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Advances in Therapeutics for Neurodegenerative Tauopathies: Moving toward the Specific Targeting of the Most Toxic Tau Species

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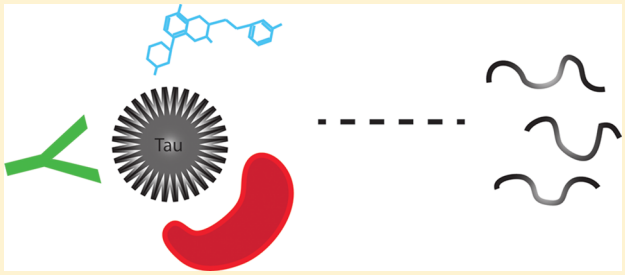
ABSTRACT: Neurodegenerative disease is one of the greatest health concerns today and with no effective treatment in sight, it is crucial that researchers find a safe and successful therapeutic. While neurofibrillary tangles are considered the primary tauopathy hallmark, more evidence continues to come to light to suggest that soluble, intermediate tau aggregates—tau oligomers—are the most toxic species in disease. These intermediate tau species may also be responsible for the spread of pathology, suggesting that oligomeric tau may be the best therapeutic target. Here, we summarize results for the modulation of tau by molecular chaperones, small molecules and aggregation inhibitors, post-translational modifications, immunotherapy, other techniques, and future directions.



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TAU IN NEURODEGENERATION

Neurodegenerative disease is a leading cause of death and disability in the elderly population. There are currently no effective therapeutics for any neurodegenerative disorders, and as life expectancy continues to rise, the number of those affected will only grow. A number of neurodegenerative disorders are associated with the microtubule-associated protein, tau. These diseases are collectively termed tauopathies and include Alzheimer's disease (AD), Parkinson's disease (PD), Lewy body dementia (LBD), progressive supranuclear palsy (PSP), Pick's disease, frontotemporal dementia (FTD), corticobasal degeneration, and even traumatic brain injury. A common histopathological hallmark of these diseases is the aggregation of tau protein, leading to accumulation of fibrillar tau deposits or neurofibrillary tangles (NFTs).[1]

Currently, the only therapeutics on the market for the most prevalent neurodegenerative tauopathy, AD, are acetylcholinesterase inhibitors and *N*-methyl-D-aspartate (NMDA) receptor antagonists. These drugs are capable only of treating the symptoms of AD, but they are ineffective at slowing the progression of the disorder. Much of the research investigating therapeutics for AD thus far has been focused primarily on amyloid-β (Aβ); however, tau pathology correlates with disease symptoms far better than Aβ. Even more striking was the discovery that Aβ treatment does not induce toxicity in the absence of tau protein in neuronal culture and in vivo.[2−6] Aside from Aβ toxicity, tau knockdown also reversed cognitive deficit associated with diabetes[7] and reduced seizure activity.[8,9] Therefore, tau represents one of the most promising therapeutic targets for AD and a number of other neurodegenerative diseases.[10] However, while tau knockout mice are able to survive and reproduce without obvious impairment,[11] the nonspecific knockout of total tau protein has been shown to cause deficits of its own. Tau knockdown can induce microtubule disorganization,[12] axonal growth deficiencies and degradation,[13,14] and may even induce a form of neurodegeneration.[15,16] These results highlight the importance of specifically targeting only the most toxic forms of tau for the treatment of tauopathies.

TAU OLIGOMERS AS A THERAPEUTIC TARGET

In its native state, tau exists as an unfolded monomeric protein, primarily bound to microtubules. It has an important role in the cell, both in stabilizing microtubules and in controlling neurite growth.[17,18] In addition, recent studies suggest that tau may be involved in learning during development.[19] *Tau* is alternatively spliced in exons 2, 3, and 10, forming six different tau isoforms. Exon 10 splice products are particularly prone to mutations, which may affect the aggregation of tau, forming three isoforms with three microtubule binding repeats (3R tau) and three isoforms with four microtubule binding repeats (4R tau).[20] While NFTs have historically been considered the main hallmark in tauopathies, they do not appear to be the main toxic species in disease. Cell death occurs in disease prior to the formation of NFTs,[21−24] and NFT-containing neurons have been shown to be functionally intact in vivo.[25] Moreover, NFTs do not affect signaling cascades involved in long-term potentiation and memory formation.[26] Tau transgenic animal models acquire behavioral deficits, synaptic dysfunction, and cell death in the absence of NFT formation.[27−33] Therefore, it

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is likely that intermediate tau species that form between tau monomers and NFTS (tau oligomers) are responsible for the onset of disease.

Tau oligomer levels have been shown to be elevated and correlate with the onset of clinical symptoms in AD and PSP brains.[34−38] Additionally, when administered to wild-type mice, tau oligomers, but not tau monomers or tau fibrils, induce synaptic and mitochondrial dysfunction and cognitive deficits.[39] The disordered, high energy state of monomeric tau, when released from binding to microtubules, allows for the exposure of hydrophobic patches and the formation of intermolecular contacts, leading to the misfolding and aggregation of tau monomer in disease. Tau fibrils that comprise NFTs are composed of hyperphosphorylated tau[40,41] in cross β-sheet conformation common to amyloid fibrils[42] and take on two major forms: paired helical filaments (PHFs) and straight filaments. Tau fibrils are known to be capable of seeding the fibrillization of tau monomer by template-assisted growth, whereby monomers that come into contact with the tau filament are integrated into parallel β-sheet structure.[43] Experimentally, fibrillization does not occur spontaneously and therefore the mechanism of aggregation onset in disease has remained mysterious. However, the formation of tau fragments composed of only the microtubule binding repeat region does lead to spontaneous aggregation in vitro,[44] as does the addition of polyanionic compounds and free fatty acids.[42,45−49] Evidence suggests that tau oligomers, rather than fibrils, may actually be the most efficient seeds for tau aggregation, through a process called oligomer-nucleated conformational induction[50−53] in which the tau monomer is first converted to an oligomeric state prior to the formation of fibrils. Post-translational modifications and the formation of disulfide bridges increase the ability of tau to form oligomers.[54,55]

The seeding of tau with brain-derived tau oligomers results in highly toxic species capable of propagating from affected to unaffected regions in cells and mice; however, fibrils are not able to propagate or induce toxic effects.[37,56,57] These results suggest a prion-like mechanism for the spread of tau pathology dependent upon oligomeric tau.[58] In spite of evidence suggesting that tau fibrils are not the most toxic species in disease and may even be neuroprotective, much of the current research on tauopathy treatment has been focused on fibrillar tau. Nevertheless, new avenues for treatment of tau oligomers, in particular tau therapeutics targeting extracellular tau, are a novel and exciting route for treatment as they may be able to slow or stop the spread of disease.[58−60]

