 3 curves have a single left-hand asymptote, no inflection point, and a response >3SD the mean activity of the sample field. Class 4 defines those samples showing no activity across the concentration range.

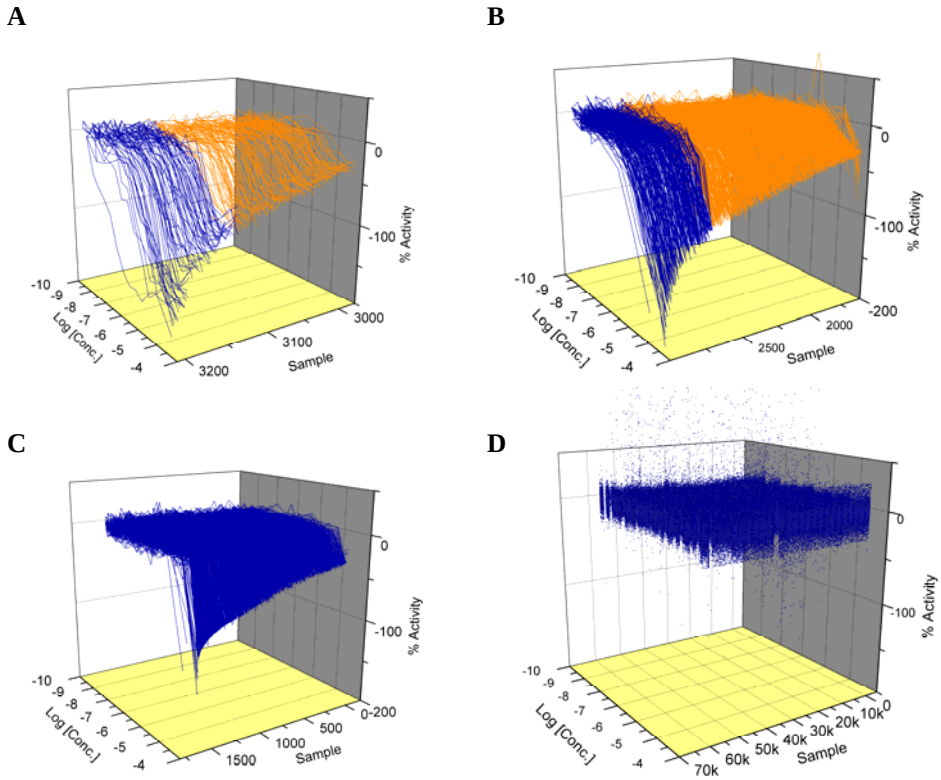


Figure 2: Inhibitors from qHTS data (AID 596) binned into curve classifications 1-4 based on curve-fitting classification criteria. A) class 1a (navy) and class 1b (orange) curves. B) class 2a (navy) and class 2b (orange) curves. C) class 3 curves (navy). D) data with no apparent dose-response, curve class 4 (navy points).

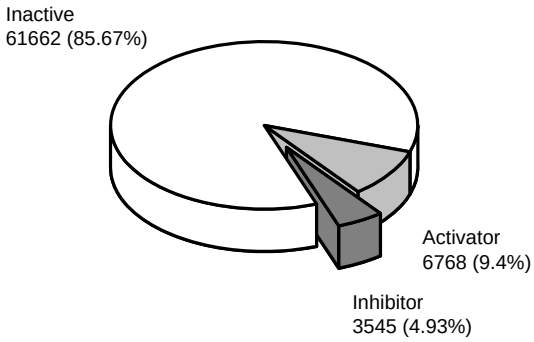
qHTS summary of assay results:

A

Screen Performance

Plates	448
Total wells	683,904
Compounds	71,975
Average Z’	0.48
Average S:B Ratio	2.9

B



C

	Class	1.1	1.2	2.1	2.2	3
Inhibition	# compounds	93	223	316	1133	1780
	% library	0.12%	0.30%	0.43%	1.6%	2.5%
Activation	# compounds	173	105	1692	872	3926
	% library	0.24%	0.14%	2.4%	1.2%	5.5%

Figure 3: Tau qHTS performance and classification of actives. A) Performance statistics of the Tau qHTS. B) Pie chart indicating overall activity of screened compounds. Inhibitors (dark grey) and activators (light grey) comprised 4.9% and 9.4%, respectively, of the collection while the remainder inactive (unshaded). C) Classification of qHTS actives. Tau assay inhibitors and activators were categorized by curve class number and percentage of library screened.

