Current state of tau aggregation inhibitors

By

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ABSTRACT

Tau is an intrinsically disordered, heat stable, and highly soluble protein found primarily in the axons of the central nervous system. It belongs to the family of structural microtubule-associated proteins, the major function of which is promoting and stabilizing microtubule assembly. However, modifications to the protein generate insoluble toxic oligomers of aggregated tau which are amyloid in nature. This modification renders them ineffective in binding to tubulin to stabilize microtubule assembly. Aggregates of tau are pathological hallmarks of many neurodegenerative disorders, collectively known as tauopathies. Some of the most well-known tauopathies are Pick's disease (PiD), progressive supranuclear palsy (PSP), corticobasal neurodegeneration (CBD), and Alzheimer's disease (AD). AD is the 6th leading cause of death in the USA and is the most prevalent tauopathy. There are only five FDA approved drugs that can arrest the cognitive decline and other symptoms of AD temporarily, but they do not reverse or inhibit the pathology of the disease. The disease therefore continues to progress, and cognitive functions decline faster when the drugs lose their effectiveness. There is a general consensus that therapies that reduce pathology would be beneficial for treating the disease. AD is characterized by two neuropathological hallmarks – extracellular senile plaques, which are aggregates of amyloid β peptide, and intracellular neurofibrillary tangles, which are composed of tau aggregates. Initially, much of the emphasis for drug discovery for AD was focused on inhibiting or reversing the amyloid senile plaque pathology. This focus was due to the observation that familial forms of early onset AD are associated with mutations which enhanced the formation of senile plaques. Additionally, the number of dementia cases involving senile plaques outnumbers dementia cases with pure tauopathies. Recent failures of drugs targeting amyloid accumulation for the treatment of AD and

new evidence further strengthening the association of tau pathology with neurodegeneration and AD cognitive impairment have placed importance on the development of tau-based therapeutics. This report discusses the current state of therapies and drugs that are available and are being developed to find an effective cure for AD and other tauopathies.

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TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION 1

1.1. MICROTUBULES AND MICROTUBULE ASSOCIATED PROTEINS 1
1.1.1. TAU PROTEIN STRUCTURE 2
1.1.2. TAU FUNCTION 6

CHAPTER 2. TAU AND DISEASES 7

2.1. MODIFICATION OF TAU SEEN IN NEURODEGENERATIVE DISORDERS. 7
2.1.1. HYPER PHOSPHORYLATION OF TAU 8
2.1.1.1. KINASES INFLUENCE TAU PHOSPHORYLATION IN VITRO, IN VIVO AND IN CELL-CULTURE BASED MODELS. 9
2.1.1.1.1. GSK3β 9
2.1.1.1.2. CDK5 10
2.1.1.1.3. MARK 10
2.1.1.1.4. OTHER KINASES 11
2.1.1.2. PHOSPHATASES 11
2.1.2. TAU AGGREGATION 12
2.1.3. OTHER POST-TRANSLATION MODIFICATIONS - TRUNCATION OF TAU 14
2.2. ARE THE MODIFICATIONS OF TAU PROTEIN TOXIC OR PROTECTIVE? 16
2.3. TAUOPATHIES 19
2.3.1. FRONTOTEMPORAL DEMENTIA WITH PARKINSONISM AND PROGRESSIVE SUPRANUCLEAR PALSY 20
2.3.2 PICK'S DISEASE (PID) 20
2.3.3. CORTICOBASAL NEURODEGENERATION (CBD) 21
2.3.4. ALZHEIMER'S DISEASE (AD) 21

CHAPTER 3. CHALLENGES IN TREATING AD AND OTHER TAUOPATHIES 25

3.1. LACK OF PROPER DIAGNOSIS 25
3.2. LACK OF EFFECTIVE THERAPIES 26

CHAPTER 4. CURRENT TREATMENTS 26

4.1. CURRENT APPROVED THERAPIES FOR AD AND OTHER TAUOPATHIES 26
4.1.1. ACETYLCHOLINESTERASE INHIBITORS 27
4.1.2. NMDA RECEPTOR ANTAGONIST - MEMANTINE 28
4.1.3. SELECTIVE SEROTONIN REUPTAKE INHIBITORS (SSRI) 29
4.1.4. COMBINATION OF THE AVAILABLE FDA APPROVED DRUGS 29
4.2. CURRENT EXPERIMENTAL TAU-BASED THERAPIES FOR AD 29
4.2.1 TAU CENTRIC ACTIVE AND PASSIVE IMMUNOTHERAPIES 30
4.2.2 MICROTUBULE STABILIZING AGENTS 31
4.2.3 TAU PROTEIN KINASE INHIBITORS 32
4.2.4 TAU AGGREGATION INHIBITORS (TAI) 33
4.2.4.1. IN VITRO TAU POLYMERIZATION 33
4.2.4.2. TAU AGGREGATION INHIBITOR-BASED THERAPIES 34
4.2.5. OLIGONUCLEOTIDE TREATMENTS FOR TAU 43

CHAPTER 5. CONCLUSION 44

CHAPTER 6. REFERENCES 49

Chapter 1. Introduction

1.1. Microtubules and microtubule associated proteins

Microtubules are essential for a variety of cellular functions such as cell motility, transport, cell shape and polarity and mitosis. Microtubules are built from heterodimers of α and β tubulin monomers. γ-tubulin belongs to the family of tubulins but does not assemble into microtubules, and instead is required for nucleating the polymerization of microtubules *in vivo* [1]. There are many classes of proteins which interact with tubulins, some of which are listed below.

The microtubule associated proteins, or MAPs, whose primary function is to stabilize and promote microtubule assembly by binding to microtubules are known as structural MAPs. Representatives structural MAPs are MAP1a, MAP1b, MAP2a, MAP2b, MAP4, tau protein, and a 205 kDa MAP [2].

A second broad classification of MAPs are the motor proteins kinesin and dynein. The primary function of motor proteins is generation of movement along microtubules using chemical energy generated from ATP hydrolysis [2]. Kinesin transports cargo of membrane bound vesicles, proteins and other organelles along the microtubules towards the positive end, and dynein transports towards the negative end of the microtubule. Microtubules grow from their positive end [2] and therefore, kinesin transports proteins which are necessary for it to grow from the positive end. Dynein can transport different organelles from the endoplasmic reticulum to the golgi and such transport can transmit signals to different parts of the cell [3].

The third major category of microtubule associated proteins are the microtubule plus-end tracking proteins (+tips) [4].They are a structurally and functionally diverse groups of proteins that are distinguished by their specific accumulation at the plus end of the microtubule tips [5-7]. Plus

end tip-proteins mainly stabilize the plus end of the microtubules. They help maintain the shape of the microtubule network and play an essential role during cell motility and morphogenesis [5].

Another category of microtubule associated proteins is the γ tubulin ring complex (γTuRC) and the proteins associated with it [8]. γTuRC is a ring shaped structure which is comprised of γ-tubulin and associated proteins that nucleate microtubule assembly *in vivo* [9-11] and *in vitro* [12].

1.1.1. Tau Protein Structure

In 1975, "tau" was first identified by Weingarten and his colleagues when it co-purified with tubulins from porcine brain extracts. Tau, an intrinsically disordered and heat stable protein, was found to promote and stabilize microtubule polymerization [13].

In humans, tau is encoded by a gene on chromosome 17 [14] and it is expressed as different isoforms due to alternative splicing of the pre-mRNA in the central nervous system (CNS). Tau is primarily localized in the neurons of the CNS in the adult human brain. Full length tau (2N4R) is comprised of 441 amino acids and is the longest isoform of tau found in the adult human brain [15-17] (Figures 1 and 2). Within the 441 residues, about 85 of them can be phosphorylated (45 of which are serines, 35 are threonines and 5 are tyrosines [18-20]).