TAU MOLECULAR CHAPERONES

Proteostasis is largely mediated by the presence of proteins known as molecular chaperones, which aid in the folding and stabilization of protein conformation. As chaperones are often upregulated in stress, they are frequently referred to as heat shock proteins (Hsps).[61] Hsps have been found to be present in increased levels in the brains of AD patients.[62−64] In a healthy system, misfolded, nonfunctional proteins will generally either be targeted for refolding by chaperones or targeted for degradation by the ubiquitin proteasome system (UPS). The Hsp90, Hsp70, and small Hsp families are three classes of chaperones commonly implicated in protein folding relevant to neurodegenerative tauopathies. Hsp70 proteins interact with hydrophobic patches in proteins early in the folding process,[65] while Hsp90 proteins act in the late stages of the folding pathway.[66] The carboxyl terminus of the Hsp70-interacting protein (CHIP) has a dual role in proteostasis, both as a cochaperone for Hsp70 and as an E3 ubiquitin ligase, ubiquitinating Hsp70-bound proteins and allowing it to control the balance between folding and degradation in protein quality control.[67,68] Tau has been shown to be a substrate for Hsp70, Hsp90, and CHIP.[69]

A wealth of research has been completed investigating the function of Hsps in regulating tau, in the context of potential therapeutics. CHIP has been found to mediate the ubiquitination of tau, acting in opposition to Hsp70. While CHIP leads to increased levels of insoluble tau aggregates, Hsp70 reduces levels of hyperphosphorylated, insoluble tau.[69] In addition, both Hsp70 and Hsp90 were found to decrease levels of tau accumulation, while increasing the association of the protein with microtubules in cells. Moreover, in mice expressing mutant tau protein and in AD brains, levels of the chaperones were inversely correlated with levels of insoluble tau aggregates.[70] However, the results from these studies inspire the question of whether the targeting of large fibrillar aggregates affects levels of toxic soluble tau intermediates.

Using P301L mutant tau mice crossed with CHIP-knockout mice, Dickey et al. showed that while levels of soluble phosphorylated tau and caspase-3 activity, which leads to increased tau aggregation, were both increased when CHIP ubiquitination function was lost, NFTs do not accumulate in these mice, suggesting that CHIP may function to target soluble, oligomeric tau species and that NFT accumulation may actually be neuroprotective.[71,72] CHIP has also been shown to lower levels of histone deacetylase 6 (HDAC6), which, when inhibited, increases the ubiquitination of Hsp90-bound proteins.[73] Direct inhibition of HDAC6 with tubastatin lowered levels of total tau, correlating with memory improvement in tau transgenic mice.[74] Hsp27 was also found to induce clearance of aberrant phosphorylated tau by a ubiquitin-independent mechanism.[75] Hsp90 can both stabilize tau binding to microtubules and lead to the degradation of tau. Therefore, while Hsp90 was initially considered to be beneficial for inhibiting tau aggregation, evidence that it may induce the selective degradation of nontoxic insoluble tau aggregates suggests that it may lead to stabilization of toxic, disordered conformations of tau.[76] This hypothesis led to the testing of the therapeutic potential of inhibiting Hsp90 activity, allowing for the release of toxic tau species from the chaperone, thereby allowing improved targeting for degradation as well as upregulating CHIP and Hsp27 activity.

As Hsp90 inhibitors are frequently too large for adequate penetration through the blood−brain barrier (BBB), multiple low-molecular-weight inhibitors were derived for testing. In cellular models, inhibitors were shown to lower levels of tau phosphorylated at specific sites and in a misfolded conformation associated with toxicity.[77] Additionally, peripheral administration of the Hsp90 inhibitor, EC102, in human tau (Htau) mice (overexpress non-mutated human tau with no endogenous mouse tau expression) led to targeted degradation of phosphorylated tau.[78] The most thoroughly investigated Hsp90 inhibitor, geldanamycin, was shown to decrease levels of insoluble tau through targeting to the proteasome[69,79] as well as to inhibit tau phosphorylation through downregulation of extracellular-signal-related kinases (ERK).[80] However, clinical testing of geldanamycin was halted due to the finding that it induced liver toxicity,[81] leading to the development of the analogue, 17-AAG, which was shown to lower levels of tau as

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well as its phosphorylation in vitro.[80,82] In tau-expressing drosophila larvae, 17-AAG and another Hsp90 inhibitor, radicicol, which also increases Hsp70 and Hsp40 activity, were shown to decrease levels of total tau. However, treatment had no effect on tau-dependent locomotor deficits,[83] although it has been shown to reverse cognitive deficit in mice by lowering soluble Aβ.[84] Other Hsp90 inhibitors, novomiocin and KU-32, which were found to be capable of crossing the BBB and inducing fewer toxic side effects, mediate neuroprotection against Aβ in cells, suggesting they may be effective against tau, but this has yet to be evaluated.[85−87] Alternatively, FK506 binding protein, which complexes with Hsp90 to prevent the degradation of tau yields increased oligomerization of tau and accelerated onset of neurodegeneration in tau transgenic mice, suggesting that therapeutics inhibiting this interaction may be worth investigation.[88]

A challenge in the therapeutic application of Hsp90 inhibitors is the wide range of effects that these drugs may have, as Hsp90 is ubiquitously expressed. Inhibiting Hsp90 leads to upregulation of the heat shock transcription factor, Hsf1, which in turn leads to increased expression of multiple different Hsps[89,90] and could be beneficial,[91] but it could also lead to unintended side effects that could act in opposition to the inhibition of Hsp90. Therefore, much of the upcoming research is focused on targeting cochaperones of Hsp90 in an attempt to yield more specific effects.[92] Withaferin A, which inhibits the cochaperone of Hsp90, Cdc37, was shown to decrease tau aggregates in mice as well as to increase Hsp70 and Hsp27 levels.[83] Additionally, the cochaperones FKBP51, BAG2, and CHIP may be effective targets, although pharmacological agents targeting them have yet to be thoroughly tested.[69,93,94] Hsp90 and Hsp70 appear to compete for binding to tau,[95] and most Hsp90 inhibitors also modulate Hsp70, which may underlie beneficial effects. Therefore, the specific modulation of Hsp70 has also been investigated.

Similar to that for Hsp90, the interaction of tau with Hsp70 is complex. Hsp70 has been shown to reduce tau fibril accumulation,[70] but it has also been found to preferentially bind to oligomeric tau,[96] inhibiting fast axonal transport deficiencies in squid axons.[97] Therefore, the therapeutic potential of both Hsp70 inhibitors and activators has been investigated. In contrast to studies showing that decreases in tau correlated with increased activation of Hsp70,[69,70,83] Hsp70 inhibitors, YM-08 and methylene blue, were shown to decrease tau levels and toxicity both in vitro and in vivo.[95,98−100] The systematic evaluation of both activators and inhibitors of Hsp70 shed some light on these apparently contradictory results. Activating Hsp70 may lead to an apparent reduction in levels of tau due to increased tau bound to the chaperone, while inhibiting the ability of Hsp70 to refold tau may lead to increased degradation of tau complexed to the protein, thereby suggesting that using a combination treatment in which Hsp70 is first increased in order to sequester tau and then its ATPase activity is inhibited in order to target tau−Hsp70 complexes for breakdown may be most effective.[101] Moreover, different members of the Hsp70 family appear to process tau differently, with Hsp72 targeting tau for degradation and Hsc70 retaining tau and aiding in its native function stabilizing microtubules.[102,103] Tau clearance may also depend upon the DnaJ-binding domain of Hsp70 chaperones.[104] Therefore, cochaperones that guide tau to be degraded, rather than sustained, may be useful.

