

Targeting tau oligomers for therapeutic development for Alzheimer's disease and tauopathies

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ABSTRACT

Tau protein has a causative role in Alzheimer's disease and multiple other neurodegenerative disorders exhibiting tau histopathology collectively termed tauopathies. The primary function of tau protein is to facilitate assembly and maintenance of microtubules in neuronal axons. In the disease process tau protein becomes modified, loses its affinity to microtubules and accumulates in the cell body where it forms aggregates. The large neurofibrillary tangles formed from tau protein assembled into filaments were thought to be the pathological structure of tau. However, more recent work indicates that smaller, soluble oligomeric forms of tau are best associated with neuron loss and memory impairment. Development of therapeutics targeting tau oligomers may be achieved by several strategies. However, a better understanding of the structure of pathological tau oligomers and their mechanisms of toxicity will facilitate the drug discovery process.

KEYWORDS: tau, oligomer, Alzheimer's, tauopathy, therapeutics, drug discovery, neurodegenerative disorder, neurofibrillary tangle, tau isoforms

INTRODUCTION

There remains a major, growing, unmet medical need for the pharmaceutical industry to find drugs that halt or reverse Alzheimer's disease (AD). The

classical hallmarks of AD are inter-neuronal plaques consisting of precipitates or aggregates of amyloid beta protein (A β), and intra-neuronal neurofibrillary tangles (NFTs) of tau protein. However, the concept that soluble oligomers (small soluble aggregates) of amyloid proteins are the acutely toxic structures of these proteins, and not insoluble aggregates like plaques and tangles, is now accepted for multiple neurodegenerative diseases [1, 2].

The major target for drug discovery for AD has been A β protein that aggregates and forms extracellular insoluble senile plaques. Although the etiology of AD is not fully understood, the A β amyloid cascade hypothesis has been the most common view of the pathological pathway of AD. It holds that the generation of A β and accumulation of A β aggregates in the brain initiate the disease process. It is supported by genetic evidence that mutations leading to increased accumulation of A β aggregates leads to familial AD. However, A β is not a sufficient target for AD therapeutic development as there are a number of weaknesses in the A β cascade hypothesis and it does not address the importance of other pathways that can cause neurodegeneration [3].

The accumulation and distribution of NFTs in the brains of AD patients is highly correlated with disease progression and can be used to stage AD by post-mortem brain histopathology, whereas the poor correlation between AD and the accumulation of neuritic plaques composed of beta amyloid has been used to challenge the amyloid hypothesis [4]. Failure to show efficacy for A β directed

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therapeutics in late stage clinical trials has increased interest in exploring alternative targets for drug discovery such as tau.

Genetic evidence has shown that abnormal forms of tau are sufficient for neurodegeneration causing memory loss and other neurological deficits in frontotemporal dementia and other tauopathies. Although no mutations in the gene for microtubule associating protein tau (*MAPT*) have been linked to AD specific *MAPT* haplotypes may be a risk factor for AD. Post-transcriptional mechanisms involved in increasing the 4R/3R tau ratio may also play a causative role. At the post-translational level there are multiple modifications to tau protein in AD and other tauopathies that cause both loss of function and gain of toxicity.

Recent advances in research in AD have highlighted the importance of tau in pathogenesis [5] and its use as a target for the development of disease modifying therapeutics. Evidence from mouse models indicates that tau reduction reverses disease phenotypes [6, 7, 8] and is necessary for the development of cognitive deficits in AD models caused by over- expression of A β [9]. The pathological structures of tau most closely associated with AD progression are tau oligomers in mouse models and also accumulate in human disease [10, 11, 12]. This review briefly describes tau protein structure and function, summarizes evidence demonstrating a causal role for tau in neurodegenerative diseases, and describes work demonstrating that soluble tau oligomers are the acutely neurotoxic structures of tau. Mechanisms of tau self-association are summarized. Publications representing the study of tau oligomers *in vitro*, in cell culture, in animal models, and in AD patients are reviewed in greater detail to provide some of the techniques and approaches used to study tau oligomers. The final section focuses on tau oligomers as a target for AD therapeutic discovery and development.

Overview of tau structure and function

Tau protein is normally localized most abundantly in the axons of neurons where it facilitates the assembly of tubulin into microtubules (MTs) and maintains their stability. A single *MAPT* gene on human chromosome 17q21 encodes tau protein.

Alternative splicing of the second and third exons in the N-terminal portion of tau (generating 0N, 1N or 2N isoforms) and the tenth exon yields a total of six protein isoforms (Table 1). The assembly domain in the carboxyl-terminal portion of the protein contains either three or four repeats (3R or 4R) of a conserved tubulin-binding motif depending on alternative splicing of exon 10 [13]. Adult brain has a ratio of about 1:1 for 3R to 4R isoforms, but the ratio of 0N:1N:2N isoforms is about 37:54:9 [14, 15]. Tau is an intrinsically unstructured protein due to its very low hydrophobic content and has been characterized to have a projection domain, a basic proline-rich region, and an assembly domain (Figure 1). Hexapeptide motifs PHF6* and PHF in the second and third repeats, respectively, have propensity to form β -sheet structures which are involved in tau interaction with tubulin to form MTs and tau self-interaction to form pathological aggregates such as paired helical filaments (PHF) [16, 17]. Tau 4R isoforms have greater MT stabilizing ability than the 3R isoforms. Only the 3R/0N tau isoform is expressed during fetal development where there are dynamic changes in the cytoskeleton. Expression of 4R/2N in the hippocampus of tau knockin/knockout mice suppresses proliferation and promotes neuronal differentiation [18]. Hyperphosphorylation of tau, particularly in the assembly domain, decreases the affinity of tau to MTs to regulate MT dynamics and axonal transport [19, 20, 21]. Additional regions in the basic proline-rich domain and the pseudo-repeat (Figure 1, small rectangles above bar) also stabilize MTs by interacting with its negatively charged surface [22]. The projection domain facilitates interaction with the plasma membrane [23, 24]. Interaction of tau with membranes is also thought to facilitate tau aggregation [25]. For recent reviews of tau structure and function see [26, 27].