In an adult human brain, alternative splicing of exons 2, 3 and 10 generates 6 different isoforms of tau ranging in length from 352 to 441 amino acids [16, 17] (Figure 1). These differ in part by the presence of either three or four carboxy terminal tandem repeat sequences of 31-32 amino acids each that are encoded by exons 9, 10, 11, and 12 (R1-R4 in Figure 1).

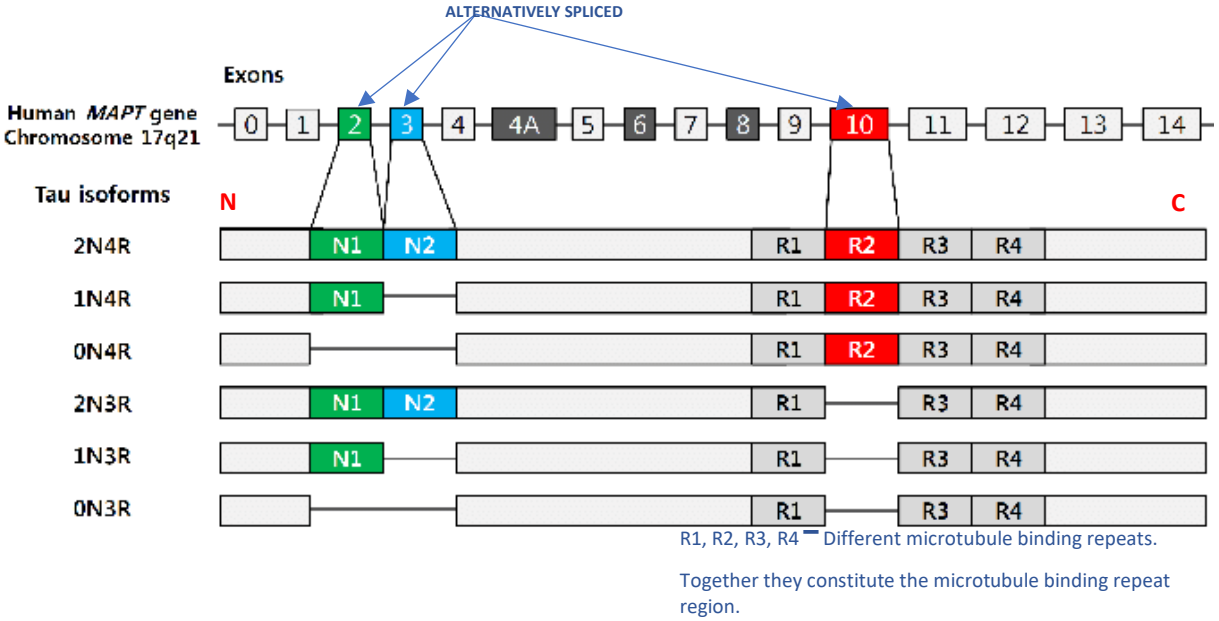


Figure 1: Structure of human tau gene and the 6 different isoforms that are generated by alternative splicing in the CNS. Figure modified from "*Tau mis-splicing in the pathogenesis of neurodegenerative disorders Sun et.al. (2016)"*

A tau protein can either have three microtubule binding repeat regions (3R) or four microtubule binding repeat regions (4R). Three isoforms of 3R tau are generated by the exclusion of exon 10 during alternative splicing. The other three isoforms of 4R tau are generated when they have four binding repeat regions including the one encoded by exon 10 [21] (Figure 1). The microtubule binding motifs are highly conserved structures which are 18 amino acids long. They are separated by less conserved 13-14 amino acid long inter-repeat sequences [21].

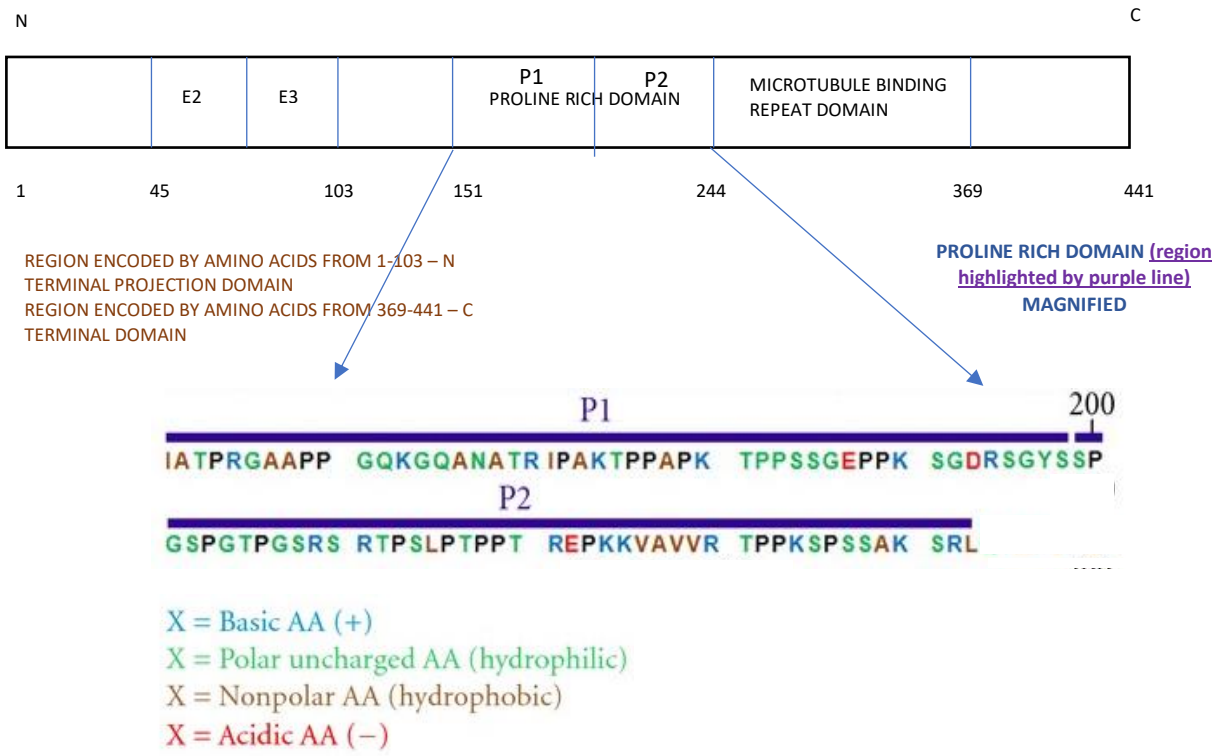


Figure 2: Structure of a 2N4R tau isoform found in the adult human brain. Illustrated are the N terminal region, Proline rich region, Microtubule binding repeat region and the C terminal region. The bold black P in the magnified proline rich region are the prolines. E2 and E3 denotes the stretch of amino acids encoded by exons 2 and 3 respectively; Modified from "*Structure and pathology of tau protein in Alzheimer's disease, Krestova et al. (2012)"*

Additionally, the isoforms of 3R and 4R tau isoforms differ as a result of alternative splicing of exons 2 and 3. Exclusion of the region encoded by exons 2 and 3 generates 0N tau. Inclusion of 29 amino acids encoded by exon 2 generates 1N tau and inclusion of 58 amino acids encoded by both exons 2 and 3 generates 2N tau (Figure 1) [22]. The N terminal region of tau is dominated by the presence of hydrophilic and charged residues that are mostly acidic in nature. The acidic nature of the N terminal region is due to the polypeptide sequences encoded by exons 2 and 3.

