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High throughput screening for small molecule inhibitors of heparin-induced tau fibril formation

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Abstract

A library of ~51,000 compounds was interrogated by high throughput screening (HTS) using a heparin-induced tau fibrillization assay. HTS was conducted with bacterially expressed recombinant tau fragment K18 and the reaction was monitored by thioflavine T fluorescence. Hits meeting criteria set for selection in HTS were further evaluated in a panel of assays designed (a) to confirm the initial results and (b) to identify possible false positives arising from non-specific mechanisms or assay-dependent artifacts. Two 2,3-di(furan-2-yl)-quinoxalines were confirmed as inhibitors of tau fibrillization with IC_{50}s in the low micromolar range (l–3 μM). Among false positive hits, members of the pyrimidotriazines, benzofurans, porphyrins, and anthraquinone, inhibited tau fibrillization by generating peroxides via catalytic redox cycles due to the reducing agent dithiothreitol (DTT) in the assay. This study delineates focused strategies for HTS of tau fibrillization inhibitors that are relevant to drug discovery for Alzheimer's disease and related tauopathies.

Keywords: Microtubule associated protein tau; Fibrillization; FTDP-17; Alzheimer's disease; High throughput screening; Inhibitor

Despite the fact that the precise mechanisms underlying neurodegenerative diseases remain to be fully elucidated, a growing body of evidence clearly indicates that protein misfolding, fibrillization, and aggregation can produce detrimental effects through (a) toxic effects directly mediated by the aggregates; and/or through (b) the loss of the normal function of the sequestered proteins [1]. As such, protein misfolding, fibrillization, and aggregation have emerged as new potential targets for therapeutic intervention [2]. One important example of pathologically relevant aggregates is the neurofibrillary tangles (NFTs) made of paired helical filaments (PHFs), which are aggregated forms of hyperphosphorylated protein tau. NFTs comprise, together with A-β amyloid plaques, the defining lesions of Alzheimer's disease (AD), and NFTs also are pathological hallmarks of other neurodegenerative diseases known as tauopathies. Importantly, the aggregation of tau fibrils to form tangles correlates with the degree of cognitive impairment in AD.

The tau protein is a microtubule-associated protein (MAP) particularly abundant in axons of neurons. The normal tau function is to stabilize the microtubules (MTs) thereby modulating the plasticity of the cytoskeleton. Since the MT-network is a key component of axonal transport, changes in the MT-dynamics caused by a loss of tau function can have profound effects on the transport of protein and other cargo to and from the cell body of neurons [3]. In AD, upon hyperphosphorylation tau becomes sequestered into NFTs thereby losing its MT-stabilizing function. The consequence of this process is the disruption of axonal transport, which ultimately leads to neurodegeneration. For this reason, tau is now recognized as an important therapeutic target for potential new treatments of AD and related tauopathies. Indeed, efforts have

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been made to identify agents that could prevent tau aggregation and/or promote the dissolution of aggregates [4–7]. One of the major obstacles encountered in the study of tau fibrillization was the difficulty in effectively generating tau fibrils *in vitro*. However, the discovery that anionic cofactors (i.e., heparin, arachidonic acid) efficiently induce the fibrillization process, and the observation that different truncated forms of tau are more prone to fibrillization compared to the full-length protein, enabled the development of *in vitro* assays amenable to high-throughput screening (HTS) of large compound libraries. This resulted in the identification of some structural classes of compounds including anthraquinones [6], polyphenols [7], and phenothiazines [4,7] that were found to inhibit tau fibrillization. However, despite these findings, clinical candidates have not yet been identified, and further screening as well as additional assay development are clearly needed. In fact, the tau fibrillization assays reported thus far differ in important ways, such as the tau isoforms or anionic cofactors employed, or concentrations of reducing agents in the reaction mixture etc. The significance of such differences are not yet known and it is unclear which of the reported tau fibrillization assays best reproduces the *in vivo* situation. However, these differences presumably account for the fact that some compounds (e.g., daunorubicin) generated opposite results in different assays. In the present study, we report HTS of a library of ~51,000 compounds in a heparin-induced tau fibrillization assay.

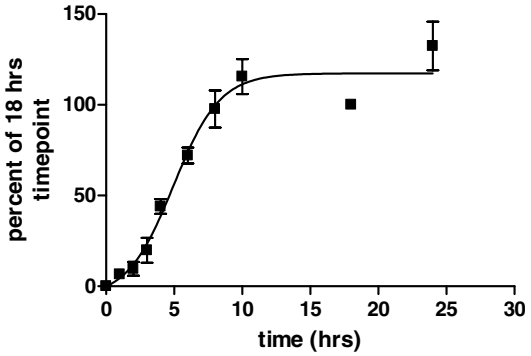


Fig. 1. Time course for tau fibrillization on a 384-well plate format. ThT assay was performed in triplicate and expressed as a percentage of the 18 h time point used in the screen.