While animal Hsps are useful in the degradation of amyloids, they are ineffective at rapid disaggregation. Hsp104 is a chaperone specific to bacteria, fungi, protozoa, chromista, and plants and has been shown to be efficacious at disaggregating amyloid proteins, including toxic oligomers.[105,106] Hsp104 has also been shown to be safe for use in animal models and is capable of disassembling α-synuclein oligomers and protecting dopaminergic neurons in a rat model of Parkinson's disease.[107] Hsp104 could theoretically be optimized for use against any amyloid protein, including tau,[108] as it dissolves aggregates from different proteins using varied mechanisms.[109] Therefore, it may be an exciting option for specific targeting of toxic tau in future therapeutics.

In addition to the clearance of tau by the UPS, autophagy and lysosomal pathways may also degrade toxic tau protein.[110] Trehalose is a molecular chaperone present in invertebrates and is an autophagy enhancer in vertebrates. The disaccharide was found to effectively decrease soluble tau levels through the upregulation of autophagy as well as to directly inhibit aggregation, resulting in a decrease in toxicity.[111] The use of other non-Hsp chaperones has also been tested for tau clearance. Protein disulfide isomerases (PDIs) are molecular chaperones specific to the endoplasmic reticulum that have been shown to be upregulated in neurodegenerative disease. PDIs induce proper protein folding through the breakage and formation of disulfide linkages.[112] PDI has been shown to be colocalized with NFTs in AD brains.[113] Analysis of PDI effects in vitro show that it inhibits tau fibrillization.[114] However, the problem remains that the effects of the chaperone on oligomerization are unknown. A molecular chaperone identified in drosophila, nicotinamide mononucleotide adenylyltransferase (NMNAT), degraded phosphorylated tau oligomers through the UPS as well as decreased apoptosis and reversed behavioral and morphological deficiencies in a drosophila tauopathy model.[115]

NATURALLY-OCCURRING SMALL MOLECULES AFFECTING TAU

Research of the health benefits of naturally occurring products has led to the discovery of multiple small molecules that affect tau, known as polyphenols. Polyphenols appear to interfere with the misfolding of multiple amyloid proteins through the disruption of π-stacking and β-sheet formation important for fibrillization.[116] Polyphenols include a class of chemicals known as flavonoids, of which myricetin, curcumin, and (−)-epigallocatechin-3-gallate (EGCG) have been demonstrated to interact with tau as well as with Hsps. Myricetin lowers tau levels through inhibition of Hsp70,[117] while curcumin, a compound found in turmeric, may act independently of Hsp activity. Curcumin was found to inhibit Aβ aggregation as well as to decrease levels of tau hyperphosphorylation in cells and mice through the Akt/GSK-3β pathway and the upregulation of proteins shown to clear tangles, BAG2 and LAMP1.[118−120] Curcumin was shown to be capable of binding to fibrillar tau in brain sections from multiple tauopathies with comparable binding to that of common markers of NFTs, Thioflavin S, AT8, and others.[121] However, none of these studies specifically assessed the effects of curcumin on toxic tau oligomers. A recent study showed that Htau mice treated with curcumin exhibited improvement in cognitive tasks, synaptic dysfunction, and alterations in Hsps associated with a decrease in tau dimers, but not tau monomer or insoluble tau,[122] suggesting that curcumin's effects may be specific to toxic intermediates. Curcumin's ability to cross the BBB makes it a potential candidate for therapeutics.[123] However, it does have limitations,

including low bioavailability and poor solubility in water. In a clinical trial of curcumin in AD patients, no evidence of improvement was seen.[124] In order to overcome these shortcomings, curcumin derivatives were created, showing increased efficacy in inhibiting tau fibrillization at a 10−1000-fold lower concentration than that of curcumin alone. The addition of a sugar group was able to increase solubility in water.[125,126] EGCG, a flavonoid found in green tea, has also been found to decrease tau phosphorylation, cognitive deficit, and toxicity in APP transgenic mice.[127]

In cellular and mouse models of tauopathies, resveratrol, a polyphenol found in grapes and red wine, reverses cognitive deficits and toxicity[128] through the activation of SIRT-1, a deacetylase that inversely correlates with tau accumulation in disease.[129] Resveratrol inhibits tau hyperphosphorylation and prevents memory deficits in vivo through the inhibition of GSK-3β activity.[130−133] Resveratrol has also been shown to be capable of converting other amyloid oligomers into nontoxic conformations and inducing their degradation.[134,135] While resveratrol appears to be safe for use in human subjects, its bioavailability is very low.[136]

Olive oil intake has been known to be associated with health benefits for many years, leading to the investigation of polyphenols found in olives, including oleuropein, oleocanthal, oleuropein aglycone, and hydroxytyrosol. All four were capable of inhibiting tau aggregation in vitro, likely through the binding of aldehydes to the microtubule binding repeat region of tau responsible for aggregation, thereby locking tau in an unfolded monomeric state incompetent to fibrillization.[137,138] While oleuropein does not appear to be capable of crossing the BBB, its metabolite hydroxytyrosol efficiently enters the brain.[139]

■ TAU AGGREGATION INHIBITORS

It has been suggested that aggregation inhibitors and molecular chaperones could work synergistically in therapeutics, creating a more effective treatment together than individually. Alone, aggregation inhibitors may lead to a dramatic increase in total monomeric tau, which could potentially mediate toxicity separately from aggregation by disrupting axonal transport and destabilizing microtubules.[140] By allowing the two treatments to work synergistically, aggregation inhibitors could increase levels of low-molecular-weight tau species, which may bind more efficiently to chaperones, allowing them to be targeted for degradation.[141]

Multiple tau aggregation inhibitors have been discovered due to their established inhibitory properties on the aggregation of other amyloid proteins. Porphyrins, including pthalocyanine tetrasulfonate (PcTS), have been studied for therapeutic and anti-aggregatory effects in prion diseases.[142] PcTS was proposed as a viable therapeutic in AD, having been shown to inhibit Aβ oligomer formation[143] as well as to block formation of tau aggregates and disassemble tau filaments.[144] However, research into the mechanism of PcTS interaction with tau fibrils showed that the compound stabilizes soluble oligomeric tau species, suggesting that the compound may actually increase toxicity.[145] Additionally, Exebryl-1, created by Proteotech, was approved to enter phase I clinical trials and was found to inhibit the aggregation of both Aβ and the microtubule-binding repeat domain of tau, apparently through the binding to tau monomer and inhibition of the conversion to β-sheet structure from random coil.[146] A third class of inhibitors, benzothiazole aniline (BTA) compounds, was originally recognized for their protection against Aβ toxicity but has recently been suggested for potential anti-tau aggregation efficacy.[147]