The microtubule binding repeat region (MTBR) of tau is flanked by a basic proline rich domain (Figure 2) [23]. The proline rich region is subdivided into P1 and P2 [24]. Tau is developmentally regulated. In the fetal brain only 0N3R isoforms are observed [15, 21]. In the

adult human brain, roughly similar ratios of 3R to 4R tau isoforms are found [23]. The 1N, 0N and 2N isoforms account for 54%, 37% and 9% of the total amount of tau, respectively [25].

Although tau is a natively unfolded protein with an overall low content of secondary structure, there is evidence that tau has the propensity to fold into a paperclip conformation when it is free in the cytoplasm [26]. The paperclip conformation generally occurs when tau dissociates from microtubules [16]. In this conformation, the C terminus of tau folds over the microtubule binding domain and the N terminus folds over the C terminus, thus the N and C terminals are near each other (Figure 3) [27]. This conformation has been speculated to protect the MTBR of tau from getting abnormally modified.

An isoform of tau with about 400 additional amino acids is found exclusively in the peripheral nervous system (PNS) and is known as "big tau". The higher molecular weight is a result of the inclusion of exon 4a in the amino terminal half of the tau protein [28]. Since its discovery more than 30 years ago, many functions of tau have been characterized based on its particular structural features.

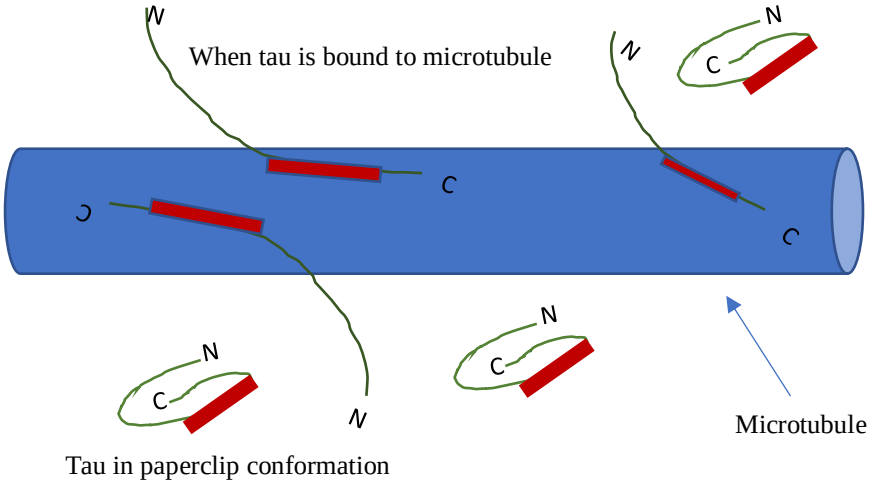


Figure 3: Structure of tau when bound to microtubules and when it is free in the cytoplasm. The red box denotes is the microtubule binding repeats regions.

1.1.2. Tau function

Dimers of alpha and beta tubulin are required to polymerize and form microtubules. Tau binds to the microtubules at the interdimer interface through repetitive short sequence motifs (R1, R2, R3 and R4 shown in Figures 1 and 2) in the C terminal region and promotes their nucleation to form microtubules *in vitro* [29, 30]. Tau can be crosslinked to Lys 336 and Lys 338 of alpha tubulin [18].

The N terminal region of tau is also referred to as the projection domain. It is so called because it projects out from the surface of the microtubule and interacts with other cytoskeletal elements and the neuronal plasma membrane [31]. Although the projection domain does not directly bind to microtubules, it influences attachment and spacing between microtubules and other cell components [23, 32].

The proline rich region (Figure 2) is involved in interactions of tau with actin and it also is associated with the bundling of F-actin (filamentous actin) [33]. F-actin has a structural, mechanical, and enzymatic role inside the cells and is involved in cell migration. Thus, tau influences bundling of filamentous actin and cross linking of cellular cytoskeleton components [33]. The Src kinases Lck, Fgr and Fyn bind to the proline rich region of tau protein. Certain other proteins such as bridging integrator (BIN1) phospholipase C (PLC) γ1, and PLC γ2 also bind to this region [34]. Tau has been observed to bind to cSrc and Fyn in cell culture models. This interaction of the proline rich region of tau with cSrc drove platelet-derived growth factor stimulation followed by cSrc mediated-actin rearrangements [35]. Tau binds phospholipase C in human neuroblastoma cells SH-SY5Y [36]. Activation of PLCγ is facilitated in the presence of fatty acids such as arachidonic acid [37]. However, under cell free conditions and in the presence of unsaturated fatty acids it has been observed that tau activates PLCγ [37]. Higher levels of

arachidonic acid are observed in the brain of AD patients and related mouse models [38]. Thus, together tau and arachidonic acid may increase signaling through the PLCγ pathway and hence, may have a role to play in neuronal cell signaling. Apart from structurally stabilizing the microtubules, tau also interacts with histone deacetylase 6, HDAC6 [39]. Tau can bind to as well as inhibit histone deacetylase 6 which deacetylates tubulin and may regulate microtubule stability [40]. Inhibition of histone deacetylase 6 also prevents induction of autophagy by inhibiting proteasome function [40]. In AD brains, the level of HDAC6 is significantly decreased [39]. Inhibition of HDAC6 interacting with tau protein reduced phosphorylation of tau at Thr231 [41]. These data suggest that inhibition of HDAC6 leads to modifications of tau [30, 31].

Tau plays a major role in promoting and stabilizing microtubule assembly. However, modifications to tau such as hyperphosphorylation and deacetylation results in the formation of toxic species, and influences tau to dissociate from the microtubules which might result in many neurodegenerative disorders such as Alzheimer's disease, Pick's disease and other related dementias [42].

Chapter 2. Tau and diseases

2.1. Modification of tau seen in neurodegenerative disorders.

Although tau has a low propensity to form secondary or tertiary structure, under pathological conditions it forms highly ordered aggregates with high β sheet content. Abnormally phosphorylated aggregated tau has been observed in the core of paired helical filaments (PHFs) which are components of neurofibrillary tangles (NFTs) (Figure 4). Modified forms of tau have been observed in AD patients and other tauopathies [43-45]. Aggregates of tau are found in many other neurodegenerative disorders such as Pick's disease (PiD), progressive supranuclear palsy

(PSP), chronic traumatic encephalopathy (CTE), and corticobasal neurodegeneration (CBD). The conversion from tau monomers to pathological aggregated tau is believed to be a multistep process [42, 46]. It has been hypothesized that abnormally high levels of modifications of tau leads to neurodegeneration as it enhances the formation of pathological tau as found in diseases [42].

