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Theoretical Article

Targeting the ensemble of heterogeneous tau oligomers in cells: A novel small molecule screening platform for tauopathies

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Abstract

Objective: Understanding the heterogeneous pathology in Alzheimer's disease and related tauopathies is one of the most urgent and fundamental challenges facing the discovery of novel disease-modifying therapies. Through monitoring ensembles of toxic and nontoxic tau oligomers spontaneously formed in cells, our biosensor technology can identify tool compounds that modulate tau oligomer structure and toxicity, providing much needed insight into the nature and properties of toxic tau oligomers.

Background: Tauopathies are a group of neurodegenerative disorders characterized by pathologic aggregation of the microtubule binding protein tau. Recent studies suggest that tau oligomers are the primary toxic species in tauopathies.

New/Updated Hypothesis: We hypothesize that tau biosensors capable of monitoring tau oligomer conformation are able to identify tool compounds that modulate the structure and conformation of these tau assemblies, providing key insight into the unique structural fingerprints of toxic tau oligomers. These fingerprints will provide gravely needed biomarker profiles to improve staging of early tauopathy pathology and generate lead compounds for potential new therapeutics. Our time-resolved fluorescence resonance energy transfer biosensors provide us an exquisitely sensitive technique to monitor minute structural changes in monomer and oligomer conformation. In this proof-of-concept study, we identified a novel tool compound, MK-886, which directly binds tau, perturbs the conformation of toxic tau oligomers, and rescues tau-induced cytotoxicity. Furthermore, we show that MK-886 alters the conformation of tau monomer at the proline-rich and microtubule binding regions, stabilizing an on-pathway oligomer.

Major Challenges for the Hypothesis: Our approach monitors changes in the ensemble of assemblies that are spontaneously formed in cells but does not specifically isolate or enrich unique toxic tau species. However, time-resolved fluorescence resonance energy transfer does not provide high-resolution, atomic scale information, requiring additional experimental techniques to resolve the structural features stabilized by different tool compounds.

David D. Thomas holds equity in and serves as an executive officer for Photonic Pharma LLC, a company that owns intellectual property related to technology used in part of this project. These relationships have been reviewed and managed by the University of Minnesota in accordance with its conflict-of-interest polices.

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Linkage to Other Major Theories: Our biosensor technology is broadly applicable to other areas of tauopathy therapeutic development. These biosensors can be readily modified for different isoforms of tau, specific post-translational modifications, and familial Alzheimer's disease–associated mutations. We are eager to explore tau interactions with chaperone proteins, monitor cross-reactivity with other intrinsically disordered proteins, and target seeded oligomer pathology.

Keywords: Tau oligomerization; Heterogeneous tau oligomers; Conformational ensembles; Fibrillation kinetics; Small-molecule inhibitors; Time-resolved FRET

1. Objective

This article emphasizes the need for targeting the heterogeneous ensemble of toxic tau oligomers in Alzheimer's disease (AD) and other tauopathies based on emerging biosensor technology. We report on two novel cellular fluorescence resonance energy transfer (FRET) biosensors that monitor tau oligomer and monomer conformations and can be used as a high-throughput screening (HTS) platform to identify novel tool compounds that modulate tau oligomer conformations, thereby attenuating their toxicity. With our biosensors and HTS platform we are poised to (1) study the conformational ensemble of tau oligomers; (2) identify novel tool compounds capable of targeting tau species, both monomer and oligomer, to disrupt oligomer formation or stabilize different tau conformations; (3) develop a biophysical fingerprint that delineates toxic and nontoxic oligomers, allowing us to better stage tau pathology, improve biomarker development, and reduce the heterogeneity present in clinical trials; and (4) provide a novel therapeutic pipeline to identify lead compounds that target spontaneously formed, early stage oligomers, instead of late-stage neurofibrillary tangles (NFTs).

2. Background

2.1. Historical evolution

Tauopathies, including AD, are a group of neurodegenerative disorders characterized by the presence of tau inclusions in affected brain regions [1]. Despite decades of rigorous and focused research, there are currently no significant disease-modifying therapies for AD or related tauopathies [2]. Furthermore, there is a dearth of compounds that target tau, with only five of the 105 small molecules currently in clinical trials being tau-focused [3,4]. Hence, there is a desperate need for technologies that enable the identification of tau-focused disease-modifying therapies [4–6].

Tau is an intrinsically disordered protein (IDP) that plays an important role in the regulation of microtubule stability and axonal transport [7]. Under pathological conditions, tau is hyperphosphorylated and detaches from microtubules, accumulating in the cytosol [8]. These pathological conditions have been correlated with upstream mitochondrial dysfunctions in the Krebs cycle and/or the electron transport system, oxidative stress [9,10], and defects in neuron morphology and axonal transport [11]. Unbound tau can misfold, initiating the tau fibrillogenesis cascade with an initial formation of tau oligomers that subsequently nucleate into paired helical filaments (PHFs), and eventually intracellular NFTs (Fig. 1) [12]. NFTs have been the primary histopathological hallmark of tauopathies, with their presence in the brain showing significant correlation with the degree of cognitive impairment [13]. However, recent studies suggest that these large insoluble NFTs are not the principle toxic species, implicating soluble oligomeric tau—intermediate tau assemblies formed before PHFs—in the induction of neurodegeneration [14,15]. Tau oligomers promote cytotoxicity in vitro and are linked to neurodegeneration and cognitive phenotypes in vivo [15–21]. They exist as an ensemble of distinct assemblies, which include both toxic and nontoxic, on- and off-pathway species along the fibrillogenesis cascade (Fig. 1) [22–28]. Critically, no specific toxic tau oligomer species has been isolated or identified to date [29–31].

2.2. Rationale

Recent efforts to target toxic tau oligomers have yielded compounds with low micromolar half maximal inhibitory concentration (IC_{50}) [32–38]. Of these small molecules, only methylene blue advanced to phase III clinical trials, albeit with unsuccessful results [39]. One commonality among these molecules is that they were initially identified using in vitro purified protein assays [32–38]. These systems do not recapitulate the cellular environment, lacking the numerous chaperone proteins that may be required to produce the ensemble of tau oligomers that populate the fibrillogenesis cascade. In addition, purified protein assays are only capable of identifying hits that directly perturb tau and are wholly naive against indirect mechanism of action (MOA). Furthermore, many of the small molecules discovered in purified protein assays disrupted both fibrils and tau oligomers, the former having recently been suggested to be potentially inert and neuroprotective [40]. Therapeutic development for tauopathies has thus begun to shift from targeting large fibrillar aggregates to inhibiting or disrupting the formation of these toxic tau oligomers [14,31,41,42]. The complex