Another promising class of aggregation inhibitors, phenothiazines, has been tested for therapeutic potential in a variety of medical fields. Investigations of the aggregation inhibitory properties of the phenothiazine, methylene blue, have yielded conflicting results that may be due to its pleiotropic nature. Many studies have shown anti-aggregation effects of methylene blue through the stabilization of monomeric conformation by modulation of cysteine residues.[148−150] Methylene blue also decreases tau pathology in vivo when administered to P301L mice[151] and attenuates toxicity in a *Caenorhabditis elegans* tauopathy model.[152] However, behavioral deficits and tau pathology were unaffected in a P301L zebrafish model treated with methylene blue.[153] Notably, in contrast to many other aggregation inhibitors, when administered to multiple tauopathy mouse models methylene blue was shown to reduce levels of tau oligomers specifically, without affecting levels of NFTs.[100,154,155] The pleiotropic nature of methylene blue may explain conflicting results and calls into question whether benefits are solely due to inhibition of aggregation or whether they may also be due to other factors, including antioxidant, energy metabolism, and inflammation benefits.[156] In addition, methylene blue has been shown to modulate levels of tau through increasing autophagy[100] and proteasome activity.[157] Methylene blue has progressed to phase II clinical trials under the name Rember, showing evidence of cognitive improvement.[158]

A number of other aggregation inhibitors have yielded promising preclinical results for decreasing tau toxicity. *N*-Phenylamines inhibit tau aggregation as well as dissolve aggregates in cellular models, leading to reduction in toxicity. Because toxicity in this cellular model was found to correlate best with levels of oligomeric tau, it is possible that *N*-phenylamines are capable of interfering with tau aggregation prior to the formation of fibrils; however, this has not been directly tested.[159,160] Phenylthiazolylhydrazides attenuate tau fibrillization and disassemble aggregates in vitro and in cells, leading to increased cell viability.[161,162]

Anthraquinones, including emodin, daunorubicin, adriamycin, and others, are effective tau aggregation inhibitors, both preventing the formation of filaments and dissolving preformed filaments. The abundant ring structures in these compounds are thought to interfere with the formation of β-sheet structure. Furthermore, anthraquinone compounds do not interfere with native tau microtubule binding and protect against cytoxicity in cellular models of tauopathy.[163]

Aminothienopyridazines (ATPZ), including a class of compounds derived based on their anti-aggregatory properties as well as their ability to cross the BBB when orally administered in mice, have been proposed for treatment. ATPZ compounds effectively inhibit the formation of tau fibrils in vitro similar to that of methylene blue, through cysteine oxidation and the inhibition of the formation of disulfide linkages, rendering tau incapable of fibrillization, and they also reverse the motor phenotype in a *C. elegans* tauopathy model.[149,152,164,165] However, analysis of levels of specific tau species treated with ATPZ compounds shows that they may be more effective at preventing fibrillization than oligomerization and may actually increase tau oligomer levels due to breakdown of larger aggregates.[166]

Rhodanines are a group of compounds that are well-tolerated in humans[167] and may have clinically relevant anti-tau

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Table 1. Tau Kinase Families

Proline-Directed Protein Kinases (PDPK)	Non-PDP Kinases	Tyrosine Kinases
Glycogen synthase kinase-3 ß (GSK3ß)	Tau-tubulin kinase ½ (TTBK½)	Src family kinases • Src • Fyn
Cyclin-dependent kinase-5 (CDK5)	Casein kinase 1a/1d/1e/2 (CK1a/1d/1e/2)	c-Abl
Mitogen-activated protein kinases (MAPK) • ERK • JNK	Dual-specificity tyrosine phosphorylation and regulated kinase 1A (DYRK1A)	
	Microtubule affinity-regulating kinases (MARKs)	
	Protein kinase cAMP-dependent A/B/C/N (PKA, PKB/Akt, PKC, PKN)	
	Ca2+/Cal-Modulin-dependent protein kinase II (CaMKII)	

aggregation properties. Rhodanines were shown to both inhibit tau aggregation and disassemble tau filaments in vitro, without yielding any toxic effects in cells.[168] The rhodanine, bb14, prevented tau pathology development in a hippocampal slice model as well as protected against Ca^{2+} dyshomeostasis, dendritic spine loss, and cell death.[169]

Intermolecular disulfide bonds formed by tau cysteine residues have repeatedly been shown to induce tau oligomerization.[170−173] Mutations inhibiting the formation of disulfide bridges prevented the formation of oligomers,[49] suggesting that disulfide cross-linking may be a valid target for blocking tau aggregation. A rosamine derivative specific for tau cysteine thiol groups, TR-2, effectively inhibits the formation of disulfide bonds necessary to oligomer and fibril formation in vitro,[174] suggesting that small molecules inhibiting disulfide cross-linking bear further study using cellular models and in vivo experiments for their efficacy as aggregation inhibitors. Targeting the paired edges of β sheets in fibrils has also been suggested as an anti-aggregation therapeutic approach,[175] although this would be ineffective in the disassembly of oligomers.

The use of aggregation inhibitors for the treatment of neurodegenerative tauopathies appears to be promising, but it should be approached with caution. Because the large majority of identified aggregation inhibitors stabilize soluble oligomeric tau species,[176] when used alone, they could result in more toxicity and cognitive impairment. Therefore, more focus on the compounds that stabilize unfolded monomer or potentially large filamentous species at the expense of the toxic oligomers may be warranted. Adaptation of approaches used for Aβ to accelerate the fibrillization in order to decrease levels of tau oligomers may be an important alternative to approaches targeting the degradation of fibrils.[177] Alternatively, approaches in which aggregation inhibitors are combined with molecular chaperones in order to first dissolve filaments into small aggregates that are more effectively recognized by chaperones, and subsequently targeting these complexes for degradation, may be useful.

TAU POST-TRANSLATIONAL MODIFICATION MODULATORS

Post-translational modifications have previously been implicated in the toxic processing of tau, leading to its aggregation. The most prevalent post-translational modification of tau is phosphorylation, which is an important modulator of tau's native function. Increased phosphorylation is known to decrease tau's affinity for microtubules.[178] However, phosphorylation has a complex relationship with tau. While many studies have found that aberrant phosphorylation is associated with tau aggregation, unphosphorylated tau aggregates are also present and induce toxicity. Additionally, some researchers show that phosphorylation may not increase tau aggregation and may even inhibit aggregation.[159,179] However, these conflicting results could be explained by the multitude of tau phosphorylation sites, which can yield different effects. Studies investigating tau phosphorylation are inconsistent in the analysis of various phosphorylation sites. Evidence suggests that phosphorylation at certain sites preceding residue 208 may actually inhibit aggregation, while phosphorylation at the C-terminal region likely increases aggregation.[180−182] Phosphorylation at certain sites is believed to induce tau toxicity both by inhibiting tau affinity for microtubules and by promoting its aggregation.

Tau kinases can be divided into three groups: proline-directed protein kinases (PDPK), non-PDP kinases, and tyrosine kinases (Table 1).[183] Inhibition of tau kinases is one of the best-studied therapeutic approaches in neurodegeneration. GSK-3β inhibition is thought to be the most promising tau kinase target for therapeutics. Blocking GSK-3β with either lithium or NP12 treatment leads to decreases in tau pathology, toxicity, cognitive deficits, and neuronal death in tau transgenic mouse models.[184−188] Tau hyperphosphorylation could also result from an inhibition of tau phosphatases in disease. Levels of the most prevalent phosphatase, protein phosphatase-2A (PP2A), are diminished in AD,[189] and treatment with PP2A leads to a restoration of tau binding to microtubules,[190] making PP2A a viable therapeutic target to reduce toxic tau phosphorylation.