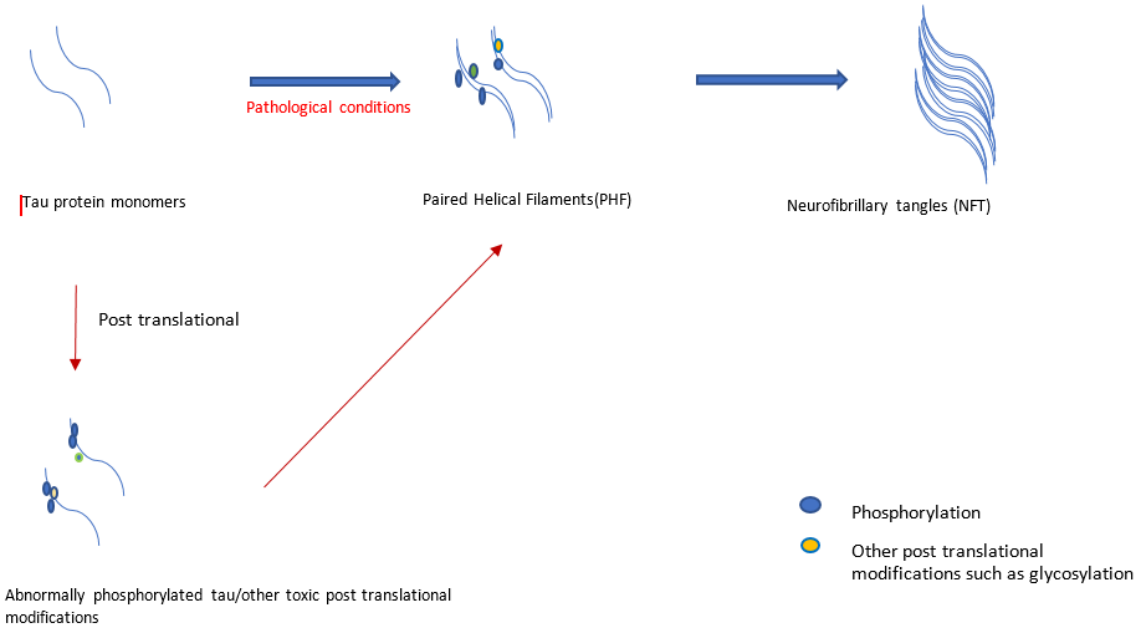


Figure 4: Schematic of abnormal modification of tau leading to the formation of NFTs in AD.

2.1.1. Hyper phosphorylation of tau

Tau binding to microtubule is modulated by phosphorylation at Y394 and S396 [14]. However, phosphorylation of the KXGS motifs within the microtubule binding motifs strongly reduces the binding of tau to the microtubules *in vitro* [37] and *in vivo* [38-40]. Since dynamic site-specific tau phosphorylation strongly influences tau function and localization, aberrant phosphorylation of tau might be the key event in tau pathology. Binding of hyperphosphorylated tau with normal tau has been revealed by *in vitro* kinetic studies [41, [47]. Phosphorylation at Ser199/Ser202/Thr205, Thr212, Thr231/Ser235, Ser262/Ser356, and Ser422 converts tau into an

inhibitory molecule that interacts with and sequesters normal non-phosphorylated tau away from microtubules [41, 47]. Hyperphosphorylated tau will bind with more affinity to non-hyperphosphorylated tau as phosphorylation reduces the total positive charge on the tau protein. Therefore, similarly charged tau will repel each other and hyperphosphorylated tau will bind to normal tau with more affinity because of its more positively charged character. Aggregation of tau into filaments is encouraged by phosphorylation of the three sites, Thr231, Ser396 and Ser422. *In vitro*, phosphorylation of Ser 396 and Ser 404 generates pro-fibrillogenic tau [48].

2.1.1.1. Kinases influence tau phosphorylation *in vitro*, *in vivo* and in cell-culture based models.

2.1.1.1.1. GSK3β

Out of the many kinases present in the brain, several have been reported to interact with tau [49]. One such kinase is glycogen synthase kinase 3β (GSK-3β). This kinase is expressed at high levels in the brain and it localizes in neurons [50]. GSK3β associates with microtubules and when it is overexpressed, phosphorylation of tau is seen to increase [41, 51, 52]. Lithium has long been used to treat bipolar disorders. It was hypothesized by Hong et al., that lithium would inhibit GSK3β and therefore reduce the hyperphosphorylation of tau observed in AD [53]. Treatment of NT2N neuronal cells with lithium not only decreased tau phosphorylation, but it also enhanced binding of tau to microtubules [53]. In studies on transgenic mouse expressing mutant tau, inhibition of GSK3β has also resulted in reduction of tau phosphorylation [54].

2.1.1.1.2. cdk5

Another kinase potentially associated with tau *in vivo* is cyclin dependent kinase 5 (cdk5) [55]. cdk5 is a unique member of the cdk family that is activated by interactions with non-cyclins p35 and p39, which are regulatory proteins expressed in post-mitotic neurons [56]. *In vitro*, tau is a substrate for cdk5, and cdk5 can phosphorylate most of the phosphorylation sites of tau which are phosphorylated by GSK3β [57]. Overexpression of cdk5 and p25 (a product formed by the truncation of p35 that accumulates in the brains of AD patients), but not p35, increases cdk5 activity, but it does not in turn increase tau phosphorylation *in situ* [58].

There are conflicting reports regarding the role played by cdk5 in tau phosphorylation. Studies with transgenic mice overexpressing mutant tau have shown that cdk5 can phosphorylate tau [59]. However, in studies conducted with triple mutant transgenic mouse models overexpressing human cdk5, its activator p35 and human protein tau, cdk5 does not result in increased tau phosphorylation [60]. Knocking out p35 significantly decreases cdk5 activity, but increased tau phosphorylation is observed [61]. In the same knockout mouse model GSK3β was increased by 50% which explains the increased tau phosphorylation [61].

2.1.1.1.3. MARK

Microtubule affinity regulating kinase (MARK) might play an important role in phosphorylating tau in models of transgenic mice expressing tau. MARK phosphorylates all the four KXGS motifs particularly Ser 262, Ser 293, Ser 324, and Ser 356 [62], which are present in and around the microtubule binding repeat of tau as well as in other microtubule associated proteins, and this results in detachment of tau from microtubules and their destabilization [62]. In

cell culture models, MARK appears to regulate tau phosphorylation and influence the growth of neurites and neuronal polarization in N2a cells [63].

2.1.1.1.4. Other kinases

CK1 is a casein kinase and it has been seen to localize with tau aggregates in AD [10, 57]. Several isoforms of CK1 exists which can phosphorylate tau. CK-1 δ (an isoform of CK1 generated by splicing) phosphorylates tau at Ser202/Thr205 and Ser396/Ser404 *in vitro* [58, 59] and colocalizes with NFTs in AD, PSP, and PiD [60]. Moreover, the mRNA of CK1δ is upregulated in AD brains [60]. The 5 tyrosine phosphorylation sites of tau are phosphorylated by SFKs (Src family kinases) such as Src, Lck, Fyn and c-Abl kinase [61].

2.1.1.2. Phosphatases

Phosphatases interacting with phosphorylated tau resulted in the reduction of aggregates and restoration of the microtubule binding function of tau, promoting microtubule stabilization and polymerization [64]. In general, PP2A (protein phosphatase 2A) is responsible for 70% of the cellular phosphatase activity [64] in the human brain. In AD brains, a significant reduction of the PP2A activity can be observed [64], which suggests that an imbalance of phosphatases and kinases plays a major role in the generation of abnormally phosphorylated tau isoforms. It has also been hypothesized that downregulation of phosphatases might be due to phosphatase inhibitors present in the brain. The PP2A inhibitor I_1^{PP2A} purified from bovine kidney [65] and human brain [66] was found to inhibit purified preparations of PP2A [66]. Tanimukai et al., found a significant increase in the mRNA expression of I_1^{PP2A} and I_2^{PP2a} at neocortical levels in AD, as compared to control cases, by performing *in situ* hybridization and immunohistochemical studies [67]. On performing double histochemical studies, they also found that inhibitors of PP2A and PP2A colocalized with

abnormally phosphorylated tau [67] suggesting that inhibition of phosphatase PP2A results in abnormal phosphorylation of tau protein as seen in AD.

In summary, evidence collected over the years indicates that overexpression of tau kinases such as GSK3β and cdk5, and inhibition of phosphatases such as PP2A results in the hyperphosphorylation of tau, which subsequently results in formation of toxic tau oligomers (discussed below). Although aggregated tau is heavily phosphorylated in human brains, it is not yet proven that all phosphorylated tau is in aggregates or will eventually aggregate [68]. The ability of tau to interact with microtubules is reduced upon phosphorylation. However, it is still unknown whether phosphorylation acts as a trigger for tau aggregation.