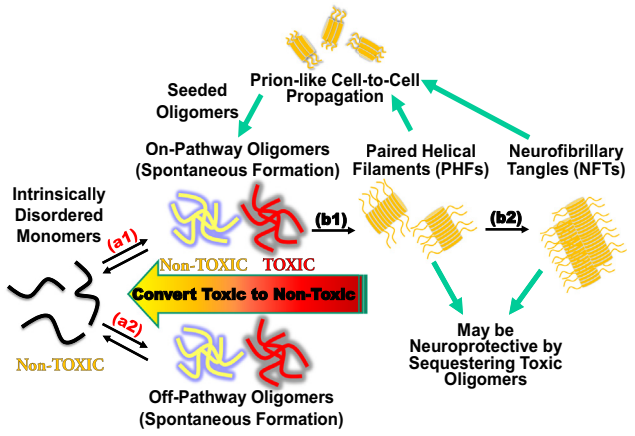

Fig. 1. Tau fibrillogenesis cascade for tauopathies and Alzheimer's disease (AD). The intrinsically disordered tau monomer is capable of misfolding into spontaneously formed oligomers, producing toxic assemblies implicated in AD (arrows a1 and a2). Although oligomers are metastable and difficult to monitor with high precision and accuracy, the large assemblies (PHFs and NFTs, arrows b1 and b2) form irreversibly with β-sheet structures. The fibrillar species can be excreted via exosomes leading to a prion-like cell-to-cell propagation of pathology and may induce seeded oligomerization. NFTs may be neuroprotective by sequestering toxic oligomers, and disruption of NFTs may induce toxicity from increased concentrations of toxic oligomers. Our cellular time-resolved fluorescence resonance energy transfer (FRET) biosensors target reversible spontaneous oligomerization (a1 and a2), while also monitoring seeded aggregation and downstream processes such as fibrillization (b1 and b2) with high sensitivity.

heterogeneity of tau oligomers likely requires the cellular environment (e.g., post-translational modifications [PTMs] and chaperone proteins) to produce the ensemble of toxic and nontoxic tau assemblies. Hence, a cellular biosensor approach capable of monitoring this ensemble holds promise as a novel HTS platform to discover more effective therapeutics.

Building on the groundbreaking biosensors developed by the Diamond group (which were engineered to detect pathogenic species in patient biofluids as a biomarker for AD diagnosis) [43], we have developed a technology platform that directly monitors spontaneous tau oligomerization in cells, enabling therapeutic targeting of early stage tau pathology. Our robust assay can be easily modified to accommodate additional tauopathy cell models and new pathologic phenotypes as they continue to be elucidated. Through our approach, we will develop two unique classes of tool compounds, direct tau binders and indirect tau effectors (modifying tau oligomers through orthogonal pathways). The interplay between direct and indirect MOA and corresponding changes in tau oligomer conformations and toxicity will provide much needed insight into tau pathology.

We engineered two distinct FRET biosensors to monitor tau oligomerization. These biosensors were used for HTS of the National Institutes of Health Clinical Collection (NCC) library using our state-of-the-art fluorescence lifetime plate reader (FLT-PR) [44]. Fluorescence lifetime (FLT) detection increases the precision of FRET-based screening by a factor of 30 compared with conventional fluorescence intensity detection [45], and provides exquisite sensitivity to resolve minute structural changes within protein ensembles. This sensitivity allows direct detection of conformational changes within an ensemble of oligomers (e.g., conversion from toxic to nontoxic oligomers), the dissociation of oligomers, or even changes in monomer conformations [46–48]. The FRET biosensors express full-length 2N4R wild-type (WT) tau and fluorescent protein fusion constructs in living cells, allowing us to monitor *inter*molecular or *intra*molecular tau interactions. Using full-length 2N4R WT tau ensures the targeting of spontaneously formed ensembles of tau oligomers, not fibrils, as the 2N4R isoform of WT tau does not fibrillize without seeding [49–52].

After first establishing that the new technology platform specifically monitors tau oligomer formation (using known tau aggregators), we identified a small molecule, MK-886, that directly binds tau and strongly attenuates FRET with a half maximal effective concentration (EC_{50}) of 1.40 μM in human embryonic kidney 293 cells (HEK293) used in our HTS (and a FRET EC_{50} of 1.06 μM in SH-SY5Y cells). The compound rescues tau-induced cell cytotoxicity with an IC_{50} of 0.523 μM. To elucidate the MOA, we used an advanced single-molecule FRET (smFRET) technique to show that MK-886 perturbs the folding of purified, monomeric tau in the proline-rich and microtubule binding regions. This effect is recapitulated in our cellular intramolecular FRET biosensor and indicates an unfolding of the two termini of tau.

To further explore MOA of MK-886, we used a heparin-induced thioflavin-T (ThT) aggregation assay with purified tau. MK-886 reduces the lag phase of tau fibrillization and is unable to nucleate tau fibrillization without the presence of heparin. It has been shown that overexpression of P301L tau does not induce fibril formation in SH-SY5Y cells [52], suggesting that P301L tau-induced toxicity is because of toxic oligomer. Because MK-886 rescues tau-induced cytotoxicity although not fully ablating tau oligomer-associated FRET, we hypothesize that the rescue of P301L tau-induced cytotoxicity by MK-886 is through conversion of toxic tau oligomers into nontoxic oligomers. Whether these new oligomers are on- or off-pathway species is difficult to determine in our cellular system, without the use of inducers. Thus, the rescue of P301L tau-induced toxicity could be through an accelerated conversion of toxic tau oligomers into neuroprotective fibrils. Nevertheless, our new technology is well suited to identify novel compounds capable of remodeling tau oligomers and rescuing tau-induced cytotoxicity.

3. New or updated hypothesis

We hypothesize that the spontaneously formed ensemble of tau oligomers includes early stage toxic tau species and by resolving conformational differences between toxic and nontoxic oligomers, we can target toxic tau assemblies,