Materials and methods

Fibrillization assay. The fibrillization assay adopted here was modified from the one reported by Mandelkow et al. [6] with the main difference that recombinant myc-tagged truncated tau fragment K18 was employed rather than K19. The myc tag was added in order to evaluate antibody-based assays, such as the DELFIA and Alpha Screen, in comparison with the thioflavine T (ThT) fluorescence assay. Since the ThT assay proved to be generally simpler and more reproducible than the antibody-based assays, particularly under automated HTS conditions ($Z' = 0.85$; Cv = 4.4%), we selected it as the assay of choice for the primary screening. The total volume of the reaction mixture was 25 μl, which included 20 μM myc-tagged K18 (1:1: N-terminal myc:C-terminal myc tag), 20 μM heparin, and 2 mM DTT in 100 mM sodium acetate, pH 7.0. After 18 h of incubation at 37 °C, an addition of 25 μl of a 25 μM solution of ThT was made and incubation continued for 1 h at room temperature prior to fluorescence reading (Fig. 1). The non-fibrillizing mis-sense K18 mutant obtained by substituting lysine 311 with aspartate (K18-K311D) was used as negative control, while K18 in DMSO provided the positive control. The criterion set for selection in the primary assay was 40% inhibition.

Secondary assays. Sedimentation of the ThT reaction was performed at 186,000 g for 30 min through a 25% sucrose cushion and the supernatant removed from the pellet. Pellets were resuspended in a volume equal to the supernatant and equal amounts of supernatants and pellet were analyzed by SDS–PAGE on a 12.5% acrylamide gel. N-terminal and C-terminal myc-tagged K18 migrated slightly differently and showed up as a double band. Negative staining with uranyl acetate followed by electron

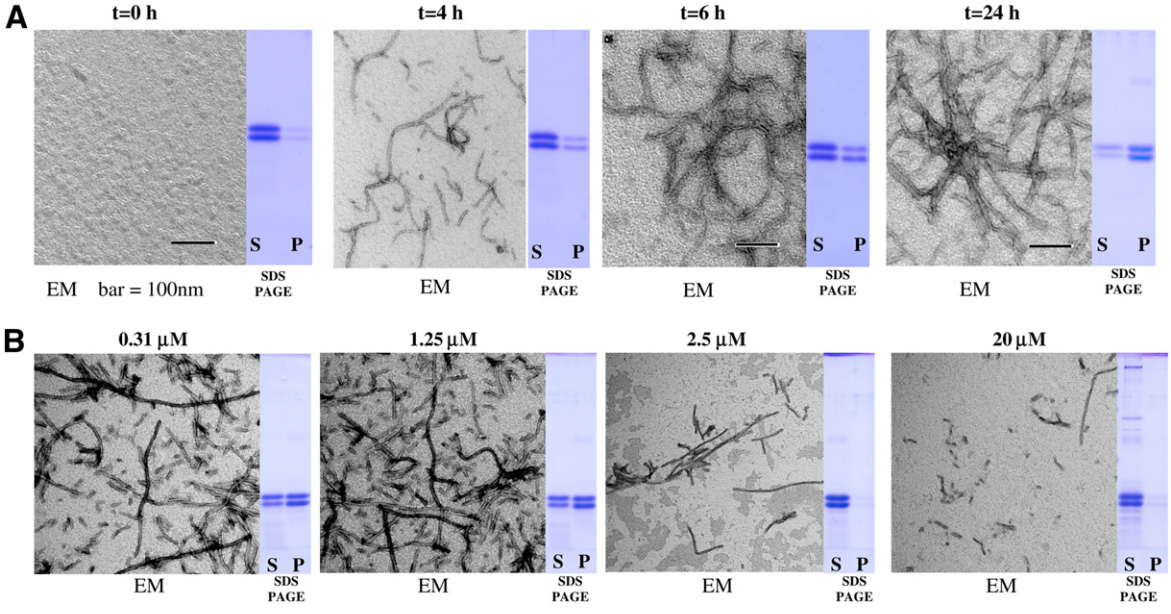
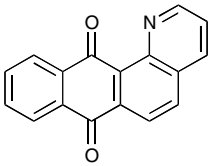
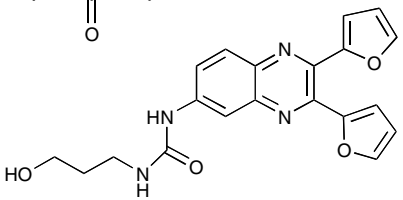
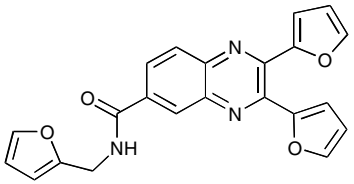
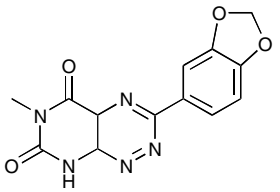
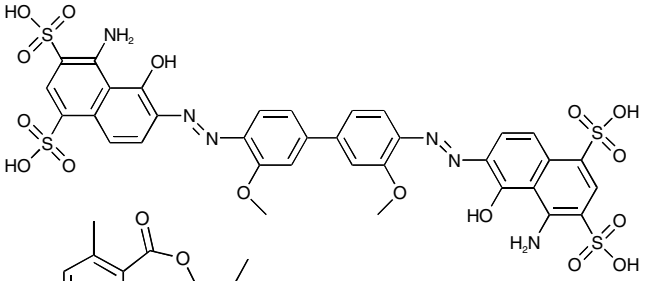
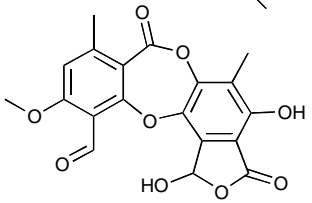