Table 1. Tau protein isoforms.

| Residues | Spliced exons | Nomenclature |
|----------|---------------|--------------|
| 352 | - | 3R/0N |
| 381 | 3 | 3R/1N |
| 410 | 2, 3 | 3R/2N |
| 383 | 10 | 4R/0N |
| 412 | 3, 10 | 4R/1N |
| 441 | 2, 3, 10 | 4R/2N |

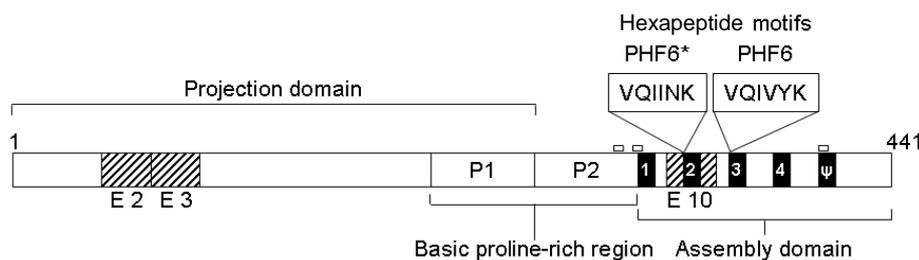


Figure 1. Bar diagram of tau protein structural features. A schematic of the full-length tau protein isoform 4R/2N (441 amino acids) is shown. The projection domain, basic proline-rich region (subdivided into P1 and P2 regions) and assembly domains are indicated by brackets. Alternative spliced exons 2, 3 and 10 are indicated by hatched rectangles. Black rectangles indicate repeats 1-4 and pseudo-repeat in the assembly domain. Hexapeptide motifs PHF6* and PHF6 involved in microtubule binding and tau self-association are detailed. The small rectangles above the bar indicate additional regions involved in microtubule binding.

A causal role for tau in neurodegenerative diseases

That tau dysfunction is sufficient for neurodegeneration and dementia, even in the absence of other disease processes, comes from direct evidence that mutations in *MAPT* cause frontotemporal dementia with Parkinsonism linked to chromosome-17 (FTDP-17) [28]. The 32 different mutations found in the study of over 100 families can be grouped into categories influencing splicing of the primary transcript and causing changes in amino acid sequence of tau. Most missense mutations are located in the assembly domain and generally reduce the affinity of tau to MTs. Several of these mutations promote aggregation of tau *in vitro* and *in vivo* such as P301L and P301S. Mutations in the stem-loop structure at the border of exon 10 and the following intron alter splicing causing aberrations in the ratio of 4R to 3R isoforms demonstrating that maintenance of the proper ratio of tau isoforms is necessary to prevent neurodegeneration and dementia.

Sporadic tauopathies such as progressive supranuclear palsy, corticobasal degeneration, Pick's disease and argyrophilic grain disease are characterized by pathology with tau filaments composed predominantly of 4R isoforms and are linked to *MAPT* mutations [28]. Recent reports indicate aberrant splicing of tau transcripts in AD demonstrating a common defect with other tauopathies. Increases in the ratio of 4R to 3R tau mRNAs were found in individual human cholinergic basal forebrain neurons in nucleus basalis and CA1 hippocampal neurons in AD [29]. Aberrant

alternative splicing in sporadic AD was also shown using polymerase colony, a single-molecule-based technology. A *trans* mechanism involving the reduction of splicing factor htra2-beta-1 in AD was linked to the increase in four-repeat tau isoforms [30]. Further genetic support for a causative role for tau in AD comes from the observation that tau haplotypes driving higher levels of tau expression increase AD risk [31] and from the report that linkage of tau haplotypes with increased CSF tau in people with A β deposition accelerated AD progression [32]. Similarly, increased expression of 4R tau and linkage to *MAPT* haplotypes has been reported for Parkinson disease [33], the second most common form of neurodegenerative disease following AD.

Multiple mouse models have been developed in which the expression of human tau isoforms or human tau constructs with mutations found in FTDP-17 caused development of neurodegenerative phenotypes supporting a causal role for aberrant tau expression in memory impairment and neuron loss [34, 35, 36, 37, 38, 39, 40]. A remarkable publication implicated tau in mediating A β memory loss and excitotoxicity. Erik Roberson, Lennart Mucke, and colleagues reasoned that since higher levels of tau may cause AD reducing tau levels might be protective [9]. They tested their hypothesis by reducing endogenous tau expression in transgenic hAPP mice with cognitive deficits caused by overproduction of A β . Tau knockout mice were crossed with hAPP mice to generate mice with two, one, or no endogenous tau alleles with or without the hAPP transgene. Learning and

memory were tested using the Morris water maze [71]. Strikingly, hAPP mice with both tau alleles took longer to learn and had difficulty remembering the position of the submerged platform in the water maze, whereas mice with one or no tau alleles performed at control levels. Reduction of tau alleles also protected mice from premature death and decreased sensitivity to excitotoxins suggesting a role for tau in regulating neuronal activity. The protective effects of reducing tau were not caused by reduction of A β pathology signifying that tau potentiated downstream pathogenic mechanisms of A β . The authors suggest that tau reduction could protect against AD and other neurological conditions associated with excitotoxicity [9, 41].

Tau oligomers, not tangles, are the primary pathological aggregates

NFTs have been implicated in mediating neurodegeneration in AD and tauopathies as it correlates well with cognitive deficits and neuron loss [4, 42, 43, 44, 45]. However, the study of animal models of tauopathy has shown that memory impairment and neuron loss is dissociated from accumulation of NFT. Strong support for this contention came from the analysis of transgenic mice rTg4510 that express tau P301L in the forebrain under control of a tetracycline-regulated promoter. These mice developed memory impairment, neuron loss and NFT when the construct was expressed. However, suppression of expression caused improvement in memory and reduction in neuron loss even as NFTs continued to accumulate clearly demonstrating that pre-tangle tau species were responsible for the neurodegenerative phenotype [6]. Additionally, there was regional dissociation of neuron loss and NFT pathology in this model [46] and in another mouse model expressing all six human isoforms [47] showing that tangles are not acutely neurotoxic.

Incongruence between tangle formation and neurodegeneration and behavioral deficits were found in other mouse models of tauopathy and AD. Transgenic mice expressing a human mutant tau P301S construct prone to aggregation developed hippocampal synapse loss and dysfunction, as well as, microglial activation months before the accumulation of filamentous tau inclusions [37].