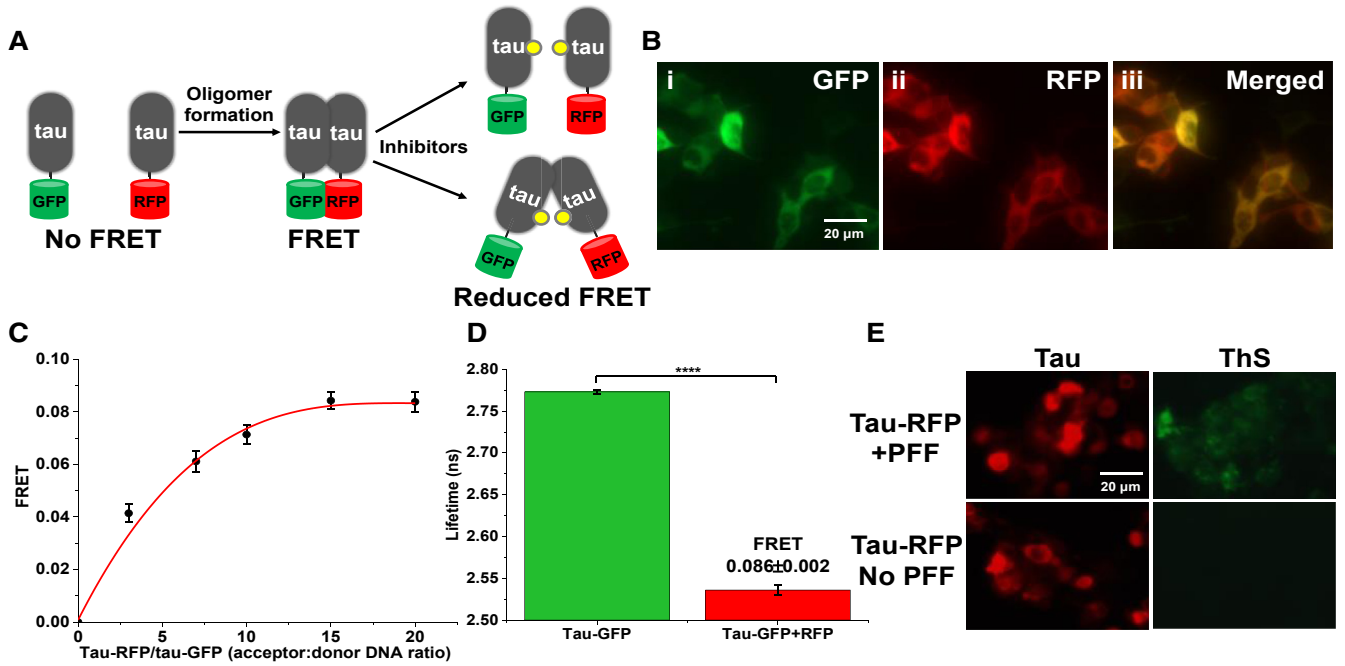

Fig. 2. The tau intermolecular FRET biosensor and fluorescence lifetime technology enable direct monitoring of tau oligomerization in cells. (A) Schematic representation of live-cell–based tau intermolecular FRET biosensor. FRET signal is observed when tau oligomers form, which can be modulated by small-molecule inhibitors. Tau oligomer is drawn as a dimer for illustration but it can be any species more than a dimer (≥2-mers). (B) Fluorescence microscopy images of (i) GFP channel, (ii) RFP channel, and (iii) merged channel of HEK293 cells expressing tau-GFP/RFP (tau FRET biosensor). (C) Titration of tau-RFP (acceptor) to tau-GFP (donor) illustrates that the FRET efficiency of the biosensor follows a hyperbolic dependence on acceptor concentration. (D) Fluorescence lifetime measurements of the tau biosensor show efficient FRET, indicating tau self-association. (E) Thioflavin-S (ThS) staining of HEK293 cells expressing tau-RFP (in same total DNA concentration as used in the FRET biosensor) in the presence and absence of the positive control of tau preformed fibrils (PFF). Data are represented as the means ± SD of three independent experiments. ****$P < .0001$ by two-tailed unpaired t test. Abbreviations: FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; RFP, red fluorescent protein; SD, standard deviation.

thereby rescuing tau-induced pathology. Small-molecule modulation of tau conformations can be correlated with changes in the FRET signal and tau-induced cytotoxicity. Finally, through investigating tau oligomerization in the cellular environment, we include other protein machineries (e.g., chaperone proteins), which may play significant roles in tau pathogenesis.

3.1. Early experimental or observational data

3.1.1. Intermolecular FRET biosensor directly monitors structural changes in tau oligomers in cells

To develop an in-cell HTS platform that can detect small-molecule modulation of tau oligomerization and/or perturbation of tau conformational states, we engineered two cellular tau FRET biosensors. We expressed full-length 2N4R WT tau fused to green fluorescent proteins (GFPs) or red fluorescent proteins (RFPs) (tau-GFP/RFP or "tau FRET biosensor") in HEK293 cells (Fig. 2A). Expression and homogeneity of the FRET biosensor were determined by fluorescence microscopy and immunoblotting. Fluorescence microscopy images showed that the tau proteins were evenly distributed in the cytosol of the cells, with no discernible puncta (which would have indicated more progressive aggregation, e.g., fibril formation) or other nonuniformities (Fig. 2B). Western blot analysis of the tau biosensor cell lysates confirmed the expression of fluorophore-tagged tau (Supplementary Fig. 1A).

We next tested the functionality of the tau FRET biosensor by measuring FRET efficiency using the FLT-PR [44]. The value of FRET efficiency reflects the ensemble-averaged intermolecular proximity between tau molecules, which is derived from the distance between the donor and acceptor fluorophore fused to the tau proteins. FRET between tau-GFP (donor) and tau-RFP (acceptor) in live cells showed hyperbolic dependence on acceptor concentration (Fig. 2C), with a maximum energy transfer efficiency (E) of 0.086 ± 0.002, illustrated through a substantial decrease in the donor FLT in the presence of the acceptor (Fig. 2D), indicating the formation of tau oligomers in cells. The kinetics of formation of the tau-tau assemblies was also measured by FRET, showing that the WT tau biosensor has an optimal FRET after 48 hours of expression (Supplementary Fig. 1B). We confirmed that the FRET observed from cells expressing tau biosensor arises from specific tau-tau interactions and not from nonspecific interactions between the free fluorophores (Supplementary Fig. 1C and D). Furthermore, we showed that the FRET biosensor is sensitive to the addition of forskolin, a small molecule known to induce tau

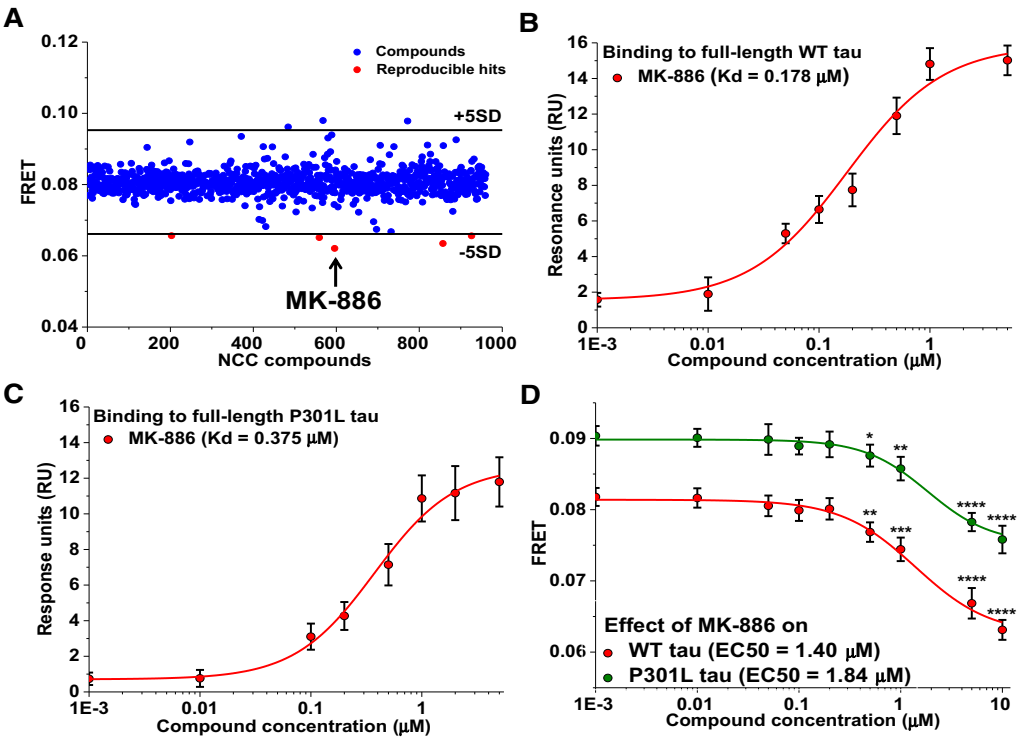

Fig. 3. Identification of MK-886 as a small molecule that directly perturbs conformational ensemble of tau oligomers. (A) Representative pilot screening with NCC library containing 727 compounds as well as in-plate DMSO negative controls. A FRET efficiency cutoff threshold was applied at a change in FRET efficiency of 5 SD (black lines). Five reproducible hits decreased FRET by more than 5 SD less than the mean of all cells (red) and MK-886 induced the largest FRET change (arrow). Surface plasmon resonance (SPR) binding curve for MK-886 to purified recombinant full-length 2N4R (B) wild-type (WT) and (C) P301L tau proteins. (D) FRET analysis of the dose response of MK-886 in both WT and P301L tau intermolecular biosensors indicates EC_{50} values of 1.40 and 1.84 μM, respectively. Data are represented as the means ± SD of three independent experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ by two-tailed unpaired *t* test. Abbreviations: EC_{50}, half maximal effective concentration; FRET, fluorescence resonance energy transfer; DMSO, dimethyl sulfoxide; NCC, NIH Clinical Collection; SD, standard deviation.