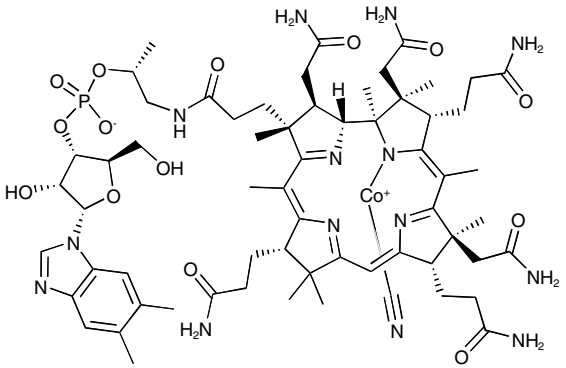
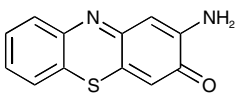
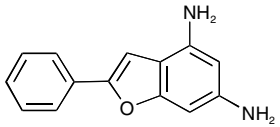
Fig. 2. A Sedimentation assay and EM examination of the pellets: (A) positive control (K18 + DMSO) at different time points; (B) after 18-h incubation at different concentrations of 113F08. N-terminal and C-terminal myc-tagged K18 migrate slightly differently and give rise to double bands. EM, electron microscopy; S, supernatant; P, pellets.

Table 1
Hits and false positives obtained from the screening

Cpd class	Cpd name	Structure	% Inhib. (ThT)[a]	Sed. assay[b]	% Inhib. (ThT) no DTT	Sed. assay no DTT	IC_{50} (μM)
Anthraquinones	31G03		76	++	39	–	0.63
Quinoxalines	113F08		69	++	70	+++	2.4
	330B06		27	+++	85	++	3.1
Pyrimidotriazines	300C04		94	+++	20	–	0.36
Sulfonated dyes	6C06		102	+++	51	–	3.2
Depsidones	15B02		98	+++	100	+++	1.6

(*continued on next page*)

Table 1 (*continued*)

Cpd class	Cpd name	Structure	% Inhib. (ThT)[a]	Sed. assay[b]	% Inhib. (ThT) no DTT	Sed. assay no DTT	IC_{50} (μM)
Porphyrins	06D04		75	+++	−2.6	–	4.5
Phenothiazines	30H07		100	+++	99	+++	0.29
Benzofuran	348E10		84	+++	9.8	–	0.61

[a] Percentage inhibition of K18 fibrillization followed by ThT assay.

[b] Sedimentation assay data: "+++", 100% inhibition; "−", 0% inhibition, IC_{50} determined in the presence of DTT. Additional hits from the pyrimidotriazines, 17H03 and 93D02, behaved similarly to 300C04 and are not shown.

microscopy (EM) was performed on the resuspended sedimentation pellets (Fig. 2A).

Compound library. The chemical library screened in this study was designed to maximize structural diversity within chemical space while favoring the representation of "drug-like" structures. The library consisted of 1120 off patent FDA-approved compounds from the Prestwick Chemical Library™ (Prestwick Chemical, France), 240 pure natural product compounds from the Greenpharma Natural Compound Library (Prestwick Chemical, France), a combinatorial library of 30,000 compounds probing 125 different scaffolds from the NOVACore library (Chembridge, San Diego, California), and 21,000 compounds maximizing sampling of chemical space from the DIVERSet™ library (Chembridge, San Diego California).