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The DYRK1A kinase has been shown to directly phosphorylate tau as well as to interact with phosphorylation activity of GSK-3β and phosphatase activity of calcineurin to increase tau hyperphosphorylation and NFT formation.[191−193] DYRK1A is also involved in the regulation of tau alternative splicing, potentially leading to toxicity due to an imbalance in the ratio of 3R/4R tau.[194,195] These results, combined with the ability of DYRK1A to interact with regulators of Aβ cleavage[196,197] and evidence of its elevation in neurodegenerative disease,[198] have made its inhibition a promising therapeutic target for AD. However, due to the fact that DYRK1A plays a critical role in the modulation of multiple signaling pathways, its inhibition should be approached with caution, and the goal of treatment must be to decrease levels of DYRK1A only to those seen in healthy controls.[199] The most potent and bioavailable known inhibitor of DYRK1A is harmine, a natural compound derived from tropical plants. However, harmine results in hallucinogenic and psychoactive side effects that may make its clinical application difficult.[200] The polyphenol, EGCG, has also been shown to inhibit DYRK1A activity and may result in fewer side effects.[201,202] Quinalizarin 3 is a potent inhibitor of protein kinase C that was also found to inhibit DYRK1A, but its low promiscuity score suggests it may not be useful for targeting DYRK1A.[203] Other potential natural DYRK1A inhibitors that may be useful as starting points for therapeutic agents include the peltogynoid flavonoids, benzocoumarin 5a, staurosporine, and analogue 8. Additionally, synthetic compounds, pyrrazolidine-3,5-diones, quinazolines, benzothiazoles, meriolins, meridianins, 3-(6-hydroxyindol-2-yl)-5-(phenyl)-pyridine and pyrazine analogues, chromenoindoles, and 3,6-diamino-1*H*-pyrazolo[3,4-b]-pyridines, have all shown anti-DYRK1A activity.[199]

The Src kinase Fyn is known to be associated with Alzheimer's disease and is well-documented to phosphorylate tau at tyrosine residues associated with disease.[204] Moreover, Aβ oligomers have been shown to bind cellular prion protein, PrP^c, complexed with Fyn, increasing tau dysfunction.[205] Tau has also been shown to bind to fyn in oligodendrocytes[206] and in neurons.[207] Disease-related missense mutations increase tau association with Fyn.[208] Because the PrP^c complex is associated and endocytosed with caveolin[209] and because under membrane stress, such as that induced by tau oligomers, caveolin dissociates and flattens, leading to an increase in endocytosis following relaxation of stress,[210] Fyn could potentially be involved in uptake of toxic tau. Therefore, targeting of Fyn could alleviate toxicity in disease by multiple mechanisms. Currently, there are a few pharmacologic compounds targeting Fyn that have shown promising results preclinically and clinically. Tyrosine kinase inhibitor, masitinib, was effective in reducing cognitive deficits in AD patients in a phase II clinical trial.[211] Inhibition of Src family kinases with saracatinib is another possibility. It is currently in phase I clinical trials and has been shown to be well-tolerated in cancer patients.[212]

The p75 neurotrophin receptor ($p75^{NTR}$) is abnormally modulated in AD, leading to the activation of tau kinases and Aβ toxicity. Small molecule inhibitors of $p75^{NTR}$, LM11A compounds, inhibit the induction of multiple different tau kinases in vitro, including GSK-3β, cdk5, and c-Jun,[213] and lower tau phosphorylation, correlating with cognitive benefits in an AD mouse model.[214]

Disruptions to insulin signaling are associated with neurodegeneration and heightened phosphorylated tau levels in AD.[215,216] This led to the testing of a type 2 diabetes drug, the glucagon-like peptide, liraglutide, for the modulation of tau phosphorylation. Liraglutide successfully protects against cognitive deficit in an AD mouse model, accompanied by a decrease in levels of phosphorylated tau, likely through the inhibition of mitogen-activated protein kinases, ERK and JNK.[217]

Phosphorylation of tau can also be inhibited by another post-translational modification, O-GlcNAcylation,[218] a form of glycosylation involving the transfer of β-*N*-acetylglucosamine, which is a glucose metabolism sensor reduced in AD brains.[219] A balance between O-GlcNAcylation and phosphorylation has been shown to exist for tau protein, impacting its cellular localization.[220] Inhibition of *N*-acetylglucosaminidase (NGase), the enzyme catalyzing the removal of β-*N*-acetylglucosamine, leads to decreased hyperphosphorylation of tau in mice.[221] O-GlcNAcylation also directly interacts with the aggregation of tau, inhibiting oligomerization and fibrillization, without affecting monomeric conformation, likely either by increasing the solubility of tau monomer or by destabilizing aggregated tau,[222] and protecting against cognitive deficit in an AD rat model.[223] Inhibition of deglycosylation therefore may be a viable therapeutic target.[224]

Another post-translational modification that appears to be important to tau pathology in neurodegenerative disease is glycation with glucose and ribose. Filamentous tau in AD brains has been found to be glycated.[225] While glycation does not induce fibrillization, it does shift the equilibrium toward increased fibril formation.[226,227] Glycated tau induces toxic effects, including the production of reactive oxygen species and Aβ.[228] Ribosylation was shown to induce oligomerization and eventual formation of globular aggregates that are toxic to neuroblastoma cells.[229] Therefore, the inhibition of glycation may effectively modulate toxic tau aggregation.

Recently, the possible acetylation sites of tau were mapped, and the effects of acetylation on heparin-induced fibril formation were determined. Although acetylation of tau was found to inhibit aggregation, it is incapable of preventing fibrillization entirely.[230] In addition to its role in the modulation of Hsps, HDAC6 is capable of modulating tau acetylation, which has also been found to inhibit tau phosphorylation and aggregation. Therefore, a BBB-permeable HDAC6 inhibitor was tested in vivo, yielding promising results for the inhibition of tau aggregation.[231] Furthermore, HDAC6 inhibition rescues defects in microtubules due to tau toxicity in drosophila models through increases in microtubule acetylation.[232] The non-specific HDAC inhibitors, crebinostat and BBB-permeable sodium 4-phenylbutyrate, enhance memory and neuroplasticity.[233,234] Importantly, tau also has self-acetyltransferase activity, indicating that modulating the ability of tau to acetylate may be an important therapeutic target.[235]

However, similar to that for phosphorylation, contradictory results for acetylated tau have been seen. This may be due to the heterogeneity of acetylated sites in tau samples used in different studies. Opposing results showed that inhibition of HDAC6 increased both acetylation and hyperphosphorylation of tau, leading to slower degradation of aggregates.[236] Moreover, acetylated tau is associated with tau aggregates in human tauopathy tissue as well as tauopathy mouse models. Acetylation at specific lysine sites also impaired tau stabilization of microtubules and increased tau fibrillization.[237] In another study, acetylation was shown to inhibit phosphorylated tau degradation.[238] Collectively, these results suggest that while