Similarly, a transgenic mouse model expressing human tau protein with two mutations found in FTDP-17 (P301S and G272V) exhibited axonopathy before tangle formation [48]. The triple transgenic AD mouse model accumulating both tau and A β pathology was used to study the effects of immuno-reduction of tau and A β . Antibodies against both proteins were needed to improve learning and memory behavior in these mice. Soluble tau, but not NFT, was reduced by the treatment again showing the dissociation between the neurodegenerative phenotype and insoluble tau aggregates [7]. A *Drosophila* model of tauopathy expressing wild-type and mutant forms of human tau also exhibited neurodegeneration without NFT formation [49].

Professor Avila and colleagues have brought attention to a neurotoxic role of soluble extracellular tau to help explain the reproducible spread of tau pathology in the brains of AD patients [50]. Levels of tau protein are thought to rise in AD CSF [51] due to release of intracellular tau associated with neuronal degeneration [52]. Using cultured neuroblastoma cells, soluble tau containing oligomers was shown to be more toxic than tau filaments [53] and that the mechanism of toxicity involved promotion of increased intracellular calcium through M1 and M3 muscarinic receptors [54]. Based on this research, a cyclical model was proposed to explain the spread of tau pathology. Intracellular tau is released in the extracellular space during neurodegeneration where it causes neighboring cells with muscarinic receptors to degenerate and expose additional cells to extracellular tau [53]. Taken together it appears that pre-fibrillar/oligomeric tau plays a key role in neurodegeneration and behavioral impairments [55].

Characterization of tau self-interaction

Tau aggregation *in vitro* is facilitated by anionic molecules such as heparin, arachidonic acid [56], RNA [57], and anionic detergents [25] that have been used to facilitate the assembly of tau monomers into filaments. Anionic polyanions may function as a scaffold bringing multiple cationic tau proteins into close proximity and by shielding the repulsive positive charge of tau, thus favoring kinetics of interaction [58]. The mechanism of tau polymerization is not fully understood, and has

been modeled as a nucleation-elongation reaction [59, 60, 61, 62, 63] or as an allosteric regulation model in which facilitator molecules induce conformational change in the protein, effectively eliminating the thermodynamic barrier to polymerization [64]. It is unclear whether tau oligomers are stable end-products or intermediates in tau filament assembly [65]. Multidimensional NMR studies on a C-terminal fragment of human tau were performed to clarify the formation of tau oligomers in solution caused by addition of heparin. Although the interaction site of heparin with tau was not defined, it was apparent that heparin induced tau self-interaction primarily by motifs VQIINK and VQIVYK in the third and second repeat domains, respectively, (Figure 1) to form a heterogeneous oligomeric population [65]. These hexapeptide motifs have been previously found to be important sites for tau interaction and were termed PHF6 and PHF6*, respectively [22, 66, 67, 68]. NMR characterization of a tau fragment composed of the four tandem repeats of the assembly domain and flanking regions led to the identification of additional motifs in the basic proline-rich domain and first repeat and in the pseudo-repeat in the assembly domain (Figure 1) that contribute to the association of tau with the acidic surface of MTs and other polyanions like heparin [22].

Tau oligomer formation *in vitro*

To understand the process of tau oligomerization *in vitro* Sahara, Takashima and colleagues [12] used constructs of tau protein to determine the elements in the microtubule binding repeat domain involved in heparin-facilitated formation of SDS-stable, tau oligomers. Tau isoform 4R/2N and variants were incubated at high concentration (10 μ M) with an equimolar amount of heparin (average MW 6000 kDa) at 37 °C. The formation of SDS-stable oligomers was visualized by SDS-PAGE of samples removed from the reaction over time and mixed with Laemmli sample buffer without heating.

Fluorescence spectroscopy of reactions containing thioflavin T (ThT) was used to indicate relative levels of β -sheet structure in tau during oligomerization [62] in combination with SDS-PAGE analysis of tau oligomer formation. Fluorescence levels over time produced a typical

sigmoidal plot. β -sheet formation gradually increased until 4 hrs. at which time large aggregates unable to enter the stacking gel began accumulating concomitant with rapid linear increase in fluorescence until 25 hrs. The curve leveled as the aggregation reaction reached completion. These results suggest that β -sheet content increases with aggregate size and that a higher rate of conversion to β -sheet structure occurred after tau association into oligomers of six to eight tau proteins. However, it is unclear how many times these experiments were repeated and what deviations from these results were observed.

Silver staining of incubated tau samples resolved by SDS-PAGE clearly showed the progression of formation of higher order oligomers and aggregates over 24 hrs. Tau protein used in the reactions was mostly monomer but also contained some dimer, which may have formed due to the high concentration of tau in the protein preparation (70 μ M) prior to incubation. The level of dimer peaked between 0.5-8 hrs. but decreased with time as the amount of larger aggregates accumulated. A ladder of trimer-hexamer oligomers was visible after incubation for 0.5 hr., and the level of higher order oligomers steadily increased with time. The effect of 1 mM DTT in the reaction was studied to help assess the role of disulfide bond formation in tau oligomerization. Addition of 1 mM DTT to the incubation mix lowered the amount of dimer in the starting material and slowed the rate of accumulation of tau aggregates trapped in the well while increasing the amount of lower size oligomers. In the absence of heparin, 1 mM DTT lowered the amount of dimer in the samples and no higher order oligomers were detected during incubation for 24 hr.

To determine the role for disulfide bond formation in tau oligomerization the aggregation of tau 4R/2N was compared to the aggregation of a cystless tau 4R/2N construct with conversion of both cysteines at positions 291 and 322 to alanine in repeats 2 and 3 of the microtubule binding domain. The absence of both cysteines slowed the rate of dimer formation and prevented the formation of SDS-stable higher order oligomers. However, AFM indicated that cystless construct could form filaments suggesting that intramolecular disulfide

bond formation is not required for tau assembly. The 3R/2N tau isoform, lacking exon 10 encoding repeat 2 containing cysteine 291, formed SDS-stable dimers, but levels of higher order oligomers and aggregates were reduced indicating that the second repeat confers a greater propensity for tau to oligomerize. It has been suggested that 4R tau isoforms may have a greater propensity to aggregate which may be part of the molecular mechanism for the causation of tauopathies by intronic mutations causing aberrant over-expression of 4R isoforms relative to 3R isoforms [26].