Structure-activity relationships (SAR) of the inhibitors was performed by hierarchical agglomerative clustering with a 0.7 Tanimoto cutoff using Leadscope (Leadscope Inc., Columbus, OH) fingerprints. For each cluster, maximal common substructures (MCS) were extracted, a manual step of MCS trimming was performed to create a list of scaffolds, and any overlapping scaffolds were abridged to a canonical set. Each scaffold was then represented as a precise definition to indicate descriptors such as the number of attachment points or the ring size variability. All filters were then relaxed to include the entire negative (class 4) assay data.

SAR of series:

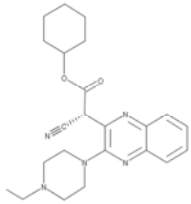
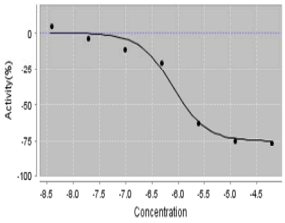
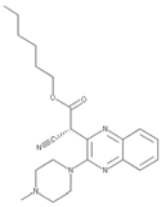
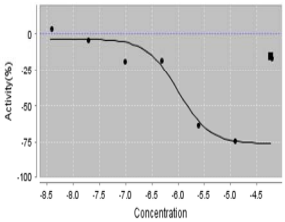
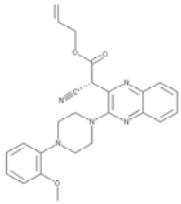
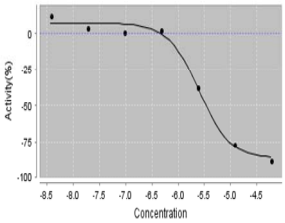
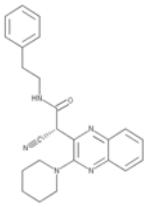
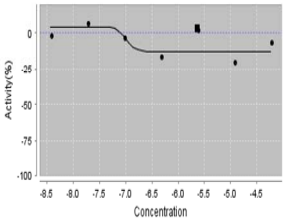
Structure	PubChem SID	PubChem CID	MW	qHTS Curve Class	qHTS AC50 (uM)	Followup AC50 (uM)	QC Method	% Purity	qHTS Titration Response Curve
	862054	663281	408	-1.2	0.8	2.7	LC-MS-ELS	>90	
	861194	662451	396	-1.2	1.0	2.8	LC-MS-ELS	>90	
	859416	660709	444	-1.1	2.5	4.0	LC-MS-ELS	>90	
	858027	659384	400	4	>57	>57	LC-MS-UV	>90	

Table I: Activity of Tau Probe and related compounds in primary qHTS and follow-up tau assay. Curve fit of qHTS titration response data for each compound is shown on the right.

Probe Characterization

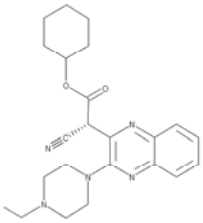
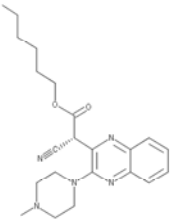
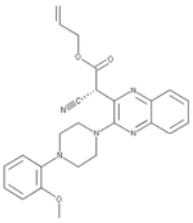
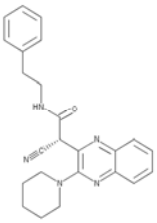
Structure	PubChem SID	PubChem CID	tau % Efficacy	tau uM IC50	alpha-synuclein % Efficacy	alpha-synuclein uM IC50	beta-amyloid % Efficacy	beta-amyloid uM IC50
	862054	663281	91	2.8	92	1.8	90	1.4
	861194	662451	90	2.8	98	1.8	92	0.8
	859416	660709	90	4.0	90	1.4	106	1.1
	858027	659384		>10		>10		>10

Table II: Activity of probe and analogs on inhibition of alpha-synuclein and beta-amyloid filament formation (See assay descriptions below and ref (2).).

Has this compound been provided to the MLSMR?