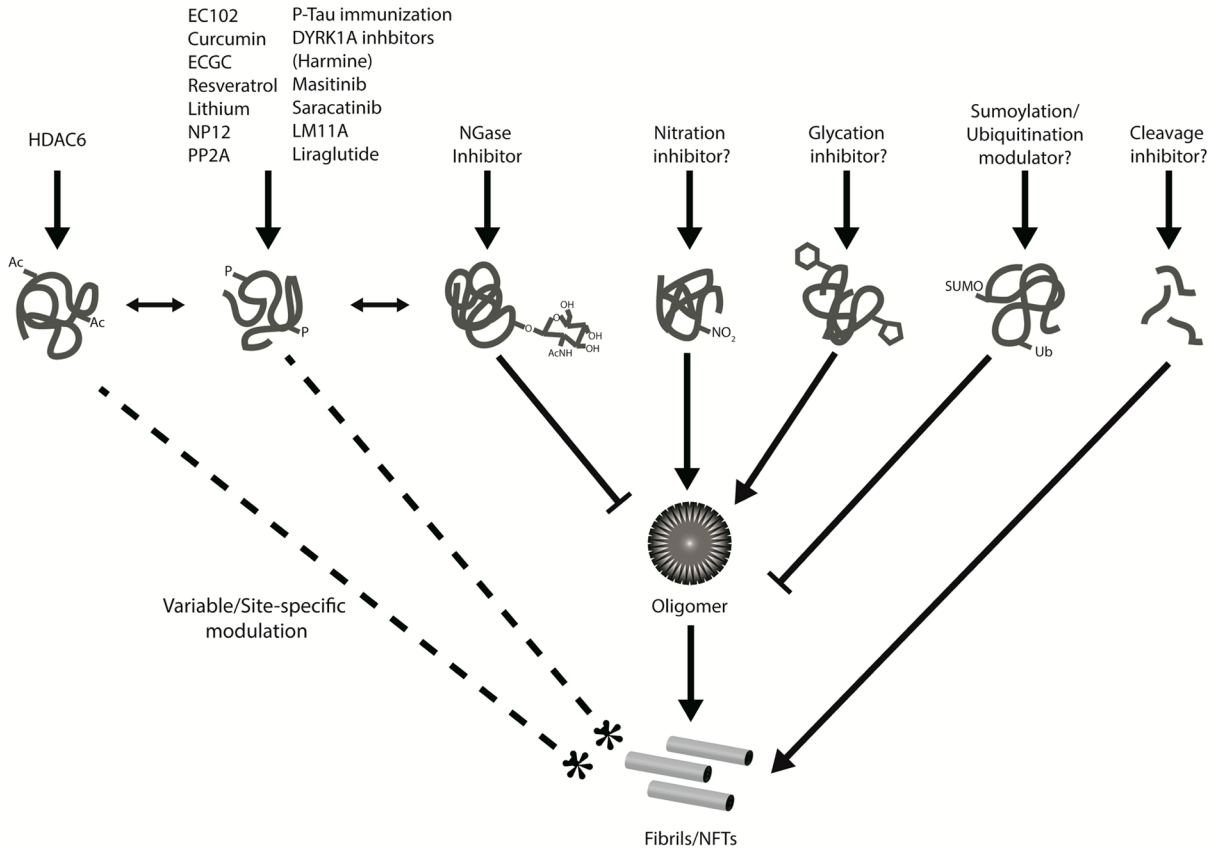

Figure 1. Summary of post-translational modification effects on tau and potential therapeutic agents. Post-translational modifications of tau appear to be important for the modulation of tau misfolding and aggregation in disease. Important modifications identified include (left to right) acetylation, phosphorylation, O-GlcNAcylation, nitration, glycation, sumoylation and ubiquitination, and tau fragmentation. Both acetylation and phosphorylation have shown conflicting results regarding the fibrillization of tau, likely due to site-specific variations in the effects on aggregation. Therefore, agents that either inhibit or activate acetylation and phosphorylation may be useful for treatment, depending on the target epitope. O-GlcNAcylation has been shown to inhibit the oligomerization of tau, leading to the development of inhibitors of NGase, the enzyme that catalyzes the removal of *β-N*-acetylglucosamine. Moreover, both acetylation and O-GlcNAcylation also modulate tau phosphorylation. Nitration and glycation appear to increase oligomerization, suggesting that inhibiting these modifications would be effective. Sumoylation and ubiquitination may process toxic tau for degradation, which could be modulated in disease. The cleavage of tau into fragments induces its aggregation and may be involved in disease.

acetylation does appear to be important in modulating tau in disease, the effect may differ drastically based on undetermined factors, including the residue. Further investigation is needed to determine the best way to target toxic acetylation. Additionally, it may be important to differentiate between effects of acetylation on fibrillization versus oligomerization, which may account for some discrepancies in results.

It has recently been reported that sumoylation (binding of small ubiquitin-like modifier proteins) is associated with tau in neurodegenerative disease.[239] While the relationship has not yet been solved, alterations in sumoylation and ubiquitination may affect degradation of tau aggregates and prove to be a viable therapeutic target in the future. Likewise, tau nitration, the addition of nitrogen dioxide to tyrosine, has been implicated in tau aggregation. Peroxynitrite, which induces nitration at specific tau residues, inhibits the ability of tau to stabilize microtubules and increases the formation of tau oligomers.[240,241] Therefore, strategies inhibiting tau nitration may reduce tau toxicity in disease.

Tau Fragmentation. Proteolytic cleavage of tau was suggested as a potential mechanism for tau aggregation early on, as the minimal component required for the formation of tau filaments is the microtubule binding repeat fragment. However, the exact mechanism of tau cleavage is unknown.[242] Tau contains many caspase cleavage sites. Caspases are known to be upregulated in neurodegenerative disease,[243] making them a likely candidate. The formation of tau fragments has been shown to precede the aggregation of tau, in a more clearly defined manner than phosphorylation.[159,160] Errors in the lysosomal processing of tau can lead to the accumulation of tau fragments, which form toxic oligomers that interact with the lysosome and inhibit its ability to degrade aggregated tau.[110] Therefore, therapeutics that aim to decrease fragmented tau, possibly through inhibition of caspases and other enzymes, as well as upregulate lysosomal processing, may be effective in neurodegenerative tauopathies.

Post-translational modifications clearly play an important role in the processing and aggregation of tau, although much more clarification is needed to understand exactly how to alter the process to reverse or prevent toxicity (Figure 1).

■ ANTI-TAU IMMUNOTHERAPY

Immunotherapeutic approaches for the treatment of AD and other tauopathies are perhaps the most promising disease-

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modifying intervention.[60] Despite the fact that tau pathology correlates better with brain atrophy,[244] most studies have been concentrated on the removal of Aβ pathology from the brain. However, the lack of positive results obtained by targeting Aβ has stimulated a growing interest in tau pathology. Several lines of investigation highlight the importance of tau protein in the pathogenesis of the disease. One of the most convincing arguments comes from previous findings indicating that Aβ toxicity is mediated by tau pathology.[30,245,246] This could explain why removal of Aβ is insufficient to stop the disease progression. Therefore, immunotherapy against abnormal tau protein seems to be an important therapeutic alternative. In the past few years, preclinical studies have shed some light on the use of tau pathology as an immunotherapeutic target. However, most of the studies thus far have been directed for specific phosphorylated tau epitopes rather than the oligomeric tau that has been demonstrated to be the most toxic species.