The 4R/2N tau construct with deletion of hexapeptide 306VQIVYK311, shown to be involved in tau self-interactions [68, 69], formed dimers trimers and tetramers only after prolonged incubation with heparin confirming the importance of this sequence in tau oligomerization. Interestingly, this construct also formed aggregates too large to enter the gel but AFM showed that they did not have filamentous structure and did not bind ThT indicating lack of β -sheet structure. Thus, tau can aggregate in the absence of the hexapeptide but can not form β -sheet structure and assemble into filaments.

Treatment of incubated samples from all the constructs with high levels of reductant (50 mM DTT) and heat (70 °C) caused tau aggregates to disassemble to monomers and dimers suggesting that disulfide bond formation stabilizes tau oligomers. However, the aggregates formed from the constructs lacking the hexapeptide or the cysteines that were too large to enter the gels were more resistant to reductant than the large aggregates formed from wild-type 4R/2N and 3R/2N.

The cysless construct was used to determine the regions involved in tau self-interaction in the absence of disulfide binding. Size exclusion chromatography (SEC) was used to purify monomers and dimers of the cysless construct. Comparison of the gel filtration profiles of cyanogen bromide fragments of the monomers and dimers suggested that the fragment involved in tau self interaction corresponded to amino acids 251-419 containing the microtubule binding domain, and immunoblot of the fragment with an antibody against the microtubule domain confirmed this conclusion. Further studies are needed to

characterize the core region for dimer formation in the absence of cysteine; the hexapeptide 306VQIVYK311 in this fragment is a strong candidate.

Transient transfection of COS-7 cells with tau constructs was also used to evaluate the role of different domains of tau involved in the formation of SDS-stable oligomers in cells. Cell lysates prepared by homogenization, boiling and high speed centrifugation were analyzed by immunoblot. Tau appeared predominantly monomeric in all the samples, but the level of dimers varied. Higher order oligomers were not apparent, possibly due to sample preparation methods or the relatively short amount of time tau was allowed to aggregate post-transfection. Dimer levels found using the wild-type 4R/2N tau sample were similar to levels of dimer found in constructs with cysteine to alanine conversions of either C291A or C322A, but conversion of both cysteines strongly inhibited the accumulation of dimer. Similarly, deletion of the entire repeat region (amino acids 251-377) prevented dimer formation. Further deletion of the third repeat containing β -sheet prone hexapeptide 306VQIVYK311 strongly inhibited dimerization. The 3R/2N isoform lacking the second repeat, containing β -sheet prone hexapeptide 275VQIINK280, caused moderate inhibition of dimer formation, whereas deletion of the first or fourth repeats caused no significant difference in dimer formation. Immunoblot of lysates treated with reductant to study levels of cysteine-independent dimers indicated that dimer levels were generally low. However, analysis of tau dimer levels normalized to the 4R/2N wild type control accentuated differences between the samples. Lack of the second or fourth repeat or either cysteine did not reduce dimer levels. However, the lack of both cysteines, either hexapeptide in the second or third repeats, both hexapeptides, or the first or third repeat resulted in dimer levels about 60-70% of the control, whereas deletion of the entire repeat region reduced dimer formation by 75% relative to the control. These cell-based studies indicated that formation of total SDS-stable tau dimers minimally required the presence of the hexapeptide in the third repeat and at least one cysteine, whereas the hexapeptide in the second repeat facilitated dimer formation. Accumulation

of SDS and reductant stable dimers had a broader requirement for the entire repeat region.

SDS-stable oligomeric tau was found in brain extracts from tauopathy mouse model JNPL3 expressing human 4R2N tau with the P301L mutation. Tau soluble and insoluble fractions were resolved by SDS-PAGE, and analyzed with antibody E1 against the human tau construct. Tau soluble in Tris-buffered saline contained a low level of dimer that was dissociated by treatment with reductant. The sarkosyl-insoluble fraction contained predominantly hyperphosphorylated tau monomers and a relatively high level of dimers that were reductant stable. This fraction also contained higher molecular weight aggregates that were disrupted by treatment with reductant to form dimers of tau consistent in size with lower levels of phosphorylation.

Similar analysis of sarkosyl-insoluble extracts of human normal and AD brain specimens showed the absence of tau in the six normal extracts, whereas the six AD extracts contained high levels of sarkosyl-insoluble tau primarily as monomers and dimers but also at lower levels as trimers and higher order oligomers [22].

Tau oligomer formation in cultured human cortical neurons

The expression of tau isoforms was studied in human cortical neurons (HCNs) in culture to analyze their role in neuronal differentiation and the effects of abnormal tau phosphorylation [70]. Cultured HCNs were derived from human fetal cortical tissue and plated on synthetic substrate polylysine for cell attachment or laminin, an abundant component of basement membrane in the brain, to induce differentiation. RNA and protein levels of tau isoforms were determined by quantitative real-time PCR and immunoblot. Immunofluorescence was used to study localization of tau isoforms in cells. Localization of tau shifted from dendrites and neuronal bodies to axonal processes as HCNs developed on laminin. Levels of total tau RNA were highest at earlier time points of days in culture which decreased with progressing days in culture. However, there was a relative increase in 4R tau RNA preceding the period of maximal axonal outgrowth in early culture. Protein levels of 4R tau compared to total

tau remained high in HCNs plated on laminin during 20 days of culture, but this difference was not seen when cells were plated on polylysine demonstrating substrate-dependent differences in levels of tau protein isoforms persisting after active axonal elongation. The pattern of tau isoform expression by immunoblot of adult brain and HCNs cultured on laminin for 40 days was similar demonstrating *in vivo* relevance of this cell model. Subcellular localization of 4R tau showed a punctate pattern along axons, whereas total tau showed a smooth pattern of immunolocalization along the axon suggesting different roles for 3R and 4R tau in modulating microtubule dynamics.

To test the role of tau phosphorylation in pathological changes in HCNs inhibitors of phosphatase 2A (PP2A) and 2B (PP2B) at non-toxic concentrations. Inhibition of PP2A with 10 nM cantharidin caused a change in the expression of specific tau isoforms, whereas inhibition of PP2B with 40 pM deltamethrin did not. Inhibition of PP2A caused an increase in soluble levels of total tau and phosphorylated tau, whereas inhibition of PP2B was ineffective. Inhibition of PP2A also caused mislocalization of phosphorylated tau to cell bodies and initial neuritic segments. Tau oligomers formed in cells treated with PP2A inhibitor had apparent molecular weights of 140 and 170 kDa by immunoblot. These results are consistent with the oligomeric tau species found in the rTg4510 mouse tauopathy model [10]. Antibody AT-180 against phosphorylated tau recognized the upper band. An antibody specific for 4R tau isoforms recognized the 140 and 170 kDa tau species, and antibody A11 against a conformational epitope in oligomers [8] recognized a band consistent with the 140 kDa tau multimer. The authors conclude that 4R tau isoforms are principally involved in tau oligomerization and continue to study the consequences of tau oligomerization in protein trafficking and synaptic activity [70].