Yes, MLS000040166

Canonical SMILES: CCN1CCN(CC1)C2=NC3=CC=CC=C3N=C2C(C#N)C(=O)OC4CCCCC4

InChI: InChI=1/C23H29N5O2/c1-2-27-12-14-28(15-13-27)22-21(25-19-10-6-7-11-20(19)26-22)18(16-24)23(29)30-17-8-4-3-5-9-17/h6-7,10-11,17-18H,2-5,8-9,12-15H2,1H3

Description of secondary assays used to optimize/characterize probe structure:

Alpha-synuclein ThS binding assay

Overview: Lewy body disease is characterized by the appearance of lesions containing alpha-synuclein. The protein components in these lesions can be induced to form filaments under experimentally tractable times when incubated *in vitro* with anionic inducers such as alkyl sulfate detergents or fatty acids. Filaments formed *in vitro* possess identical morphology with those isolated from authentic diseased tissue, which adopt cross beta-sheet conformation capable of binding small aromatic molecules. Here we describe the *in vitro* fibrillization of alpha-synuclein. Alpha-synuclein was induced to form filaments by incubation overnight at 37 C in the presence of arachidonic acid. A commonly used fluorescent probe for cross#nbeta-sheet structure, Thioflavin S (ThS), was also added to the reactions. ThS exhibits excitation and emission Stokes shifts upon binding to neurofibrillary lesions *in vitro*, allowing the extent of fibrillization to be monitored. Small molecules capable of ThS displacement or antagonizing fibrillization result in a decrease in ThS fluorescence, allowing determination of binding affinities for molecules screened.

Protocol Summary: The assay was performed in 96-well round bottom opaque plates (VWR, West Chester, PA) in 100 uL final volume. Recombinant His6-alpha-synuclein was diluted to 5 uM in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) containing 50 uM arachidonic acid inducer. ThS probe was then added to a final concentration of 10 uM with test compounds assayed at nine 3-fold dilutions beginning at 10 uM final concentration. Each compound was tested at least twice in separate assays. Controls without protein or without compound were included on each plate. After shaking 2 min to promote mixing, plates were covered with self-adhesive aluminum foil and incubated overnight at 37 C. Fluorescence (ex = 440 nm; em = 485 nm) was measured after brief shaking (10 s) in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA) operated at sensitivity 10, automatic PMT.

Beta amyloid ThS binding assay

Overview: Alzheimer's disease is characterized by the accumulation of proteinacious aggregates comprised of tau and beta-amyloid. The protein components of each lesion can be induced to form filaments under experimentally tractable times when incubated *in vitro* with anionic inducers such as alkyl sulfate detergents or fatty acids. Filaments formed *in vitro* possess identical morphology with those isolated from authentic diseased tissue, which adopt a cross beta-sheet conformation capable of binding small aromatic molecules. Here we describe the *in vitro* fibrillization of beta-amyloid 1-42 (Abeta1-42). Abeta1-42 proteins were induced to form filaments by incubation overnight at 37 C in the presence of arachidonic acid. A commonly used fluorescent probe for cross beta-sheet structure, Thioflavin S (ThS), was also added to the reactions. ThS exhibits excitation and emission Stokes shifts upon binding to neurofibrillary lesions *in vitro*, allowing the extent of fibrillization to be monitored. Small molecules capable of ThS displacement or antagonizing fibrillization result in a decrease in ThS fluorescence, allowing determination of binding affinities for molecules screened.

Protocol Summary: Human Beta amyloid peptide 1-42 (American Peptide Co., Sunnyvale, CA) was resuspended in hexafluoroisopropanol (by vortexing in a glass vial) to a final concentration of 1 mM. The solvent was then completely evaporated under a slow stream of argon. The remaining residue was dissolved in DMSO to a final concentration of 2.5 mM, then sonicated for 10 min using setting 3 (model 100, Fisher Scientific). The assay was

performed in 96-well round bottom opaque plates (VWR, West Chester, PA) in 100 uL final volume. Beta amyloid peptide 1-42 was diluted to 5 uM in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) containing 50 uM arachidonic acid inducer. ThS probe was then added to a final concentration of 10 uM with test compounds assayed at nine 3-fold dilutions beginning at 10 uM final concentration. Each compound was tested at least twice in separate assays. Controls without protein or without compound were included on each plate. After shaking 2 min to promote mixing, plates were covered with self-adhesive aluminum foil and incubated overnight at 37 C. Fluorescence (ex = 440 nm; em = 485 nm) was measured after brief shaking (10 s) in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA) operated at sensitivity 10, automatic PMT.