hyperphosphorylation and self-association, but not to gossypetin, a small molecule known to inhibit or remodel of tau fibrils (Supplementary Fig. 1E) [53]. To confirm that only oligomeric species of tau, but not fibrils, were present in the tau biosensor cells, we performed a thioflavin-S (ThS) assay in cells expressing tau-RFP at the same concentration of tau-GFP/RFP dual-transfected cells (tau-GFP was not used as it will interfere with the ThS signal), with treatment of exogenous tau preformed fibril (PFF) as a positive control. Results from the ThS assay illustrate that only cells treated with PFF have a positive ThS signal (Fig. 2E) with a significant increase in the ThS intensity (Supplementary Fig. 1F), confirming that no fibrils (e.g., β-sheet tau assemblies) are present in the biosensor cells, and more importantly that the FRET signal is the result of tau oligomerization.

3.1.2. Identification of novel small molecules from HTS of the NCC library that perturb the conformational ensembles of tau oligomers

Using our cellular tau FRET biosensor, we performed an HTS of the NCC library (727 bioactive compounds) to identify compounds that perturb the conformational ensembles of tau oligomers. The NCC library is a collection of small molecules that have been previously tested in clinical trials, and have known safety profiles and details on potential MOA.

After an initial quality control check of the cells expressing tau FRET biosensor on each day of screening (fluorescent waveform signal level and coefficient of variance), they were dispensed into drug plates and incubated with the compounds (10 μM) or dimethyl sulfoxide control wells for 2 hours. FLT measurements were acquired with the FLT-PR. A single-exponential fit was used to determine the FLT from cells expressing the tau FRET biosensor (τ_{DA}) or expressing a tau-GFP donor-only control (τ_{D}) to determine FRET efficiency (Supplementary Eq. 1). As FLT measurements are prone to interference from fluorescent compounds, a stringent fluorescent compound filter was used to flag 30 such compounds as potential false-positives [46,47]. FRET efficiency values of all compounds that passed the fluorescent compound filter are plotted (Fig. 3A), and a histogram of the FRET distribution from these compounds was fit to a Gaussian curve to obtain a mean and standard deviation for the screen (Supplementary Fig. 2A).

Our initial goal was to discover compounds that alter the conformational ensembles of tau oligomers with the potential of disrupting tau-tau interactions, leading us to focus our search on compounds that reduce FRET (although other compounds that increase FRET could potentially remodel toxic oligomers and be of interest in future studies). Five reproducible hits from the library were shown to decrease FRET by more than 5 standard deviations (5 SD) less than the mean of all wells (Fig. 3A, highlighted in red) whereas not appearing as hits in the donor-only control screen (Supplementary Fig. 2B and C).

3.1.3. Binding of hit compounds to purified tau proteins

We next used surface plasmon resonance to determine if these five hit compounds bind tau, delineating a potential direct or indirect MOA with tau. Of the five hits that reduced FRET with our tau biosensor, MK-886 was the only hit to demonstrate dose-dependent binding to purified WT tau protein with K_d = 0.178 μM (Fig. 3B and Supplementary Fig. 2D). MK-886 also showed binding to purified P301L tau protein, a more aggregation prone mutant of tau [54], with K_d = 0.375 μM (Fig. 3C and Supplementary Fig. 2E). Interestingly, MK-886 also had the strongest change in FRET (Fig. 3A, arrow). The other four hit compounds did not show direct binding to immobilized tau protein (Supplementary Fig. 2F) and therefore most likely attenuate tau FRET through an indirect MOA. All subsequent analysis in this study is focused on MK-866. The indirect MOA compounds, although outside the scope of this study, are potentially useful and are briefly discussed subsequently.

3.1.4. FRET dose response of MK-886 with cellular tau intermolecular FRET biosensors

The relative EC_{50} of MK-886 was determined by in-cell FRET measurements using the WT tau biosensor. The compound decreased FRET efficiency in a dose-dependent manner with an EC_{50} value of 1.40 μM (Fig. 3D). We also performed a FRET dose response using P301L tau FRET biosensor. The FRET efficiency of this biosensor was found to be higher than that of WT tau biosensor (Supplementary Fig. 3A), which is consistent with the known tendency of P301L tau to be hyperphosphorylated and hence more oligomeric [54]. Similar to WT, we observed a dose-dependent decrease in the FRET efficiency of the P301L tau biosensor with MK-886 with an EC_{50} value of 1.84 μM (Fig. 3D), confirming that the hit compound also remodels tau oligomers in a disease-relevant model. Interestingly, we observed that MK-886 lowered the FRET level of P301L biosensor to the basal FRET level of WT tau biosensor. This may suggest that MK-886 disrupts the toxic oligomers of P301L tau and converts them to less toxic conformations that are similar to the conformations adopted by the WT tau. In addition, we confirmed that the small molecule was acting specifically on tau and was not acting on the cytosolic fluorophores (Supplementary Fig. 3B–D). Assay quality (Z') was determined using MK-886 (Supplementary Eq. 2). The Z' value of 0.72 ± 0.02 indicates excellent assay quality, validating MK-886 as a positive control tool compound for targeting tau oligomers.

We also expressed the P301L tau FRET biosensor in the SH-SY5Y neuronal cell model, and similar FRET was observed as in the HEK293 cells (Supplementary Fig. 4A). This FRET again reflects the formation of oligomers, as it is known that β-sheet fibrils do not spontaneously form in SH-SY5Y overexpressing P301L tau unless aggregation inducers are used [52]. MK-886 reduced FRET from P301L tau biosensor in the SH-SY5Y cells with an EC_{50} of 1.06 μM (Supplementary Fig. 4B). Importantly, as will now be shown, if toxicity is observed when P301L tau is overexpressed in SH-SY5Y cells, and the toxicity is rescued by MK-886, it will further support the conclusion that the small molecule converts toxic tau oligomers into a nontoxic conformational state.

3.1.5. MK-886 reduces tau-induced cell cytotoxicity in SH-SY5Y cells with nanomolar potency

We next tested the effect of MK-886 on P301L tau-induced cytotoxicity in the SH-SY5Y neuroblastoma cell model of tauopathy [20,32,55,56]. Overexpression of P301L tau showed significantly greater cell death (37%) when compared with the vector-only control (23%) after 96 hours of expression (Fig. 4A and Supplementary Fig. 5A). Treatment of cells overexpressing P301L tau with MK-886 (1 nM to 2 μM) showed significant rescue of P301L tau-induced cytotoxicity in a dose-dependent manner, with an IC_{50} of 0.523 μM (Fig. 4B), the same order of magnitude as MK-886's binding affinity for recombinant P301L tau protein. The twofold difference in IC_{50} of MK-886 in the cell cytotoxicity assay (0.523 μM) and the EC_{50} from the FRET assay (1.06 μM) may be because of the different treatment conditions and the expression of unlabeled versus fluorophore-tagged P301L tau in each assay, respectively.