Tau oligomer formation in rTg4510 tauopathy mouse model

The rTg4510 conditional mouse model of tauopathy was used to characterize the pre-tangle tau species that best correlates with memory loss [10]. This work identified tau oligomers in pre-NFT aggregates

that are necessary for neuron loss and memory impairment or at least tightly linked to the mechanism of tau toxicity. Additionally, it identified similar tau species in human brain extracts from patients with FTDP-17 and AD.

Total brain extract was prepared using low-speed centrifugation to remove debris and mature neurofibrillary tangles (NFT). Immunoblot using antibody specific for the human tau construct indicated the expected expression of the construct by multiple species at apparent molecular weight (MW) of 55 kDa (tau55) which were generated by the normal phosphorylation states of the protein. Hyperphosphorylated tau monomer migrated at an apparent molecular weight of 64 kDa (tau64). Interestingly, longer exposure revealed the presence of tau in two additional bands at 140 (tau140) and 170 kDa (tau170) indicating the presence of oligomers composed of 2 or more tau proteins. The difference in size between tau140 and tau170 was attributed to hyperphosphorylation. These oligomers were stable in SDS and did not depend on the formation of disulfide bonds. The apparent MW of these oligomers following SDS-polyacrylamide gel electrophoresis suggests that they are composed of dimers of phosphorylated or hyperphosphorylated tau protein but the presence of other proteins in these complexes has not been ruled out.

Total brain extract was fractionated by high speed centrifugation prior to the denaturing conditions used for SDS-PAGE to estimate tau oligomer size *in vivo*. The supernatant fraction (S1) contained soluble tau, whereas the pellet extracted with sarkosyl (P3) contained the insoluble fraction of tau. Mice were evaluated at different ages to study the accumulation of tau species with the progression of disease phenotype. Hyperphosphorylated tau64 and tau170, absent at one month, appeared in total extract by 4.5 months and partitioned with the P3 fraction. Tau55 monomer and tau140 oligomer were found in the S1 fraction even at one month suggesting that tau140 is found in relatively small aggregates, whereas tau170 is derived from larger structures *in vivo*. In mice 6.5 months and older tau170 was also found in the S1 fraction showing that as the mice aged tau170 was also found in smaller size aggregates.

Size exclusion chromatography (SEC) of total extract was used to better characterize the distribution of the different tau species at different ages. At 4.5 months tau140 and tau170 elution profiles did not overlap; tau140 was predominantly in fractions corresponding to MW of ~70 kDa, whereas tau170 was in fractions corresponding to a MW range from 670 kDa to 1.5 MDa. However, at 6.5 months the elution profiles of tau140 and tau170 did overlap in fractions corresponding to aggregates of intermediate size. Hyperphosphorylated species tau64 and tau170 co-eluted, whereas tau55 had broad elution profiles in the different aged mice suggesting that normally phosphorylated tau is also part of larger aggregates.

It is commonly thought that tau hyperphosphorylation is a prerequisite for the dissociation of tau from microtubules and subsequent aggregation. The authors emphasize that the earliest pathological tau species to accumulate in this animal model is tau140 which occurs prior to the appearance of hyperphosphorylated tau64 and tau170 suggesting that during the initial stages of disease tau aggregation likely occurs before pathological tau hyperphosphorylation, thus, revision of current theory regarding the role of tau hyperphosphorylation in pathogenesis may be necessary.

Importantly, there was a significant negative correlation between spatial memory in rTg4510 mice with levels of tau oligomers. The Morris water maze [71] was used to evaluate memory in individual mice at different ages. Before the development of memory deficits at one month, only low levels of tau140 were detected. At three months there was a decrease in memory that correlated with levels of tau140 in total extract, before the accumulation of tau170 and tau64. At 5.5 and 8 months there was a negative correlation between memory and levels of tau140 and tau170 in total extracts and tau64 in the P3 fraction. However, there was no correlation between memory and levels of tau140 in the soluble S1 fraction. Taken together, the results indicate that 1) a minimal amount of tau140 in total extract must accumulate before there is a negative correlation with memory; 2) accumulation of insoluble tau64 is not necessary for initial memory impairment; and 3) tau140 and tau170 in the larger aggregates

that pellet from total extract during high speed centrifugation correlate best with memory dysfunction. These aggregates are smaller than NFTs as NFTs are removed by low speed centrifugation during preparation of total extract. Furthermore, there is dissociation between NFT counts and memory following suppression of expression of the transgenic tau protein, whereas transgene suppression causes a dramatic decrease in levels of tau oligomers that correlates with memory improvement in rTg4510 mice. Although accumulation of large, pre-tangle oligomeric tau species appear to be necessary for memory impairment, the authors acknowledge that these experiments did not rule out the possibility that unidentified smaller soluble tau species may contribute to memory impairment in this model.

The relevance of tau140 and tau170 to human dementia was addressed by immunoblot of extracts from transgenic mice and humans with AD and FTDP-17. The tauopathy mouse model JNPL3, constitutively expressing human tau441 with the P301L mutation at the same level as endogenous tau, accumulated tau140 and hyperphosphorylated tau64 and tau170 species in correlation with the onset of motor impairment. However, transgenic mice with similar expression of the normal tau protein did not accumulate tau64, tau140, tau170 or NFTs and did not develop motor impairments. Thus, the accumulation of these tau species seems to depend on the P301L mutation in these models. Analysis of brain extracts from FTDP-17 patients carrying the N279K mutation indicated accumulation of the tau170 species, whereas extract from an AD patient contained multiple tau species approximately 140-170 kDa. The increased complexity of the AD sample may be partly accounted for by the incorporation of all six tau isoforms into the aggregates, whereas only three isoforms with the 4-repeat region are in the FTDP-17 aggregates. Extensive post-translational modifications including polyubiquitination may also be involved in producing the complex set of higher molecular weight tau species [10].