Immunotherapy Targeting Phosphorylated Tau. Asuni et al. demonstrated that immunization using a peptide containing the phospho-tau Ser396/404 diminishes NFT load in a transgenic mouse expressing the P301L mutation (JNPL3).[247,248] Sensorimotor deficits associated with tau pathology were reduced after treatment. Evidently, the antibodies produced by active immunization were able to enter the cell and bind to NFTs. This was demonstrated by the labeling of purified antibodies injected into other animals.[248]

In a later study, the same research group carried out preventive immunization using the same immunogenic peptide in Htau/PS1 mice. This mouse model expresses human tau and the presenilin1 (PS1) M146L mutation in a mouse tau knockout background. The early onset usually observed at 2 months of age was prevented when mice were immunized in the early stages. As demonstrated previously, a reduction of NFTs was observed in the brains of these mice.[249]

In a separate study targeting the same phospho-sites (Ser396/404) described above, a mouse model expressing the tau P301L mutation, pR5 mice, were immunized at 4, 8, and 18 months and were evaluated to determine the benefits of tau removal at different ages. Treatment at early ages slowed the progression of pathology, whereas progression was unaffected in the oldest group of mice, where NFTs appeared to be targeted. Importantly, an increase in activated astrocytes was observed only in the group of aged mice. This indicates astrocytes may be responsible for NFT clearance.[250]

A recent immunotherapeutic approach incorporated a tau peptide containing the phospho-sites (Ser396/404) into liposomes for the treatment of transgenic mice carrying the tau P301L mutation. The long-term vaccination seemed to be effective at removing phospho-tau Ser396 in the soluble and insoluble brain fractions. However, the reduction of tau could not be confirmed in tissue because the immunostaining failed to detect a difference between groups.[251]

A similar approach was conducted in a double mutant mouse (K257T/P301S) that develops NFT pathology. Mice were immunized with a combination of three peptides containing the phospho-tau epitopes, Ser202/Thr205, Ser214/212, and Thr231. The robust decrease in NFT pathology observed in the cortex, hippocampus, and brain stem was accompanied by an increase in microglial cells, although no evidence of phagocytosis was found. It seems likely that NFT reduction is mediated by a lysosomal pathway.[252]

A subsequent immunization against the pathological epitope, phospho-Ser422, was conducted in THY-Tau22 mice. This mouse model exhibits neurofibrillary tangle-like inclusions in the hippocampus and abnormal tau phosphorylation. Some of the therapeutic effects found in treated mice included a decrease in insoluble phosphorylated tau, which correlated with cognitive improvement in treated mice. Importantly, following immunization, the levels of tau were high in the blood, suggesting that a peripheral sink mechanism was involved in antibody-mediated tau clearance.[253]

Safety is one of the main concerns in immunotherapy. The use of peptides to stimulate an immunoresponse seems to have devastating consequences, especially in cognitively intact individuals. The first active immunization using full-length tau in wild-type mice induced tauopathy-like abnormalities, including NFTs, axonal damage, and gliosis, accompanied by neurological deficit.[254] However, it is not clear whether the full-length tau was responsible for the toxic effects found. In this regard, a second study addressed the safety aspects of the use of peptides for active immunization against tau. The phospho-tau epitopes Ser202/Thr205, Ser212/214, and Thr231 were administered in a NFT mouse model with E257T/P301S tau mutations and in wild-type mice. Repeated phospho-tau immunization induced encephalitogenicity in both transgenic and wild-type mice. Their findings suggest that repeated phospho-tau immunization using recombinant peptides can have safety issues.[255]

In this regard, passive immunization, the use of antibodies to treat tau pathology, seems to be superior to active immunization. Antibodies against phospho-tau (Ser396/404) have been used to immunize the JNPL3 mice described above. Repeated doses of the PHF1 (Ser396/404) antibody decreased functional impairment and also reduced levels of insoluble tau from the dentate gyrus of the hippocampus as well as the cortex.[256] In a similar manner, immunization of two tauopathy mice, JNPL3 and mice expressing the P301S mutation, using PHF1 and the conformational antibody, MC1, which detects early misfolded tau conformation, was completed. As demonstrated in previous immunizations, the treatment with both antibodies reduced a 64 kDa band from brain homogenate. The NFT burden was reduced in cortex/forebrain in the JNPL3 model but showed variable results in the P301S model. However, the spinal cord in P301S mice showed a reduction in neurospheroids in the treated group, correlating with locomotor improvement.[257]

Passive immunization against other phosphorylated sites associated with tau pathology has been completed. Recently, the phospho-tau Ser202/Thr205, recognized by AT8 antibody and known to be a marker of NFT pathology, was used to immunize the well-characterized triple transgenic (3xTg-AD) mouse. This is a good model for AD, as it contains the APP-Swedish (KM670/671NL), tau (P301L), and presenilin 1(M146 V) mutations. Treatment with AT8 reduced somatodendritic tau load with no effect on Aβ pathology.[258]

Although these immunizations seem promising, the main concern of immunotherapy against phosphorylated tau is that while these phospho-sites seem to be prominent in AD, they are not exclusive of the pathology and can also be found in functional tau.

Immunotherapy Targeting Tau Aggregates. In a recent study, three antibodies with differing abilities to block tau aggregate seeding were used to immunize P301S mice. The chronic infusion of the antibodies HJ9.3 (residues 306−320), HJ9.4 (residues 7−13), and HJ8.7 (residues 25−30) into brain lateral ventricle by osmotic pumps improved cognition in mice.

All antibodies markedly reduced hyperphosphorylated, aggregated, and insoluble tau. These findings are expected, as these antibodies have affinity for monomeric and fibrillar recombinant tau and stain neurofibrillary tangles and neuropil threads in AD brain.[259]

Immunotherapy Targeting Tau Oligomers. Our group recently studied the benefits of removing tau oligomers in the JNPL3 mouse. A single dose of our anti-tau oligomer-specific monoclonal antibody (TOMA) reduced cognitive and motor deficits associated with tau pathology in mice. Importantly, the reduction of tau oligomers had no effect on NFT load. Our findings indicated that tau oligomers were cleared from the extracellular space since the antibody did not enter the cell. It is possible that clearance of extracellular oligomers by antibodies may inhibit further uptake and thereby indirectly clear intracellular tau aggregates.[260] In a second study from our group, we found that cognitive deficit induced by injection of brain-derived tau oligomers can be prevented in an Htau mouse model. A single injection, as well as multiple doses of TOMA, was demonstrated to be effective as a preventative therapy inhibiting oligomeric tau and preserving memory function.[261] Our findings confirmed that removal of tau oligomers, without lowering levels of hyperphosphorylated NFT load or functional monomeric tau, conferred benefits to memory.

Mechanism of Antibody-Mediated Inhibition of Spreading. Although most of the immunization studies described above proved to be beneficial, many questions remain regarding the mechanism of action. While safety concerns arise from active immunization, the outlook on antibody treatments seems to be optimistic. Some of the studies indicate that antibodies are not able to enter cells, suggesting that tau pathology clearance occurs in the extracellular space (Figure 2). It seems likely that extracellular tau may function as a seed, contributing to the spread of the disease. The work from Yanamandra et al. supports this notion, as blocking the uptake of extracellular tau prevented the seeding of intracellular tau, resulting in cognitive benefits.[259] This is particularly important because immunotherapeutic approaches should be expected to stop the progression of the disease. On the other hand, the function of tau in the extracellular space is not clear. Therefore, antibodies directed for the removal of pathological tau should not compromise functional, monomeric tau. In this regard, our work has pioneered the specific removal of tau oligomers without affecting functional tau by passive immunization.