Known probe properties:

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:

Molecular Weight	407.50866 [g/mol]
Molecular Formula	C23H29N5O2
XLogP	4.1
H-Bond Donor	0
H-Bond Acceptor	7
Rotatable Bond Count	6
Tautomer Count	3
Exact Mass	407.232125
MonoIsotopic Mass	407.232125
Topological Polar Surface Area	82.4
Heavy Atom Count	30

Compound preparation:

Compound is prepared in DMSO at 10 mM stock concentration. Assays described above have 0.6% DMSO final concentration in buffer.

Probe availability:

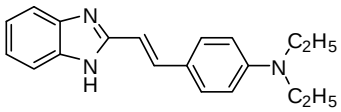
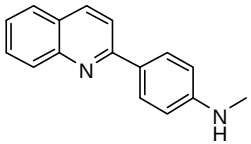
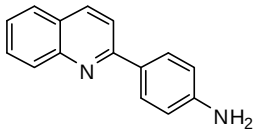
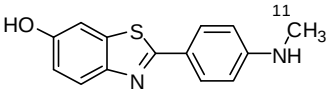
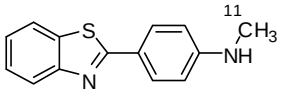
Aliquots of MLS000040166 are available from the NCGC upon request.

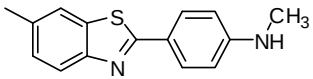
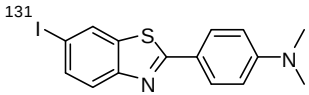
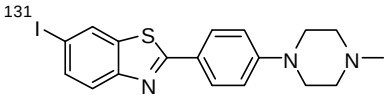
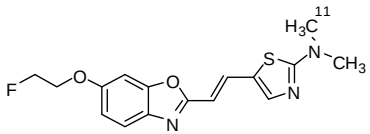
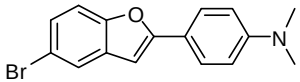
Appendices

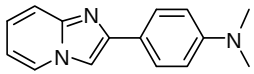
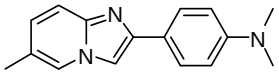
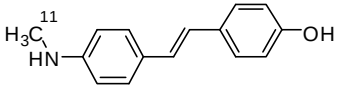
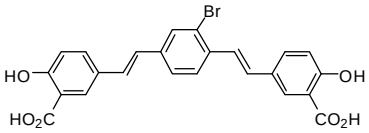
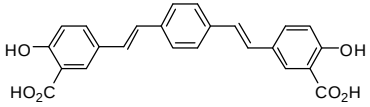
Comparative data on probe, similar compound structures and prior probes:

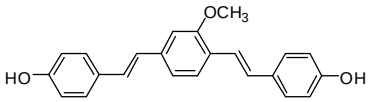
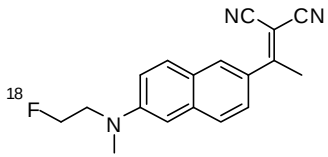
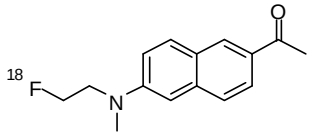
Note added in proof: Crowe et al. (3) found a similar series, 2,3-di(furan-2-yl)-quinoxaline, that inhibited tau filament formation *in vitro*.

Probes for Tau and $A\beta_{1-42}$ Filaments:

Compound	Name	Structural Class	Potency (nM)		Selectivity (fold selectivity)	Stage	Reference
			tau	Aβ			
	BF-126	benzimidazole	EC_{50} = **583** ± 2.07 (intrinsic fluor)	EC_{50} = **1280** ± 1.64 (1-42; intrinsic fluor)	stains tau over $A\beta_{1-42}$ (2.2-fold)	*ex vivo* (mice)	(4)
	BF-158	quinoline	EC_{50} = **399** ± 2.41 (intrinsic fluor)	EC_{50} = **659** ± 2.04 (1-42; intrinsic fluor)	stains tau over $A\beta_{1-42}$ (1.7-fold)	*ex vivo* (mice)	(4)
	BF-170	quinoline	EC_{50} = **221** ± 2.31 (intrinsic fluor)	EC_{50} = **786** ± 1.79 (1-42; intrinsic fluor)	stains tau over $A\beta_{1-42}$ (3.6-fold)	*ex vivo* (mice)	(4)
	6-OH-BTA-1 (PIB)	benzothiazole (Thioflavin T derivative)	n.d.	Ki BS1 = **1,865** (1-40; comp ThT fluor) Ki BS3 = 25 (1-40; comp radioassay)	stains Aβ plaques	*in vivo* (humans)	(5), (6), (7)
	BTA-1	benzothiazole (Thioflavin T derivative)	n.d.	Ki BS1 = **200** (1-40; comp ThT fluor)	stains Aβ plaques and prion deposits	*ex vivo* (mice)	(5), (8), (9)