2.1.2. Tau aggregation

Tau aggregates are formed in two steps – nucleation and elongation. Tau monomers and dimers form a nucleation center and when a critical cluster size is obtained, the result is tau oligomerization in a dose and time dependent manner [69]. Tau oligomers then form longer fibrous aggregates with a cross β structure similar to amyloid aggregates [70]. It has been demonstrated that the propensity to form aggregates is influenced by the hexapeptide motifs present in the second and third microtubule binding repeat regions of tau [71].

Tau filaments isolated from AD brains and those induced *in vitro* share a cross β structure which is similar to the amyloid fibrils [72]. It has been hypothesized that the hexapeptide motif VQIVYK (Val-Gln-Ile-Val-Tyr-Lys, also termed as PHF6) located at the beginning of the third microtubule binding repeat and encoded by residues 306-311, and VQIINK (Val-Gln-Ile-Ile-Asn-Lys, also termed as PHF6*) located at the N terminal region of the microtubule binding repeat region 2 and encoded by residues 275-280 [73] (Figure 1) play a major role in the formation of tau aggregates. PHF6 and PHF6* have the propensity to self-assemble and form cross beta sheet

structure. Solid state nuclear magnetic resonance of microtubule repeats 1, 3 and 4 showed the presence of three beta strands, one from each repeat [74, 75]. A part of the core of PHFs corresponds to residues 306-324 located in MTBR 3 [76] demonstrating that the hexapeptide repeat regions form the core of tau aggregates. The hexapeptide regions of tau self-assemble in the absence of chemical stimuli [23, 77, 78], and they form aggregates under similar conditions as does full-length tau [79].

Certain missense mutations in tau, such as P301L, enhance the propensity of tau to form beta sheets and render the tau isoforms more prone to aggregation. Similarly, it has been observed that self-assembly of tau at VQIVYK and VQIINK motifs can become accelerated if there are point mutations around them, such as are observed in P301L and ΔK280 mutants [73, 79]. At the same time, if unfavorable amino acid substitutions are made in the hexapeptide motifs, such as proline residues which disrupt the beta sheet structure, this can render tau incompetent for assembly [80]. Crystallization of fibril forming motifs has shown that the hexapeptide motifs VQIVYK and VQIINK are necessary as well as sufficient for tau fibrillization [81]. Therefore, the above data attest to the fact that hexapeptide motifs play a major role in the assembly of tau monomers into insoluble aggregates in cells.

When tau is phosphorylated at serines, threonines and tyrosines, it becomes more negatively charged. Similarly, other post-translational modifications such as acetylation neutralize the positively charged lysine residues [13]. These types of post-translational modifications result in the formation of a more negatively charged tau molecule or an overall reduction in the positive charge on tau. The charge alteration might impact overall binding of tau to microtubules, and may affect the folding capability of tau into the paper clip structure, therefore exposing the phosphatase activating domain present in the N terminal region of tau [82]. The phosphatase activating domain

activates GSK3β kinase. In AD brains higher levels of GSK3β are observed and this is also associated with abnormal phosphorylation of tau.

Mirbhaha et al. had observed that when tau aggregates are exogenously delivered or "seeded" in mice brains, propagation of those aggregates take place from one part of the brain to the other [84]. Tau inclusions at the injection sites were observed in the brain of P301S tau transgenic mice which were intracerebrally injected with brain extracts containing tau aggregates isolated from presymptomatic animals. It was suggested by the authors that when presymptomatic mice was intraperitoneally injected with brain extracts from P301S tau transgenic mice, cerebral tau inclusions were promoted in the presymptomatic mice [83]. Therefore, they concluded that aggregated tau can promote inclusion formation in the CNS of transgenic mice. These studies suggested that introducing seeds of tau aggregates into the brain results in the propagation of tau aggregates from the point of injection to other portions of the brain.

In AD, tau aggregates are found to be abnormally phosphorylated [84]. Abnormal phosphorylation of tau negatively impacts the ability of the protein tau to bind to microtubules. Conformational changes of tau can be observed as tau gets phosphorylated, and therefore this post-translational modification - along with others such as truncation and glycosylation - can influence tau aggregation [84-86].

2.1.3. Other post-translation modifications - truncation of tau

Proteolytic cleavage of tau protein has been identified in tauopathies such as PiD [87], AD [88], CBD and PSP [89]. A protease-resistant core of 12kDa and 9.5 kDa tau fragments from within the paired helical filaments (PHF) in AD brains were identified by the same antibody which recognized C terminally truncated tau at Glu^{391} position [90]. The protease resistant 12 kDa form of tau suggested that truncation might be the mechanism that leads to the formation of forms of

tau that are prone to misfolding and self-assembly [91]. This view was supported when truncated tau was observed in association with the neurofibrillary pathology in AD brains [92]. These were immunolabelled with monoclonal antibody MN423 which specifically recognized Glu^{391} truncated tau associated with neurofibrillary tangles found in AD [48]. DC11 is also a truncation dependent conformational antibody that recognizes abnormal tau in AD, but not normal functional tau in control brains [93]. Recombinant tau proteins truncated either at the N terminus or at both N and C termini were recognized by DC11 [93]. This evidence indicated that both N- and C-terminally truncated forms tau are present in AD brains and are likely involved in pathological conformations of tau.

It is well-known that abnormal proteolytic cleavage of proteins is a common observation during aging as well as being associated with neurodegeneration [94]. Caspase, which belongs to the family of serine-aspartyl proteases, is involved in a majority of such truncation of proteins. Tau protein has been observed to be cleaved by caspase proteases at multiple sites. Caspase-3 cleaves tau at the carboxy-terminal residue of aspartic acid-421 (Asp^{421}) [94]. Tau truncated at Asp^{421} by caspase 3 was recognized by a monoclonal antibody Tau C3 [88]. It was also observed that truncated tau assembled more rapidly and more extensively *in vitro* [88] suggesting that truncation of tau at Asp^{421} can result in assembly of tau monomers into filaments as observed in AD.

In vitro studies have revealed that both Glu^{391} and Asp^{421} truncated tau has a higher propensity to form aggregates than full length tau [95]. Insoluble lysates from AD brains are found to be enriched with Asp^{421} and Glu^{391} truncated isoforms of tau. Tau truncated at Asp^{421} colocalizes with tangles in AD brain as well as in neurofibrillary tangles that are induced *in vitro* [88]. Expression of truncated $tau^{151\text{-}391}$ including 3 or 4 microtubule repeats in transgenic mouse brains induces neurofibrillary pathology resembling that of human tau pathology [96, 97]. Rats which

express truncated tau$^{151-391}$ with 3R or 4R repeats exhibit pathological features such as tau phosphorylation with increasing age [97]. When sarkosyl insoluble tau from rat brains expressing Tau$^{151-391}$ was extracted, truncated forms of tau were seen to co-aggregate with endogenous rat tau [96]. This suggested that tau truncation influences misfolding of normal tau which eventually is responsible for the generation of tangles in AD and other tauopathies [13].

The mitochondria derived from synaptosomes of AD patients are enriched for truncated tau $^{26-230}$ (NH2 htau). The truncated tau might be the reason for the altered function and quality control of mitochondria at the synapses [98]. The studies conducted by the authors showed that excessive mitochondrial turnover and NH2 htau-induced *in vitro* neuronal death might be related. This suggested that truncated tau may contribute to synaptic dysfunction in AD which can be induced by the aberrant recruitment of parkin and UCHL-1 in the mitochondria. Recruitment of these proteins in the mitochindria makes them prone to unwanted autophagic clearance [98].