We note that MK-886, which was blindly identified in our HTS, has been shown to play a role in modulating AD-related tau pathology through inhibition of 5-lipoxygenase–activating protein (FLAP) [57], potentially altering the clearance and phosphorylation state of tau [58,59]. Our observations suggest that MK-886 rescues tau-induced cytotoxicity through direct binding to tau protein and not by modulating FLAP, a previously undescribed MOA. SH-SY5Y cells do not express 5-lipoxygenase or FLAP and therefore are a particularly well-suited model to evaluate alternative MOA for MK-886 rescue of tau-induced cytotoxicity [60]. We also confirmed that there were no changes in the relative levels of expressed tau (Supplementary Fig. 5B) or the phosphorylation state (Supplementary Fig. 5C) because of MK-886 treatment in the SH-SY5Y cell model, although it remains possible that MK-886 may be altering other PTMs such as acetylation or oxidation.

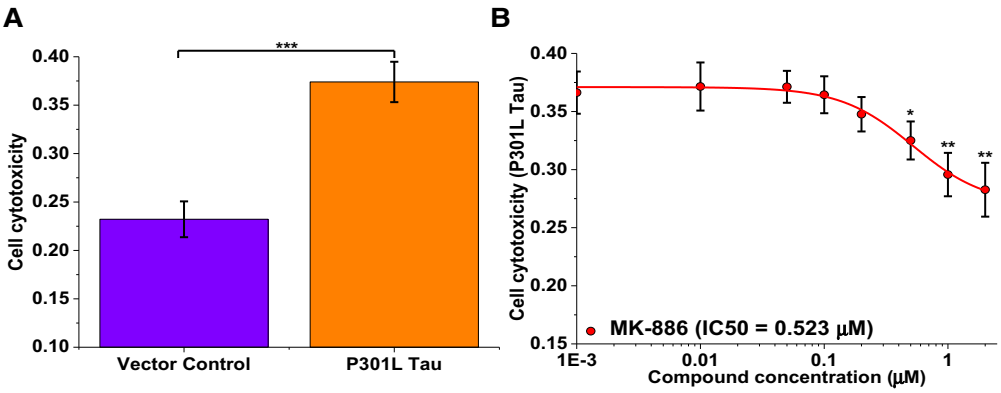

Fig. 4. Rescue of tau-induced cell cytotoxicity in SH-SY5Y human neuroblastoma cells by MK-886. (A) SH-SY5Y cells were transfected with vector control and P301L tau. Significant cell death is observed in cells transfected with P301L tau as compared with the vector control. (B) MK-886 rescued P301L–tau induced cytotoxicity in SH-SY5Y cells with an IC_{50} of 0.523 μM. Data are represented as the means ± SD of three independent experiments. $*P < .05$, $**P < .01$, and $***P < .001$ by two-tailed unpaired *t* test. Abbreviation: SD, standard deviation.

In addition, it has been shown that expression of P301L tau may also result in cell death through downregulating the expression of survivin, inhibitor of apoptosis proteins or X-linked inhibitor of apoptosis proteins [55]. Thus, we needed to rule out the possibility that MK-886 rescues P301L tau–induced cell cytotoxicity by modulating the expression of these genes, rather than by directly altering tau conformations. To do so, we used two small molecules (YM-155 and UC-112) that are potent suppressors of the expression of survivin, inhibitor of apoptosis proteins, and X-linked inhibitor of apoptosis proteins, thus mimicking the effect of P301L tau on this pathway. This allowed us to test whether MK-886 can rescue cell cytotoxicity in the absence of P301L tau, simply by upregulating these survival genes [61]. Our results showed that MK-886 did not rescue the cell cytotoxicity induced by YM-155 (1 μM) or UC-112 (1 μM) (Supplementary Fig. 6A and B). Thus, these important control experiments strongly suggest that MK-886 rescues P301L tau–induced cell cytotoxicity by directly perturbing the conformations of the toxic tau oligomers to form a nontoxic conformation.

3.1.6. MK-886 specifically perturbs the proline-rich region/microtubule binding region of tau monomer and induces conformational changes in the cellular tau intramolecular biosensor

To further investigate the MOA of MK-886, we used smFRET to examine the effect of MK-886 on monomeric tau. Using two different doubly fluorescent-labeled tau constructs (labeled at the proline-rich region/microtubule binding region (PRR/MTBR) or at the N-terminal domain) [62], we monitored the conformation of two distinct regions of tau (Fig. 5A). The smFRET shows that MK-886 causes not only a substantial increase in FRET for the PRR/MTBR–targeted construct (Fig. 5B) but also a minor decrease in FRET for the N-terminal domain construct (Fig. 5C). This suggests that MK-886 specifically binds and induces a conformational change in tau monomer at the PRR/MTBR region, resulting in a subsequent loss of interactions between the N-terminal domain and the PRR/MTBR. To determine whether MK-886 also perturbs the monomer conformation of tau in cells, we tested the compound with a cellular tau intramolecular FRET biosensor (GFP-tau-RFP). The intramolecular FRET biosensor has a basal 6% FRET signal (Supplementary Fig. 7), illustrating the intramolecular interactions arising from the paper-clip monomeric structure in which the N- and C-terminus of tau are folded to close proximity [63]. Treatment with MK-886 reduced intramolecular FRET with an EC_{50} of 2.12 μM, similar to that of oligomer modulation, suggesting that the change in conformational states of oligomers is in part because of perturbation of the tau monomer (Fig. 5D).

It has been suggested that the folding over of tau's two termini to form the classic "paper-clip" structure is because of electrostatic interactions that arise from the opposite net charges of the N-terminal and MTBR domains [63]. Although this global folding is specific, it has been shown to be a rather weak interaction [63]. We speculate that the binding of MK-886 to the PRR/MTBR of tau may shield these interactions and lead to an opening of the two termini, resulting in the observed decrease in FRET of the intramolecular FRET biosensor. From our previous observations with smFRET on tau constructs, this type of conformational change is often accompanied by the PRR/MTBR becoming substantially more compact (increase in FRET) in recombinant protein systems [62].

3.1.7. MK-886 stabilizes tau conformations that promote the formation of β-sheet–positive fibrils in the presence of aggregation inducer

We have shown that MK-886 directly binds to immobilized tau, modulates tau oligomer and monomer conformation (both in cells and purified proteins), and rescues tau-induced cytotoxicity. To further explore MOA of MK-886 and identify whether MK-886 targets on- or