Compound	Name	Structural Class	Potency (nM)		Selectivity (fold selectivity)	Stage	Reference
			tau	Aβ			
	6-Me-BTA-1	benzothiazole (Thioflavin T derivative)	n.d.	**Kd BS3 = 3.25** (1-40; comp radioassay)	stains Aβ plaques	*in vitro*	(5), (7), (9)
	TZDM	benzothiazole (Thioflavin T derivative)	n.d.	Ki BS2 = **145** (1-40; comp ThT fluor)	stains Aβ plaques	*ex vivo* (humans)	(9), (10)
	TZPI	benzothiazole (Thioflavin T derivative)	n.d.	Ki BS2 = **480** (1-40; comp ThT fluor)	stains Aβ plaques	*ex vivo* (humans)	(9), (10)
	BF-227	Benzoxazole (Thioflavin T derivative)	n.d.	Ki = **4.3** ± 1.5 (1-42; comp radioassay)	stains dense Aβ plaques	*in vivo* (humans)	(11)
	BF-1	Benzofuran (Thioflavin T derivative)	n.d.	**Ki BS2 = 110** (1-40; comp ThT fluor)	stains Aβ plaques	*in vivo* (mice)	(9), (12)

Compound	Name	Structural Class	Potency (nM)		Selectivity (fold selectivity)	Stage	Reference
			tau	Aβ			
	IMPY-H	imidazo-(1,2)-pyridine (Thioflavin T derivative)	n.d.	Ki BS1 = **1420** (1-40; comp ThT fluor) Ki BS3 = **1242** (1-40; comp radioassay)	stains Aβ plaques	*ex vivo* (humans)	(9), (13), (14)
	IMPY-Me	imidazo-(1,2)-pyridine (Thioflavin T derivative)	n.d.	Ki BS1 = **1000** (1-40; comp ThT fluor) Ki BS3 = **42** (1-40; comp radioassay)	stains Aβ plaques	*ex vivo* (humans)	(9), (13)
	SB13	styrylbenzene (Congo red derivative)	n.d.	Kd = **2.4** (comp radioassay)	stains Aβ plaques	*in vivo* (humans)	(14), (15), (16)
	BSB	stilbene (Congo red derivative)	n.d.	Ki BS1 = **600** (1-40; comp ThT fluor Ki)	stains Aβ plaques and prion deposits	*ex vivo* (mice)	(7), (8), (17)
	X34	stilbene (Congo red derivative)	n.d.	n.d.	stains Aβ plaques & neuritic lesions	*ex vivo*	(18), (19)

Compound	Name	Structural Class	Potency (nM)		Selectivity (fold selectivity)	Stage	Reference
			tau	Aβ			
	Methoxy -X04	Styrylbenzene (Congo red derivative)	n.d.	Ki = **26.8** (comp ThT fluor)	stains Aβ plaques and prion deposits	*in vivo* (mice) *ex vivo* (humans)	(20), (21)
	FDDNP	amino-naphthyl	n.d.	Ki BS1 = **335** (1-40; comp ThT fluor) Ki BS3 = **42** (1-40; comp radioassay)	stains Aβ plaques, neuritic lesions, and prion plaques	*in vivo* (humans)	(7), (22), (23), (24), (25)
	FENE	amino-naphthyl	n.d.	Kd High = **0.16** ± 0.02 Kd Low = **71.2** ± 8.6 (1-40; comp ThT fluor)	stains Aβ plaques & neuritic lesions	*ex vivo* (humans)	(22)

Note: BS1, BS2 and BS3 refer to three different binding sites present on fibrils of $A\beta_{1-40}$ (7), (9).

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