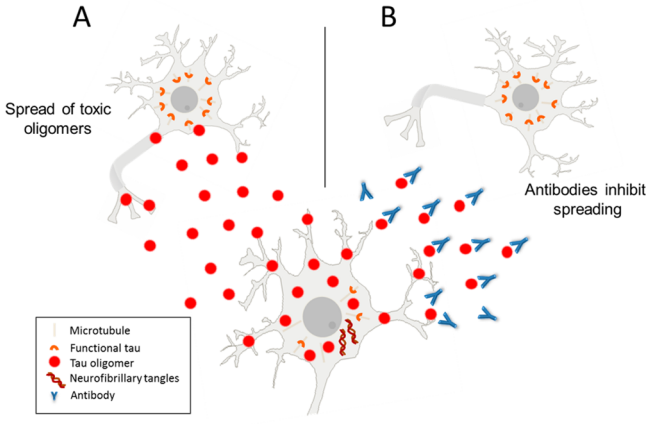

Figure 2. Mechanism of the prevention of tau oligomer spreading by antibodies. (A) Tau oligomers released from affected cells can propagate to neighboring or synaptically connected neurons, inducing the spread of disease. (B) Passive immunization with tau oligomer-specific antibodies prevents the uptake of extracellular tau oligomers responsible for the spread of pathology.

OTHER POTENTIAL STRATEGIES TARGETING TAU TOXICITY

Targeting tau loss of function, in addition to the toxic gain of function, through the use of microtubule stabilizers may be beneficial for therapeutics because neurodegeneration is likely partially dependent on aberrant axonal transport.[262] Microtubule stabilizers have previously been used clinically for cancer treatment. Drugs that stabilize microtubules have shown benefits for toxicity, cognition, synaptic function, and tau pathology in cells and in multiple tauopathy animal models.[263−273] These results suggest that the stabilization of microtubules may be a beneficial route of treatment. However, microtubule stabilizers frequently have undesired side effects. Additionally, the majority of mutations inducing FTD affect tau splicing, leading to a higher ratio of 4R/3R tau and suggesting that therapeutics targeting tau alternative splicing may also be effective.[274]

CONCLUDING REMARKS

Neurodegenerative tauopathies are a devastating health crisis affecting many millions across the globe. Current treatments are ineffective, and as life expectancy and therefore prevalence of neurodegenerative disorders continue to increase, the demand for an effective therapeutic is growing. While tau oligomers are likely the best candidate for a therapeutic target, much of the research already completed in the field of tau therapeutics has focused on the historical hallmark of the disease, NFTs. Therefore, research needs to be optimized to specifically target the most toxic tau species and the species most likely to underlie the seeding and spread of pathology. Thus far, techniques that have been studied for the treatment of tauopathies include molecular chaperones, small molecules and aggregation inhibitors, post-translational modification modulators, immunotherapy, microtubule stabilizers, and alternative splicing modulators (Figure 3). Molecular chaperones have a highly complex relationship with tau, requiring far more study into their precise mechanisms of interaction in order to optimize their use for treatment. Both naturally occurring and synthetic small molecules inhibiting and degrading tau aggregation show potential as a route for treatment. However, more systematic studies need to be completed to determine the exact species that these molecules target. Because studies from different laboratories frequently report opposing results and techniques for measuring levels of tau are inconsistent, it is difficult to determine which drugs may be the most beneficial. Moreover, very few studies that report defibrillization investigate whether this leads to an increase in smaller, toxic aggregates. Likewise, studies of the efficacy of modulating tau post-translational modifications report contradictory results, likely due to the lack of systematic testing of differences in the modification of different epitopes. Passive immunotherapy studies appear to be very promising in terms of safety when compared to active immunization and in efficacy, particularly conformation-specific antibodies for misfolded tau and tau oligomers. The most efficacious treatment will likely result from the combination of different effective therapies, possibly

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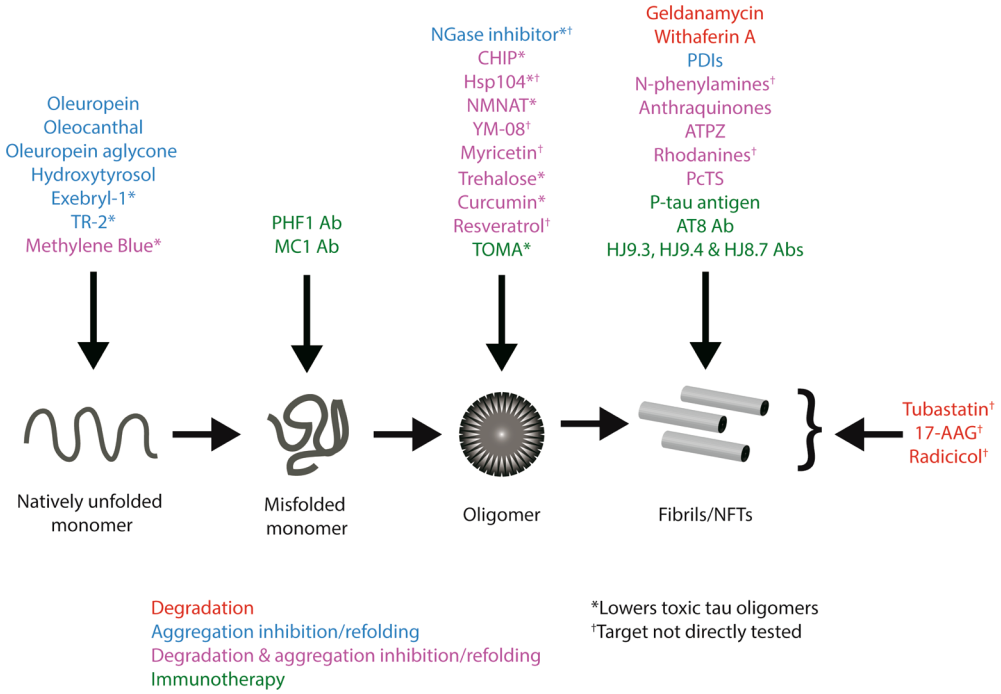

Figure 3. Summary of potential tauopathy therapeutics that have been tested and their hypothesized modes of action. Modulation of pathological tau could occur at various levels in the aggregation pathway, either interfering at the native monomer, misfolded monomer, oligomer, or at the fibrillar level. Tau can be targeted for degradation (red), aggregation could be inhibited or pathological species could be changed to a nontoxic conformation (blue), a combination of degradation and aggregation inhibition could inhibit pathological species (purple), or toxic species could be specifically targeted by active or passive immunotherapy (green). While many researchers are testing modulation of pathological tau, few studies have specifically measured whether treatment lowers levels of toxic tau oligomers specifically (*), and the tau species upon which the drug acts is uncertain in many cases (†).

through the combination of different techniques for targeting tau, as well as targets for other amyloid proteins involved in diseases with the presentation of tau as a secondary amyloidosis. While there is still a long way to go in the field of tauopathy therapeutics, the results summarized here suggest that there is reason to be optimistic for the future.

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Notes

The authors declare no competing financial interest.

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