Tau oligomer accumulation in human AD

Studies were conducted by Akihiko Takashima's group at the Laboratory for Alzheimer's Disease at RIKEN's Brain Science Institute and collaborators

to find the pre-filamentous form of tau associated with AD [11]. Tau oligomers isolated from human AD brain samples or formed *in vitro* from full length tau (4R/2N) had a granular/non-filamentous appearance when characterized by atomic force microscopy (AFM) and were termed granular tau oligomers (GTO). GTO were purified from human frontal cortices using immunoaffinity, treatment with 1% sarkosyl detergent and sucrose step gradient fractionation. AFM of isolated GTO ranged in diameter size from 5 to 50 nm with a peak at 20 nm. GTO produced *in vitro* using heparin to facilitate aggregation had a similar size and sedimentation coefficient as the GTO found *in vivo*. The approximate molecular mass of GTO determined by static laser light scattering averaged 1843.3 +/- 112.4 kDa corresponding to 40 +/- 3 full length tau molecules. However, this estimation is more speculative for the GTO found *in vivo* as other proteins may be forming heterogeneous oligomers with tau and because of the presence of six tau isoforms ranging in size from 352 to 441 amino acids in human adults. Furthermore, post-translational modifications including hyperphosphorylation, polyubiquitination and truncation create a large and diverse series of tau protein derivatives *in vivo*.

The levels of GTO were quantified in 21 frontal cortex samples with varying Braak stages of AD based on the number and distribution of NFT in the brain. Elevated amounts of GTO were found in cortical samples from Braak-stage 1 AD patients compared to Braak-stage 0 samples where NFT have not yet accumulated. Similar elevation of GTO was found in samples with Braak stages 2-5 even though the density of NFTs increased with disease progression. Thus, a general increase in GTO is correlated with AD, but the results generated with this small sample size do not show a more specific linkage between GTO levels and Braak staging of AD progression. However, the results are consistent with the notion that the GTO may be precursors of tau filaments.

The relationship between GTO levels and the accumulation of heat shock proteins (Hsp) was also studied in these frontal cortex samples as they are involved in refolding or degrading misfolded and aggregated intracellular proteins in neurodegenerative diseases [72, 73, 74]. An inverse

correlation was found by immunoblot for each sample between levels of GTO and chaperones Hsp90, Hsp40, Hsp27, α -crystallin and CHIP, whereas there was a positive correlation between these chaperones and soluble tau suggesting that chaperones help maintain tau in soluble form [75].

To further address the temporal sequence of GTO and filament formation *in vitro* tau aggregation assays with heparin facilitator were performed using thioflavin T (ThT) to monitor β -sheet formation, and AFM was used to quantify GTO and filament formation over time. During the first 4 hours of incubation there was no change in ThT fluorescence and no aggregates were detected. There was a rapid increase in GTO beginning at 6 hours that peaked at 21 hours, whereas levels of tau fibrils steadily increased until 328 hours of incubation. A temporal study of sucrose gradient purified tau species using AFM also showed that GTO were formed from monomer before the formation of fibrils. Furthermore, in the absence of heparin, concentrated sucrose gradient purified GTO formed fibrils, whereas tau in soluble fractions did not, indicating the intermediate nature of GTO in the aggregation pathway to fibrils.

Both GTO and fibril levels appeared to contribute to ThT fluorescence suggesting that both types of aggregates contained β -sheet structure. Monoclonal antibody MC1, recognizing a pathological conformational epitope in tau before the formation of NFT [76] showed affinity to tau in sucrose fractions containing GTO and tau fibrils in an immunoblot. Interestingly polyclonal antibody A11, recognizing a general conformational structure of amyloid oligomers but not fibrils [3], showed affinity to tau in sucrose fractions containing tau fibrils but not GTO. The authors claim that A11 has affinity to proteins with either β -barrel or β -sandwich structures and speculate that tau fibrils may be composed of GTO containing β -sheet structure. It is unclear why A11 antibody did not recognize free GTO as the ThT data indicate they contain β -sheet structure. However, results from the immunological approach to characterize the structures of tau aggregates do support the contention that GTO and tau fibrils have distinct conformations [11].

Targeting tau oligomers

There are a number of strategies to develop therapeutics directly or indirectly targeting tau oligomers in neurodegenerative diseases that may be generally categorized as 1) tau protein reduction, 2) inhibition of tau oligomer formation, 3) disruption of tau oligomers, and 4) inhibition of neurotoxic mechanisms of tau oligomers (Figure 2).

Tau protein reduction

A general approach to lowering levels of tau oligomers is to lower the concentration of tau protein in cells. This has been achieved in mice through genetic manipulation to provide protection from excitotoxicity [9], and by repression of a regulatable tauP301L construct to improve memory and reduce neuronal loss [6]. Although useful for laboratory experiments and proof-of-principle, these approaches may not be used directly as therapeutic strategies. RNAi reduction of total levels of tau protein, or the 4R tau isoforms disproportionately expressed in tauopathies, would require advances in the technology for delivery of RNAi to neurons *in vivo*. Manipulation of alternative splicing mechanisms is also an option for development of therapeutics targeting tau 4R isoforms [77, 78]. Upregulation of lysosomal degradation of intracellular protein aggregates by autophagy is also being considered as a therapeutic mechanism for neurodegenerative diseases involving protein aggregation [79, 80]. Currently, therapeutic approaches for reduction of tau protein appear to be most advanced in the areas of immunotherapy [8] and chaperone-mediated degradation [73, 74].