Tau also undergoes certain other post translational modifications such glycosylation [97], ubiquitination [99], SUMOylation [100], nitration [101] and oxidation, which may indirectly facilitate tau aggregation [102], potentially by modifying tau conformation.

2.2. Are the modifications of tau protein toxic or protective?

Loss of function as well as toxic gain of function of tau protein has been observed in many neurodegenerative disorders [103]. Post-translational modifications of tau, such as abnormal phosphorylation and truncation, leads to the sequestration of tau into aggregates, which results in an inability to bind to tubulin to promote microtubule assembly [102].

A study has shown that tau aggregates in frontotemporal dementia impair proteasome activity [104]. Myeku et al. working with the mouse model rTg4510 expressing the P301L mutation in tau observed that there was decrease in proteasome activity with the increase in tau

aggregation [105]. The authors crossed a tauopathy mouse model with a mouse which overexpressed a transgenic reporter for proteasome activity and verified that indeed proteasome defects were related to tau aggregation [105]. When they immunoprecipitated proteasome components with tau from the brains of the mice model they saw that association of tau with proteasomes increased with age and severity of the disease [105]. This study demonstrated that modifications to otherwise benign tau can result in impaired proteasome activity.

An example of toxic gain of function of tau has been demonstrated by a study conducted by Winkler et. al [106], in which they co-expressed human full-length tau along with truncated tau (3R $tau_{151-421}$Δtau). Co-expression of full length tau with truncated tau (3R $tau_{151-421}$Δtau) resulted in the failure of axonal transport, clumping of mitochondria, disruption of the Golgi apparatus, along with severe paralysis in mice. They generated an inducible mouse line overexpressing human 3R $tau_{151-421}$(Δtau). They co-expressed it with wild type 4R or with wild type 3R tau or with mutant full length 4R P301S tau. In all the double mutants, severe cell damage and motor palsy was observed. They also demonstrated mouse lines expressing human mutant full length P301S tau developed sarkosyl-insoluble tau inclusions, neurodegeneration, and paralysis [106]. In contrast, when truncated tau was no longer expressed in the mouse brain, functional and structural recovery was observed in mice expressing full length 4R tau. Also, double transgenic mice for full length 4R and 3R tau were unaffected [106].

NH2-tau (Tau $^{26-230}$) has been isolated from tangles in the brains of AD patients and has decreased ability to bind to tubulin [23]. Tau 35 (E187-L441) is present in PSP and CBD but not in control brain samples [25]. Expression of Tau 35 in the transgenic mouse brain resulted in tau pathology, as well as cognitive and motor dysfunction in the mice [107]. Therefore, truncated tau has been shown to be neurotoxic and capable of playing a major role in tau pathology.

A 33kDa N-terminally truncated tau (starting at residue Ser71 in 0N3R tau -Ser128 in 2N4R tau) was found in tangles purified from human brain [108]. A 17kDa tau fragment was identified in the cerebellar granule neurons undergoing apoptosis [109]. Overexpression of tau$^{45-230}$ induced apoptosis in CHO cells and neurons. In an indirect study where formaldehyde was used to induce tau aggregation, it was observed that apoptosis took place in the neurotypic cell line SH-SY5Y and also in rat hippocampal cells [110]. Therefore, it can be inferred from these observations that truncated tau has toxic properties that result in apoptosis of neurons.

Surprisingly, it has also been found that tau monomers can be toxic to the neurons. In a study led by Nadine Ait Bouziad et al, it was found that tau interacts with cell membranes forming highly stable protein-phospholipid complexes which are toxic when added to hippocampal primary neurons [111]. The same study demonstrated that monomeric, fibrillar, and tau-phospholipid complexes induce cellular apoptosis when they are added exogenously to the hippocampal region of the transgenic mice brain [111]. Studies by Gomez-Ramos et al. proposed a mechanism by which monomeric tau can induce toxicity through muscarinic receptor-induced liberation, where the ligand (tau) binds to the receptor which causes the liberation of Ca^{2+} ions. [112]. On the other hand, tau oligomers induce toxicity by interacting with cell membranes [113], pore formation [113], facilitated endocytosis [114], and deleterious interactions with neuronal spines [111, 115]. Therefore, this suggested that tau, when truncated or aggregated, as well as certain forms of monomeric tau, can be toxic to neurons.

However, the presence of tau aggregates or the "toxic" oligomers in the Alzheimer induced mice brain or even in humans who did not suffer from any tauopathy raises a doubt regarding the toxicity of tau aggregates. In most of the studies conducted with *Drosophila* models of tauopathy, NFTs are not formed even though neurodegeneration had already started to take place [106-110].

There is evidence that suggests NFTs are not sufficient for toxicity [106-110]. Mouse models which conditionally expresses human tau (tau 0N4R-P301L) do not form toxic aggregates of tau either [111]. These mouse models develop NFTs with age and consequently undergo neuronal loss and progressive motor deficits [111]. When tau expression is switched off after the formation of NFTs, memory improves, and cell loss is stabilized, however NFTs continue to accumulate [111]. Hence, this experiment suggests that the presence of NFTs does not result in neurodegeneration.

A study attempting to determine whether filamentous tau is toxic or protective, found that tau filaments appeared "healthy" in terms of nuclear morphology suggesting that polymerized protein might be neuroprotective [116].

Toxic oligomers of tau have been observed in many neurodegenerative disorders that are collectively termed as tauopathies. In the next section, we will briefly discuss a few of the known tauopathies, with more attention given to Alzheimer's disease since it is the most prevalent of all the known tauopathies.

2.3. Tauopathies

In 1907, Alois Alzheimer presented his paper 'On a peculiar disease of the cerebral cortex' in which he described neurofibrillary tangles (NFTs) and senile plaques as pathological changes associated with presenile dementia [118], utilizing a silver staining technique developed by Max Bielschowsky and Korbinian Brodmann [35]. Half a century later, ultrastructural investigations made by Kidd, Terry and Crowther revealed that the NFTs are essentially made of double helical stacks called "paired helical filaments" and to a lesser extent by straight filaments [117-119]. In the mid-1980s, hyperphosphorylated tau protein was found to be the major component of PHFs, and this was confirmed by biochemical studies conducted on isolated NFTs from AD brains [120, 121]. Subsequent studies revealed that, not only in AD, but many progressive neurodegenerative

disorders such as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP 17), chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD) are pathologically defined by tau aggregates or toxic oligomers of tau [122-124].

The diseases where toxic oligomers of tau play a major role in neurodegeneration are collectively termed as tauopathies. Tauopathies can be differentiated based on the morphology of the tau aggregates, propagation of the aggregates, cells affected and their location in the brain, the ratio of tau isoforms and the symptoms observed in the affected individuals [125].

2.3.1. Frontotemporal dementia with Parkinsonism and progressive supranuclear palsy

In the late 1990s, mutations in the gene encoding tau were demonstrated to cause frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17). FTDP-17 was found to be associated with hyperphosphorylated filamentous inclusions of tau which were sufficient to cause neurodegeneration with dementia [117, 118]. PSP is a form of Parkinsonism which is characterized by NFTs (fibrillar inclusions found inside the cytoplasm of the cells) and neuropil threads (found in the dendrites which get swollen due to the accumulation of tau filaments and also found in the distal axons/terminals). NFTs and neuropil threads found in PSP are made of straight filaments and paired helical filaments which consists of hyperphosphorylated tau in neurons and glia of subcortical and cortical structures and are mainly composed of 4R isoforms of tau [126].