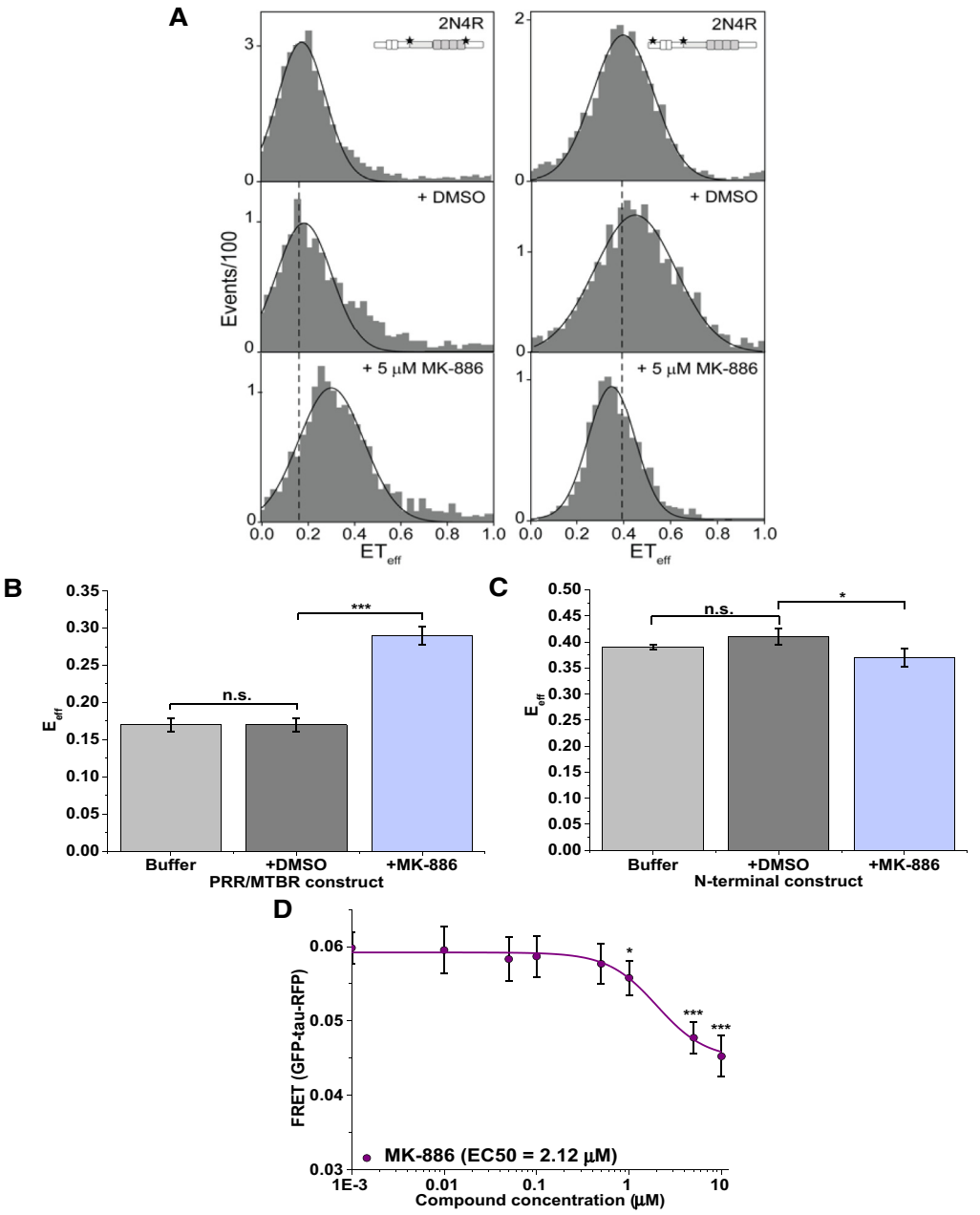

Fig. 5. MK-886 binds and perturbs tau monomer conformation. (A) Single-molecule FRET (smFRET) measurements in the absence and presence of MK-886 with WT 2N4R tau double labeled at the proline-rich region/microtubule binding region (PRR/MTBR, left) or at the N-terminal domain (right). Tau schematic represents the labeling position for each construct. The black line is drawn from the peak of the histogram in buffer for comparison with DMSO and MK-886 samples. Representative histograms are shown. (B) Quantification of the smFRET measurements indicates that the PRR/MTBR becomes substantially more compact (increase in FRET) on binding MK-886 (5 μM) (A, bottom left) when compared with tau in buffer (A, top left) or DMSO (A, middle left), whereas the N-terminal domain (C) shows only minor differences in the presence of MK-886 (5 μM) (A, right). (D) FRET analysis of the dose response of MK-886 in the cellular tau intramolecular biosensor indicates an EC_{50} value of 2.12 μM, similar to that of oligomer modulation, suggesting that the change in conformational states of oligomers is in part because of conformational changes of tau monomer. Data are represented as the means ± SD of three independent experiments. $^{*}P < .05$, $^{***}P < .001$ and n.s. indicates not significant by two-tailed unpaired *t* test. Abbreviations: EC_{50}, half maximal effective concentration; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; SD, standard deviation; WT, wild type.

off-pathway oligomers, we performed a heparin-induced ThT aggregation assay in the absence and presence of MK-886. MK-886 shortens the lag phase of tau β-sheet fibril formation in a dose-dependent manner (Fig. 6A), suggesting that it induces or stabilizes on-pathway, early stage species in the amyloidogenic cascade. We confirmed that MK-886 did not have a direct effect on ThT fluorescence (Supplementary Fig. 8A) and did not act to nucleate for fibril formation (Supplementary Fig. 8B). In addition, we tested if MK-886 disrupts PFFs. Comparison of MK-886 to gossypetin (a known remodeler of tau fibrils) illustrates that MK-886 did not reduce the ThT signal from tau PFF, whereas

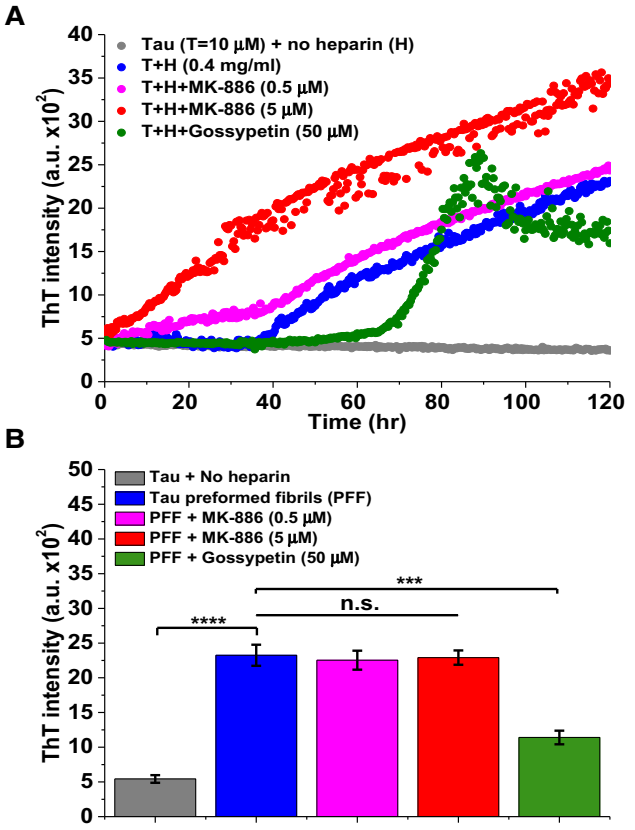

Fig. 6. MK-886 alters the ensemble of conformational states of tau oligomers by stabilizing a fibrillization promoting conformation. (A) Effect of MK-886 on the tau fibrillization cascade as characterized by thioflavin-T (ThT) assay with purified WT tau proteins. Fibrillization was induced by heparin (0.4 mg/mL) in the presence of DMSO control, MK-886 (0.5 and 5 μM) and gossypetin (50 μM, a known small-molecule inhibitor or remodeler of tau fibrils as positive control). (B) Effect of MK-886 on tau preformed fibrils (PFF) with gossypetin as a positive control. All samples were treated with DTT (5 mM). Data are represented as the means ± SD of three independent experiments. ***$P < .001$, ****$P < .0001$ and n.s. indicates not significant by two-tailed unpaired t test. Abbreviations: DMSO, dimethyl sulfoxide; SD, standard deviation; WT, wild type.

gossypetin showed a significant decrease, indicating the disruption of β-sheet fibril structure (Fig. 6B). These results, in combination with the changes in FRET and reduction in tau-induced cytotoxicity, suggest that MK-886 alters the conformational ensemble of tau oligomers favoring a subset of nontoxic, on-pathway oligomers that promote tau fibrillization.