Reduction of tau pathology has been successfully achieved using active immunotherapy against a

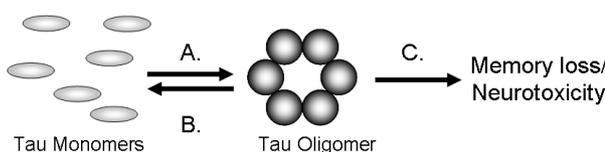


Figure 2. Drug discovery approaches. Therapeutics may be directed at inhibition of tau oligomer formation (A), disruption of tau oligomers (B) or inhibiting neurotoxic mechanisms associated with tau oligomers (C).

pathological tau conformer in transgenic mice expressing the aggregation-prone human tau P301L construct [8]. The study used active immunization against a phosphorylated tau peptide Tau379-408[P-Ser396,404] to selectively target phosphorylated tau associated with aggregates. This phosphorylated sequence also forms the phospho-epitope specifically recognized by antibody PHF-1 that was also used to evaluate treatment efficacy. The amino acids phosphorylated in the peptide are potential phosphorylation sites for kinases Cdk-5, GSK3 β and MAPKs and are C-terminal to regions characterized for formation of β -sheet structure in the repeat motifs (345) or for microtubule binding (370-380). The peptide was not fibrillogenic or toxic to cultured neuronal cells. Histology with antibodies MC1, recognizing a pathological tau conformational epitope, and PHF-1 showed that pathological tau was reduced by immunotherapy. Immunoblot analysis of insoluble and soluble tau fractions with antibody PHF-1 showed that immunotherapy increased the ratio of soluble tau to insoluble tau suggesting mobilization of tau from an insoluble form to a soluble form in treated mice. Immunotherapy improved performance of mice treated at 5 months compared to 8 months in rotarod, transverse beam and locomotor activity tests suggesting clearance of earlier-stage aggregates may be easier to clear or their clearance may be of greater therapeutic benefit. The mechanism by which intracellular tau is cleared by immunotherapy is not fully understood, but similar intracellular clearance of α -synuclein by immunotherapy was observed in a mouse model of Parkinson disease and is thought to occur by a lysosomal pathway [81].

Chaperones such as heat shock proteins (Hsp) function to maintain proteins in their properly folded state, and misfolded proteins incapable of being properly refolded are targeted by ubiquitination for proteasomal degradation. Multiple proteins interact with Hsp to regulate their activities, and recent studies have begun to elucidate mechanisms regulating the degradation of hyperphosphorylated tau protein. A protein termed 'carboxyl terminus of Hsp70-interacting protein' (CHIP) is a ubiquitin ligase that forms a complex with Hsp70 to ubiquitinate hyperphosphorylated tau for proteasomal degradation [82]. Hsp90 is involved in the folding

and stabilization of misfolded proteins. Small molecule EC102 inhibition of Hsp90 specifically reduced hyperphosphorylated tau in a mouse model through a CHIP-mediated mechanism [82, 83]. It remains to be seen whether tau reduction using this approach will modify behavior.

Inhibition of tau hyperphosphorylation

Hyperphosphorylation of tau reduces the affinity of tau to microtubules and leads to its redistribution to the somatodendritic compartment of neurons where tau can aggregate [84]. Development of tau kinase inhibitors has been the most popular strategy for targeting tau in part due to the ability to design small molecules targeting the characterized structure of the ATP-binding pockets of kinases and the availability of numerous antibodies against tau phosphoepitopes to evaluate compound efficacy, optimization and dosing using CSF samples in clinical studies [85, 86, 87]. A major challenge is the development of kinase inhibitors with an acceptable safety profile as the targeted kinases have pleiotropic functions [86]. The primary kinases being targeted are GSK3 β , Cdk5 and ERK2 that phosphorylate tau in and around the microtubule-binding domain [85]. As exposure of neurons to A β leads to activation of these kinases it is logical that upstream targeting A β may help lower their activation. However, targeting A β would be less effective if other disease mechanisms initiated or perpetuated their activation. Validation of kinases as a target is supported by the study by Le Corre and colleagues [88] that showed a promiscuous kinase inhibitor can reduce tau hyperphosphorylation and prevent motor deficits in JNPL3 mice expressing human tau P301L. Significantly, this treatment reduced soluble tau aggregates but not NFTs providing further support for the notion soluble oligomers are the toxic aggregates of tau. Similarly, tau kinase inhibitors were able to protect synapses in hippocampal slices transduced with the human tau P301L constructs despite insoluble tau accumulation [89].

Disruption of tau oligomers and inhibition of their formation with small molecules

In vitro tau aggregation assays using anionic facilitators for selection of inhibitor compounds have been developed primarily for development

of therapeutics targeting tau filaments [25, 90, 91, 92, 93]. Assays for detecting filaments include methods based on thioflavin S or thioflavin T fluorescence indicative of β -sheet structure, light scattering, sedimentation, and electron microscopy. Epitope-tagged tau protein was used to evaluate antibody-based assays DELFIA and Alpha Screen, but these assays were more complicated and less reproducible than the thioflavin T assay [93]. Thioflavin assays may also have potential for detection of tau oligomers because tau self-interaction also involves formation of β -sheet structures in the hexapeptide motifs [94]. However, light scattering and sedimentation assays would not be effective due to the small size of the oligomers. Phenothiazines such as methylene blue have been found to inhibit tau aggregation *in vitro* [90, 91, 92, 93]. Interestingly, methylene blue also inhibits oligomerization of A β but does so by promoting fibril formation [95]. Presently, methylene blue is being evaluated for treatment of AD in clinical trials. This compound was originally synthesized for use as a textile dye before AD was first described over 130 years ago but has multiple medical applications, as well. For example, methylene blue inhibits the synthesis of nitric oxide providing neuro- and cardioprotection in the treatment of vasodilatory shock [96, 97] and protects kidney from damage due to septic shock [98]. It may be likely that the observed neuroprotective activity of methylene blue derives from both its inhibition of tau aggregation and its inhibition of oxygen radical formation similarly to curcumin, which inhibits A β aggregation and oxidative damage [99].

Development of novel antibodies specific for tau oligomers would enable use of ELISAs to screen compounds *in vitro* and to evaluate their efficacy *in vivo*. These antibodies may also be useful for passive immunotherapy. Presently, there are ELISA approaches that may be helpful for detecting tau oligomers *in vitro* [100]. An ELISA technique to detect small protein aggregates may be useful for distinguishing tau oligomers from tau monomers which uses the same monoclonal antibody for capture and detection so that only multimeric tau complexes containing more than one epitope are detected [101, 102, 103]. Use of a tau-specific antibody for capture and the polyclonal

A11 antibody against protein oligomer structure [2] for detection may also be helpful for evaluating tau oligomers. However, caution is warranted when interpreting these ELISAs using biological specimens as heterogeneous protein complexes of tau with other proteins may generate false positive signal.