2.3.2 Pick's disease (PiD)

Pick's disease (PiD) is another type of tau-related dementia. PiD is characterized by difficulty with language and thinking, unwarranted anxiety and behavioral changes. Mutations of the tau gene have been observed in PiD [127]. The abnormal tau aggregates form round inclusions,

termed Pick's bodies, that are localized near the nucleus. Generally, the tau filaments found in PiD are primarily straight filaments composed of 3R tau isoforms [128].

2.3.3. Corticobasal neurodegeneration (CBD)

Corticobasal neurodegeneration or CBD is a neurodegenerative disorder that is characterized by asymmetrical cortical dysfunction. Abnormal accumulation of hyperphosphorylated 4R tau isoforms, similar to the NFTs seen in PSP and Alzheimer's disease are found in patients with CBD. Patients with CBD suffer from motor neuron degeneration which leads to akinesia, stiffness of limbs, asymmetric involuntary movements, and other complications [129].

2.3.4. Alzheimer's disease (AD)

AD is the 6th most common cause of death and is also the most expensive disease in the USA [130]. It is an irreversible progressive chronic neurodegenerative disorder that impairs memory and cognitive skills. Over time, patients find it difficult to carry out the simplest tasks such as remembering their name or trying to find their way back home from their backyard. Episodic memory deficits or short-term memory losses occur for the first three to six years in the initial stages of the disease. Difficulty in the retrieval of old memories and difficulty in speech are commonly observed in the later stages, followed by visuospatial impairment in the advanced stages of the disease [129- 133].

Approximately 5.5 million Americans are living with Alzheimer's disease today. Care for Alzheimer's disease affected patients and patients with other dementias has been estimated to cost $277 billion per year and the costs are expected to rise to $1.1 trillion by 2050 [130].

The most common form of AD is Late Onset AD or LOAD, in which patients begin showing symptoms after the age of 65 years, while in early onset AD or EOAD which accounts for about 5% of the cases, age of onset generally lies between 30 and 65 years of age [133].

Genetically, AD is divided into two groups – familial AD and sporadic AD [133]. Familial AD are cases of EOAD and are due to rare genetic mutations in one or more genes that encode proteins such as amyloid precursor protein (APP gene), presenilin 1 (PSEN1 gene) or presenilin 2 (PSEN2 gene) [129, 134]. APP is a transmembrane protein expressed at high levels in the brain. Cleavage of APP by β site APP cleaving enzyme 1 (BACE1) and γ secretase in the brain results in the formation of amyloid β peptides which are one of the pathological hallmarks of AD [135].

APP can be cleaved by two pathways [136]. In one of the pathways which is called the non-amyloidogenic pathway, cleavage of APP takes place by α and γ secretases [136]. This produces soluble APPα peptide which stimulates nerve growth factor and in turn influences the production of acetylcholine which is an important neurotransmitter dealing with memory and cognitive functions [136]. However, cleavage of APP by both β and γ secretases produces amyloid β peptide fragments. Cleavage by γ secretase at different positions of APP results in C terminal heterogeneity in the peptide population [136]. Aβ peptide cleaved at position 40, called Aβ40, is the most abundant Aβ product in the brain followed by the peptide cleaved at position 42, called Aβ42. Aβ42 is more hydrophobic, and along with Aβ40 forms long insoluble fibrils in the brain [135]. Presenilins 1 and 2 regulate the proteolytic function of γ secretase, and mutations in the genes PSEN1 and PSEN2 can disrupt the ratio of Aβ42 to Aβ40 in the brain, resulting in early onset of AD [34].

The sporadic form accounts for 95% of AD cases and is generally late onset. It most likely results from a combination of genetic and environmental factors [137]. The most common genetic

risk factor in sporadic AD is the presence of ε4 allele of apolipoprotein E [138]. ApoE is the dominant lipid and cholesterol carrier in the brain and it is involved in Aβ clearance or catabolism. The human ApoE gene consists of 3 polymorphic alleles - ε2, ε3 and ε4 - which have a worldwide frequency of 8.4 %, 77.9% and 13.7%, respectively [138]. However, in AD patients the ε4 allele is seen to be approximately 40%. Expression of ApoE ε4 increases the risk of AD [139].

AD is described by two underlying neuropathological hallmarks - NFTs formed by hyperphosphorylated aggregates of tau, and senile plaques formed by aggregation of Aβ peptide [140, 141]. There are indications that tau and β amyloid might work together to drive neurons to a diseased state which finally results in neurodegeneration [142]. Although how both proteins influence each other's functions is a matter of debate, as both of them can form aggregates and spread through the brain by themselves and function independently of one another.

The amyloid cascade hypothesis (ACH) was developed because it was noted that amyloid β peptides were the main constituents of senile plaques, and because mutations in APP and other associated proteins resulted in early onset of AD. According to amyloid beta cascade hypothesis (ACH), accumulation of amyloid beta peptide acts as the main trigger for AD. It was suggested that Aβ plaques influence tau to form toxic oligomers [143]. In one study with transgenic mice overexpressing human P301L tau, the injection of Aβ42 resulted a five-fold increase in NFTs near the injection sites [144]. In another experiment conducted by Lewis et al., JNPL3 transgenic mice expressing mutant tau were crossed with Tg2576 transgenic mice expressing mutant β amyloid precursor protein. In the progeny, Aβ deposits developed at the same age as parental controls, however NFT pathology was enhanced in the double mutant progeny strain [145]. These examples are two of many studies conducted that suggest Aβ triggers tau pathology. Because of these experiments and others contributing to the amyloid cascade hypothesis, Aβ was considered to play

a key role in AD pathogenesis upstream of tau pathology. Therefore, along with the genetic mutations in APP and other associated proteins, Aβ was considered to be the prime target for developing therapeutics for treating AD pathology. Promising immunotherapies such as monoclonal antibodies, solenazumab and bapineuzumab, that target Aβ pathology, failed to achieve cognitive and functional endpoints in phase 3 of clinical trials [146, 147]. In 1991, staging of AD-related neuropathology was described by Braak and Braak using the brain distribution of NFTs. NFTs and neuropil threads seem to follow a characteristic distribution pattern that directly correlated with cognitive impairment in the brains of deceased AD patients [148, 149]. In 2007, Roberson et. al., observed that reducing tau content blocked Aβ and excitotoxin-induced neuronal dysfunction *in vivo* [150]. In a similar study, it was demonstrated that knocking out endogenous mouse tau genes in transgenic mice expressing human mutated APP and PS1 protected the mice against memory loss, cognitive impairment and synaptic loss [151]. The tau knockout mice model also had less plaque content than APP/PS1 transgenic mice model. This study also showed that reducing tau levels prevented behavioral deficits in transgenic mice expressing APP without altering Aβ [150]. This study along with the previous work by Roberson et al., demonstrated the feasibility of the argument that tau is necessary for Aβ pathology [150]. It was also demonstrated from tau knockout mice models that knocking out tau aggregates protects against synaptic loss. Therefore, with respect to these experiments it was suggested that pathological tau is indeed toxic to the neurons. Therefore, it has been hypothesized that targeting tau pathology might inhibit the formation of tau aggregates and development of tau-targeting therapeutics can provide protection against memory impairment as well as potentially reverse AD pathology. Whether Aβ influences the production of toxic forms of tau or *vice versa* is still a much-debated topic.

AD is one of the most common causes of death in the USA, yet there are hardly any therapies which could reverse or inhibit the pathology of the disease. In the next section, we will discuss the challenges that are faced in treating AD and other tauopathies.