3.2. Future experiments and validation studies

The identity of a specific toxic tau oligomeric species remains elusive. Indeed, it is unlikely that a single unique toxic conformation exists. It is far more likely that an ensemble of toxic oligomers (differing in size, conformation, and even molecular constituency) populates the fibrillogenesis cascade [22–28]. This heterogeneity in tau oligomer targets highlights the need for an ultrasensitive screening platform capable of monitoring structural changes within the ensemble of tau assemblies. Our FRET-based platform for monitoring full-length tau oligomerization in cells is a new technology that is capable of doing this, and elucidating novel compounds, which alter conformation and oligomerization, thereby providing a new pipeline of therapeutic discovery for tauopathies.

With this technology in hand, we and others are in a position to explore multiple important issues. First, screens will now be done of larger libraries and of libraries built specifically for targeting the central nervous system (CNS) (i.e., favorable blood-brain barrier permeability). These screens will dramatically increase the statistical sampling of small-molecule–induced changes in time-resolved FRET (TR-FRET) signatures. With a larger sample, the high information content of TR-FRET can, when complemented with other structural tools (discussed subsequently), be used to cluster compounds into distinct classes based on their myriad of structural effects on the targets [47]. To more adequately generalize the pathophysiological relevance of these clustered structural motifs, we will move to inducible cell lines [43] and alternate cell lines including eventually patient-derived induced pluripotent stem cell (iPSC) neurons [64] and iPSC-derived spheroids [65,66]. Additional extensions of this technology for small molecule discovery should include using different cellular models of tau pathology including, among others, modification of the oligomerization trigger through the addition of tau seeds (fibrils, oligomers, or monomer), upregulation of specific kinases or chaperone proteins (e.g., GSK3β or HSP70), and treatment with environmental toxins [14]. This will allow us to examine multiple proposed mechanisms of induced tauopathies, providing key insight into differences between on- and off-pathway oligomerization [67]. Each of these steps will be critical to building a more complete and useful correlation between structural heterogeneity and toxicity.

In addition, as we have shown that full-length tau can be engineered as a cell-based FRET-biosensor of oligomerization (here using the 2N4R isoform), we should now explore potential nuances in how oligomerization depends on tau isoform. We will not only be able to explore the propensity of different isoforms to oligomerize, but should also be able to test the likelihood that different isoforms comingle in heterogeneous oligomers. It will be of great interest to ascertain whether lead compounds are isoform specific or can target multiple distinct isoforms. Broadly speaking, information on isoform-specific oligomerization could be useful in designing more effective, patient-stratified design of clinical trials [68]. The Diamond group pioneered the tau cellular biomarker field with their tau repeat domain FRET biosensor cells [43]. Use of full-length tau thus expands on the existing technology and should facilitate additional stratification of potential biomarkers present in AD versus other tauopathies. Following Diamond, experiments that compare the sensitivity and relative reactivity of each biosensor to different tauopathy-associated biofluids will provide new

insight into heterogeneity in tau assemblies inherent in these distinct diseases.

Ultimately, determining and validating the specific MOA by which lead compounds act—be they through direct binding to tau monomers/oligomers or indirectly by altering cellular processes that lead to alterations in oligomers—will require comprehensive approaches to link biophysical experiments with cell biological observables. One set of immediately accessible questions is how the compounds' impact on monomer folding and/or oligomerization relates to tau localization in cells, most obviously on microtubule binding. Here, future work will further probe PTMs (including hyperphosphorylation, which we started here using forskolin) and acetylation, specifically testing how compounds alter the relationship between microtubule unbinding and tau folding/aggregation. As another example, tau has recently been shown to mislocalize to dendritic spines, disrupting synaptic transmission in primary neurons [69]. Whether these or numerous other mechanisms related to mislocalization can explain the cytoprotective effects of lead compounds will require additional experiments. For example, nanoimaging modalities like fluorescence-lifetime imaging microscopy and time-correlated single photon counting can provide the necessary spatial resolution to correlate subcellular localization (e.g., microtubules, cytosol, mitochondria, and so forth.) and distinct tau conformations [70].

4. Major challenges for the hypothesis

The technology described here is based on the hypothesis that tau biosensors expressed in cells can be used to accomplish two complementary, but distinct goals: (1) they can be used to find small molecules that modulate tau toxicity; and (2) they can provide a direct, albeit low-resolution reporting of the structures and conformations of a heterogeneous ensemble of toxic and nontoxic states. For the first part of this hypothesis, namely modulation of toxicity, we have demonstrated the power of our approach with MK-886. The second part is more of a challenge. This broader and longer-term hypothesis is built on the idea that biophysical tools, such as TR-FRET, can provide key insight into the unique structural fingerprints of heterogeneous toxic tau oligomers, and that this detail can be exploited in drug discovery. This is a far more nuanced and difficult bar for the field to meet, but is nonetheless a goal that should and, we believe, can be tackled. Why is this difficult? On the one hand, unlike fibrils, oligomers are highly heterogeneous (number of monomers per aggregate, local or transient structural motifs/folds, molecular constituency, and so forth), making it improbable that high-resolution structural biology tools are or ever will be applicable to their study (they simply lack well-defined secondary or tertiary structural elements). On the other hand, the field currently relies only on low-resolution techniques that provide little to no structural information (e.g., antibody recognition, protease protection, and detergent resistance). Finding a middle ground requires a set of structural techniques that can adequately and accurately interrogate oligomers, but more crucially can stratify structural fingerprints at a quantitatively useful and reproducible resolution. Absent high-resolution structures, we should nonetheless be able to ask: what are the critical amino acids that dictate oligomer-prone monomer folds and what are the deleterious intermonomeric amino acid motifs that dictate toxicity? As an example of how this can begin to happen, we complemented our TR-FRET with smFRET and were able to isolate the region of tau impacted by MK-886 binding. But this is just a start, as far more detailed information should be obtainable using a set of creative and state-of-the-art experimental and computational approaches to interrogate these structures [71–73].

In theory, TR-FRET waveforms contain high-content information that can resolve relative species populations and protein-protein distance distributions [74]. Unfortunately, TR-FRET alone does not provide atomic structural resolution to uniquely identify specific species. The process of extracting these structural data from TR-FRET requires model fitting. The challenge is that the model must be constrained by information we do not yet have, including constraints on the stoichiometry of the tau oligomer. This highlights a current limitation in analyzing tau-tau TR-FRET as there are no well-defined structural states, and the exact toxic species, including the number of interacting tau monomers, is unknown. To begin to make progress in this regard will require additional biophysical tools. These will include, among others, analytical ultracentrifugation and analytical gel filtration for oligomer size. Despite the promise of these techniques for grouping oligomeric species into meaningful clusters, doing so in cells will be the greatest challenge moving forward. Higher resolution structural information for stratifying oligomeric species can be obtained in purified, in vitro assemblies using other spectroscopic techniques such as nuclear magnetic resonance and electron paramagnetic resonance. However, because tau oligomers in cells likely consist of other molecular constituents and are folded with the help of chaperones, there is a real danger in relying too heavily on the folding of these assemblies outside the native cellular environment. There have been recent advances that allow for the use of high-resolution techniques, such as nuclear magnetic resonance, in cells [75–77]. These and other advances in biophysical tools will be of critical importance in the coming years.