Results and discussion

Screening

Upon completion of the primary screening, 71 compounds (~1/700) met or exceeded the selection criterion of 40% inhibition by HTS. These compounds were further evaluated through: (a) retest of the primary screening and (b) sedimentation assay followed by examination of the pellets by EM (Fig. 2B). Out of the initial set of hits, eleven compounds were confirmed to inhibit the fibril formation in both primary and secondary assays (see Table 1) representing eight compound classes. Five of the eight classes had been previously published: sulfonated dyes, phenothiazines, anthraquinones, benzofurans, and porhyrins were among the classes producing hits in our assay. New compound classes included several quinoxalines and pyrimidotriazines, and a depsidone.

Among the compounds that successfully passed all assays, a sulfonated dye (06C06), a phenothiazine (30H07), and stictic acid (15B02), a natural product extracted from lichens, were both given lower priority either due to concerns over their chemical reactivity and stability (15B02), or because the class of compounds had been already studied (sulfonated dyes and phenothiazines).

Testing for false positives

Next we turned our attention towards identifying possible false positives. In order to eliminate possible false positives that could inhibit tau fibrillization through a non-specific mechanism involving the generation of peroxides, all hits were evaluated for their ability to inhibit tau fibril formation in the absence of DTT, which is known to participate in catalytic redox cycles with specific classes of compounds, with rapid generation of peroxides [10]. Seven compounds lost activity in the absence of DTT representing five compound classes (Table 1). The anthraquinone (31G03), porphyrin (6D04), benzofuran (348E10), and all three pyrimidotriazines (17H03, 93D02, and 300C04) showed dependence on the presence of DTT.

We tested several published representatives of the anthraquinones and porphyrins for dependence on the presence of DTT (daunorubicin, doxorubicin, hemin, and protoporphyrin IX). As opposed to the compounds in these classes obtained from our screen, none of these representative compounds showed DTT sensitivity, highlighting the need to be mindful of individual chemistries within any compound class.

Since non-specific binding of aggregates of small molecules onto proteins is often the source of false positives in high-throughput screenings [8,9], dose–response curves were generated at different concentrations of K18 for the remaining 2,3-di(furan-2yl)-quinoxalines (Fig. 3). It is expected that IC_{50}s of compounds that exhibit inhibitory activity through the formation of aggregates would be independent protein concentration. For both of the compounds in this class the IC_{50} varied with protein concentration, indicating that non-specific inhibition due to a compound aggregation mechanism of inhibition is not occurring.

The remaining compounds shared an identical 2,3-di(furan-2yl)-quinoxaline structure (113F08 and 330B06), exhibiting IC_{50}s in the 1–3 μM range. Interestingly, examination of the compound library revealed ~200 entries containing the quinoxaline substructure, most of them, however, completely inactive at 10 μM. This would suggest the presence of a structure–activity relationship, yet resolving the differences between test compounds that exhibit moderate or low activities, proved to be challenging in the current assay. In the course of our testing we found the activity to be dependent on the presence of the 2,3-di(furan-2yl) moieties on the quinoxoline scaffold. Of the other 2,3-di(furan-2yl) quinoxalines with low activity we found six additional compounds which show dose-dependent activity. The 2,3-di(furan-2yl)-quinoxalines represent the first examples of a new scaffold exhibiting inhibition of tau fibril formation *in vitro* and as such, they may be useful in further defining the structural requirements for inhibition of fibril formation.

In conclusion, this study delineates analytical strategies for the interrogation of compound libraries by HTS to identify tau fibrillization inhibitors that may become candidates for further structure function and *in vivo* studies in

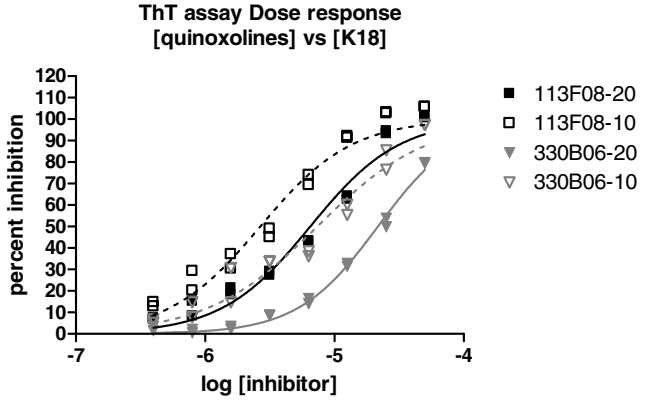


Fig. 3. Dose dependence of quinoxalines at different concentrations of K18. ThT assay result was expressed as a percent inhibition relative to controls containing DMSO alone. Suffix following compound name indicates concentration of K18 in micromolar.

drug discovery efforts to develop better treatments for Alzheimer's disease and related tauopathies.

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