Cell assays for tau oligomerization

Cell-based assays have been developed for studying the large insoluble tau aggregates [104, 105, 106]. However, as tau oligomers are becoming accepted as the acutely toxic structures of tau protein, cell assays focused on monitoring early events in tau self-interaction are being designed. Gail Johnson and co-workers recently published work on three assays to study the roles of GSK3 β phosphorylation and caspase 3 cleavage on tau oligomerization in mammalian cells. The split green fluorescent protein (GFP) complementation assay, originally used to detect protein aggregation *in vitro* or in prokaryotes [107], was applied to the study of tau aggregation in human embryonic kidney (HEK) cells [108]. The basis of the assay is generation of GFP fluorescence upon association of two GFP fragments [107] so that when tau protein linked to the small GFP fragment is co-expressed with the complimentary GFP fragment the GFP fluorophore is formed. Soluble tau oligomer formation led to increased fluorescence, whereas subsequent formation of insoluble aggregates resulted in sequestration of the small GFP fragment, inhibition of complementation, and reduced fluorescence. The aggregation-prone Δ K280 tau construct exhibited low complementation efficiency, whereas the aggregation-resistant construct, with prolines substituted for isoleucine residues in the hexapeptide motifs (I277P; I308P), showed high complementation efficiency. Phosphorylation of full-length tau by GSK3 β resulted in increased GFP signal indicating tau oligomerization without the formation of insoluble aggregates, whereas similar phosphorylation of a tau construct pseudophosphorylated at S396/S404 led to decreased GFP intensity and formation of sarkosyl-insoluble aggregates [108].

Another fluorescence-based assay for tau oligomerization was developed using the technique of fluorescence resonance energy transfer (FRET)

microscopy. In this assay, at nanometer-scale distances, energy is transferred from the donor fluorophore, cyan fluorescent protein (CFP), to the acceptor fluorophore, yellow fluorescent protein (YFP), so that yellow fluorescence can be used to measure levels of donor and acceptor fluorophores in close proximity. Fusion constructs of CFP and YFP to tau constructs were co-transfected into HEK cells and the effect of GSK3 β phosphorylation on tau self-association was monitored. Phosphorylation of the tau constructs by GSK3 β led to increased levels of tau oligomers that did not progress to insoluble aggregates [109].

The β -galactosidase (β -gal) complementation assay [110, 111] was also established for studying tau self-association in HEK cells [112]. Mutant constructs of β -gal that become active upon complementation were fused to tau constructs so that tau self-interaction reconstituted β -gal activity. Chemiluminescent detection of β -gal activity showed that inhibition of GSK3 β with lithium treatment reduced chemiluminescence indicating that phosphorylation by GSK3 β facilitated tau-tau association [112]. Although the same principles used in these cell-based assays may also be used in the design of higher throughput *in vitro* assays the cellular environment provides a better approximation of tau self-association *in vivo* and may also be formatted for high throughput analyses.

Targeting neurotoxic mechanisms associated with tau oligomers

Multiple unanswered questions remain regarding the role of tau oligomers in the etiology of AD and other tauopathies. The oligomeric structures relevant to disease still need to be defined, as well as, the mechanisms of toxicity. Tau oligomer size has been roughly estimated by sarkosyl solubility, ultracentrifugation, size exclusion chromatography, SDS-PAGE [10, 12] and AFM [11]. SDS-PAGE provides the highest resolution of oligomers but may not indicate the native oligomerization state of tau as it denatures protein structure and alters protein interactions, therefore, chemical cross-linking prior to SDS-PAGE may help to provide better characterization of the native complex [113]. Protein heterogeneity in tau oligomers

isolated from biological specimens has not been ruled out further complicating analysis.

Work highlighting the role of tau in excitotoxicity [9] begins to address the issue of mechanism. M1 and M3 muscarinic receptors mediate toxicity to cultured neurons by soluble extracellular tau [54], but most tau protein and tau oligomers are intracellular, and the mechanisms by which they mediate toxicity are not clear. Sequestration of normal tau and other microtubule associating proteins by hyperphosphorylated tau into aggregates has been proposed as a mechanism for tau toxicity [114]. Redistribution of tau from microtubules to the somatodendritic compartment enables multiple aberrant tau interactions that may have toxic effects; characterization of these interacting proteins would help determine mechanisms for tau oligomer-mediated neurotoxicity.

Targeting tau loss of function

Tau hyperphosphorylation and redistribution from the axon to the somatodendritic compartment leads to loss of tau microtubule-stabilizing function. Microtubule-stabilizing therapeutics are being developed to compensate for loss of this deficit using microtubule-stabilizing compounds such as paclitaxel derivatives [115]. Clinical trials are evaluating a microtubule-interacting peptide NAPVSIPQ in patients with amnesic mild cognitive impairment. Intranasal delivery of the peptide in the triple transgenic mouse model developing both tau and A β pathologies [116] reduced levels of both hyperphosphorylated tau and A β when administered at an early pathological stage [117] and at an increased dose enhanced cognitive function and reduced tau pathology at a later pathological stage [118]. Apparently, targeting tau loss of function also indirectly targets tau aggregates associated with tau gain of toxicity.

CONCLUSION

Multiple experimental models indicate that soluble tau oligomers are the aggregates of tau protein most closely associated with behavioral deficits and neurodegeneration. Better characterization of the specific oligomers causing neurodegenerative diseases and their neurotoxic mechanisms is necessary. High-resolution structural analysis of tau oligomers is challenging as tau is a natively

unfolded protein, but if successful would provide the basis for rational drug design to inhibit tau oligomer accumulation. Programs targeting tau hyperphosphorylation, tau degradation, and perhaps tau filaments may also yield therapeutics active against tau oligomers. Progress is now being made in developing cell assays that can be used to target tau oligomers, as well as, in animal models of tauopathy for pre-clinical evaluations of compounds. The development of tau oligomer-specific antibodies will enable biomarker assays to test compound efficacy in vivo and may have therapeutic benefit for passive immunotherapy. Targeting tau loss of function may also indirectly inhibit tau oligomers formation.

Significant advancements have been made in understanding the molecular basis for tau involvement in Alzheimer's disease and other tauopathies by collaboration of multiple laboratories worldwide. Recent work identifying tau oligomers as acute pathological structures of tau protein has led to the development of new approaches in this field. Unraveling the molecular mechanisms for tau in neurodegeneration is creating novel targets for disease intervention. Application of this knowledge for therapeutic discovery and development will significantly advance the goal of modifying the course of numerous neurodegenerative diseases based on tau pathologies.

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