5. Linkage to other major theories

Tauopathies have a vast heterogeneity in their clinical presentations (e.g., AD, frontotemporal dementia, and movement disorders, among others) [78], which is one of the major challenges that plagues current clinical trials [79]. This heterogeneity may be explained in part by strong

histopathological differences and differential laminar and regional brain distributions. For therapeutic intervention, an equally important potential source of this heterogeneity is molecular variations such as isoform composition and PTMs [78,80]. Hence, there is a need for robust tools and/or biomarkers to stage and delineate (particularly, at the molecular level) the numerous different tauopathies.

Although our biosensors and HTS technology are focused on the oligomer hypothesis of tauopathy, they are directly translatable into other areas of tauopathy research and therapeutic discovery. The presence of misfolded tau and the formation of the tau oligomers can be attributed to upstream dysfunctions in neurophysiology and axonal transport. For example, mitochondrial dysfunction and oxidative stress are believed to be a prominent early event in the pathogenesis of AD, contributing to tau phosphorylation and the formation of NFTs [81]. In particular, deterioration of mitochondrial functions such as impairments in the activity of Krebs cycle enzymes and electron transport machineries (e.g., cytochrome c oxidase) have been correlated with severity in the clinical state of tauopathies and AD [9,10]. Importantly, impaired cytochrome c oxidase activity can potentiate the generation of mitochondrial-derived reactive oxidative species, suggesting that defective mitochondrial bioenergetics and oxidative stress are coupled in a vicious cycle [10]. It has also been shown that in familial AD, tau and amyloid-beta (Aβ) can augment the pathologic deterioration of mitochondrial function [82]. In addition, there is compelling evidence that tau can play a role in controlling motor protein–driven vesicle transport along microtubules [83]. Furthermore, there is an age-dependent decline in axonal transport rates, which correlate with increases in hyperphosphorylated tau [11]. Our FRET biosensors can be used to study these effects. For example, we can use the intramolecular biosensor to study the global tau folding when it is bound to microtubules versus when it is detached from microtubules. Our intermolecular biosensor can also be used to monitor the kinetics and the extent of free soluble tau that are detached from microtubules and start to form oligomers, providing important fundamental information in understanding early stage events underlying tau pathology.

Misfolded or oligomerized tau can be a symptom, as much as a cause, of an underlying pathology. Despite our focus on disrupting oligomers, using the cellular tau biosensors to modulate upstream effectors of tau dysfunction may actually hold the most promise. The TR-FRET screen in cells does not discriminate between compounds that act directly on tau folding/oligomerization and those that operate indirectly by binding to other upstream targets. Coupling secondary biophysical assays to the screen allows us to elucidate direct versus indirect MOA. Compounds that act through an indirect MOA—for example, those that rescue dysfunctional autophagy or mitochondrial functions, endoplasmic reticulum or oxidative stress—can provide insight into specific pathways that are disrupted in tauopathy, giving rise to novel therapeutic targets and strategies. On inspection, this appears likely the case for several of the compounds we identified in our screen but whose MOA we have not yet elucidated. Two of these compounds (bumetanide and torsemide) are both loop diuretics, which inhibit the sodium-potassium-chloride cotransporter (NKCC1) in vascular smooth muscle and have been shown to reduce the risk of AD dementia in both adults with normal cognition or with mild cognitive impairments [84]. The other two hits (benzbromarone and triclosan) have been shown to attenuate oxidative stress [85] and induce autophagy [86] (respectively), both known cellular dysfunctions in AD.

Our biosensors should be useful in a variety of other contexts being pursued aggressively in the field. One example is the prion-like propagation of tau pathology [43]. We plan to use the technology in an HTS campaign targeting the uptake and propagation of toxic tau species. We have already begun to see the power of the technology in this way, observing increased FRET because of uptake of WT and truncated tau protein by our biosensor (data not shown). A second example builds on mounting evidence of cross-reactivity and comorbidity among misfolded proteins in numerous neurodegenerative diseases. These players include other IDPs, Aβ and α-synuclein [87]. Our biosensors can directly probe these types of interactions via coexpression of these constructs into the tau biosensor cells, and/or treating the tau biosensor with Aβ or α-synuclein fibrils/oligomers and monitoring FRET, cytotoxicity, and uptake. Different donor- and acceptor-labeled proteins (e.g., tau-GFP/Aβ-RFP, and so forth) can also be developed and used to study potential direct protein-protein interactions. Our strategy, combining cellular fluorescent biosensor and TR-FRET measurement in an HTS platform, is broadly applicable to other drug discovery efforts targeting IDPs involved in numerous neurodegenerative diseases.

A straightforward improvement will be to express tau in a stable or inducible cell line and in human iPSCs for physiological relevance. This will further improve conditions for screening in a native environment. In addition, we will launch further HTS campaigns using the most optimized tau FRET biosensors to screen CNS-focused libraries such as the CNS–multiparameter optimized library or the CNS-Set library to ensure small molecules have a high probability of crossing the blood-brain barrier. In conjunction with smFRET and other techniques such as analytical ultracentrifugation, the biosensors can be used to develop a clearer picture of how mutations (e.g., P301L), different tau isoforms, and post-translation modifications alter the conformations of oligomers, and what determines toxicity. Ultimately, we will need to test the functional effect of the hit compounds in

animal models with tau oligomer–induced pathology to see if they rescue the pathology. If the compounds are functional in the animal models, they will be poised to be tested in clinical trials. As for the development of biomarkers, an immediate next step is to compare our biosensor with the existing tau repeat domain FRET biosensor developed by the Diamond group to test their sensitivity in cerebrospinal fluid from patients with AD. If our biosensor is sensitive to these patient samples, we can then further investigate how different groups of patient samples may have different effects on our FRET biosensor. This will act as an initial step in potentially stratifying the patients into different groups to be tested in clinical trials for more effective targeting and drug discovery.

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Author contributions: C.H.L. designed and conducted all the experiments. C.K.W.L. and Z.D. contributed to protein purification and assisted with cell-based assays and biochemical experiments. S.W. and E.R. performed and analyzed the single-molecule fluorescence resonance energy transfer (FRET) experiment. D.D.T. provided expertise on FRET and high-throughput screening, and provided comments and edits to the manuscript. A.R.B. and K.H.A. provided comments and edits to the manuscript. C.H.L and J.N.S. wrote the manuscript.

Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jalz.2019.06.4954.

RESEARCH IN CONTEXT

1. Systemic review: Clinical heterogeneity in tauopathies and Alzheimer's disease is a major challenge that impedes the development of effective treatments.
2. Interpretation: Using an emerging fluorescent biosensor technology, we established a novel high-throughput screening platform for the discovery of small molecules that target the ensemble of heterogeneous tau oligomers in cells.
3. Future directions: Distinct identity for toxic or non-toxic tau oligomers needs to be delineated to improve biomarker development and reduce the heterogeneity present in clinical trials.

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