Chapter 3. Challenges in treating AD and other tauopathies

3.1. Lack of proper diagnosis

One of the major problems with AD or other tauopathies is the difficulty in diagnosing affected patients as no single effective tool exists that can give a definitive result indicating whether a person is suffering from any of the known tauopathies [152]. Given the similarity in the symptoms of different tauopathies, it becomes even more difficult to diagnose the type of dementia one person is suffering from. Cognitive tests such as the mini mental state examination [153], or a list of words memorized to be recollected later, or the clock drawing test [154], are not efficient methods of testing since a blind person, or a person who is deaf and mute, or does not know how to read can perform poorly in these tests despite not suffering from dementia .

Recent technology has introduced brain imaging techniques which can be used to look at the structure and function of the brain. MRI/CAT scans can be used to study the structure of the brain by measuring the brain volume and PET scans can give us data which are helpful in studying brain function [155-157]. Current molecular imaging techniques utilizing PET scans are a step towards successful diagnosis of many neurodegenerative disorders [157-160]. PET scanning makes use of small molecular weight compounds such as Pittsburg compound B (^{11}C-PIB) which can bind to Aβ peptides, and emit radiation allowing for visual and quantitative measurement of Aβ deposition [161]. ^{11}C-PIB compound is one of the most widely studied amyloid radioligands which is used to study the amyloid β plaques found in AD by PET imaging. Compared to a control

population, the propensity of this radioligand to bind in AD patients increases by 50% due to the increased levels of Aβ peptide [148, 149]. Other radioligands approved for β amyloid PET imaging include ^{18}F-florbetapir and ^{18}F-flutemetanol [160]. ^{18}F-FDDNP(2-(1-(6-[2-fluorine 18elabelled fluoroethy)methylamino]-2-napthyl) ethylidene)malononitrile) is a tracer molecule which can be used for both β amyloid and tau pathology [162]. Currently, another compound ^{18}F-AV-1451 and ^{18}F-T807 is in clinical phase development as a PET tracer for *in vivo* imaging of tau aggregates [160, 162]. These techniques will not only help elucidate the degree of pathology but also potentially allow better diagnosis.

3.2. Lack of effective therapies

The second and major drawback in treating AD or other tauopathies is the lack of any efficient, commercially available drugs that are directed towards treating the underlying causes of the disease. Due to the prevalence of AD over other tauopathies, most of the research conducted over the years has been directed towards finding a cure for AD rather than other tauopathies. Most of the drugs available for AD (Table 1) are directed towards facilitating communication at synapses [163-165] of the neurons, as destruction of the synapses is widely observed in several neurodegenerative disorders such as AD [166].

Chapter 4. Current treatments

4.1. Current approved therapies for AD and other tauopathies

The currently available drugs approved by FDA (Table 1) are the cholinesterase inhibitors donepezil, galantamine, and rivastigmine, and the NMDA receptor blocker memantine. These drugs have been used to treat the cognitive symptoms associated with AD and can be effective for

a certain amount of time [167]. The biggest drawback of these drugs is that the cognitive functions of patients gradually decline as soon as the drugs lose their effectiveness.

Table 1: FDA approved drugs for treating AD and other tauopathies

Generic	Brand	Approved for	Side effects
Donepezil	Aricept	All stages	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
Galantamine	Razadyne	Mild to moderate	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
Memantine	Namenda	Moderate to Severe	Headache, constipation, confusion and dizziness.
Rivastigmine	Exelon	Mild to moderate	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
Memantine+Donepezil	Namzaric	Moderate to severe	Nausea, vomiting, loss of appetite, increased frequency of bowel movements, headache, constipation, confusion and dizziness.

4.1.1. Acetylcholinesterase inhibitors

Acetylcholine is an important neurotransmitter involved in memory retention [168] and other memory related functions. Acetylcholine transmits signals from one cell to the other. Once the message has been delivered, acetylcholine is broken down by an enzyme known as acetylcholinesterase. Acetylcholinesterase helps remove acetylcholine from the synaptic cleft, terminating the signal [165]. In AD, cholinergic neurons producing acetylcholine are destroyed

and acetylcholine levels in the brain are reduced. Acetylcholinesterase inhibitors such as Donepezil (brand name: Aricept) was the first approved drug for AD. It was approved by the FDA in 1996. Donepezil, in common with other cholinesterase inhibitors, reduces the rate at which neurotransmitters are broken down, thereby delaying the loss of cognitive symptoms and the onset of dementia. In some of the AD patients, rivastigmine, an example of another acetylcholinesterase inhibitor, may improve memory and awareness [169]. It has been used to treat neuropsychiatric symptoms in CBD, however it does not affect cognition.

4.1.2. NMDA receptor antagonist - Memantine

In AD patients, an excess amount of glutamate has been observed [170]. Glutamate is a major excitatory neurotransmitter [171]. It functions by transmitting messages from one neuron to the other [172]. When glutamate or glycine binds at the glutamate binding site, the N-methyl-D-aspartate (NMDA) receptor gets activated and allows the transmission of positively charged ions through the ligand-receptor gated channel [172]. Therefore, the excessive glutamate present in AD binds to the NMDA receptor and the gated ion channel remains open which allows a constant flow of positively charged ions [172]. Memantine is a partial uncompetitive NMDA receptor antagonist that can reduce the action of glutamate [173, 174], and decrease glutamate excitotoxicity associated with AD [158].

All the 5 drugs (Table 1) have a variable effect on different people with AD. Most of these drugs have side effects for different patients and might not work at all for many people. Although in a few patients, they may slow down the process of disease progression or worsening of the symptoms for a while, the underlying pathology is not slowed, and the disease progression eventually goes back to its initial rate [159].

4.1.3. Selective serotonin reuptake inhibitors (SSRI)

In open label cases or in case reports, it has been demonstrated that SSRIs have been able to decrease neuropsychiatric symptoms such as disinhibition and repetitive stereotypical behavior [175]. It can also be used to treat food craving, inappropriate sexual behavior and aggressiveness. This is used to treat depressive symptoms in CBD, PSP and dementia with Lewy bodies [176].

4.1.4. Combination of the available FDA approved drugs

No evidence has been found which clearly indicates that combining all three different cholinesterase inhibitors - donepezil, galantamine and rivastigimine (Table 1) would yield better results. Rather more discomfort is apparent as more side effects have been observed in patients taking a combination all three cholinesterase inhibitors [167, 177].

However, if Aricept is taken along with Memantine the rate of disease progression is slower [177]. This is a temporary treatment and over the course of time, disease progresses, and condition of the patients worsens.

4.2. Current experimental tau-based therapies for AD

Given the inefficacy of the currently available FDA approved drugs to arrest the progression of the disease, research is focused on developing further treatments and small molecules that target the underlying cause or the pathological hallmarks of AD. The neuropathological hallmarks strongly influence the recent AD therapeutic approaches. Without drugs efficiently working against the neuropathological hallmarks - β amyloid plaques and NFTs, the cost of treatment and care for AD has been estimated to increase to 1.1 trillion dollars by the year 2050, and the health of patients will decline due to progression of the disease [130]. After the failure of 19 drugs targeting Aβ pathology in clinical trials, greater attention is focused on reducing tau pathology [146].

Treatments for tau are broadly classified to the five groups namely: (a) tau-centric active and passive immunotherapy, (b) microtubule stabilizing agents, (c) tau protein kinase inhibitors, (d) tau aggregation inhibitors, and (e) oligonucleotide treatments for tau aggregates.

4.2.1 Tau centric active and passive immunotherapies

Efforts have been made to create safe and effective active and passive vaccines for tau. In passive immunotherapies, exogenous antibodies are introduced to the AD affected patients. In active immunotherapies, the body's immune system is stimulated to produce anti-tau antibodies to destroy the toxic oligomers of tau. The first active tau vaccine to enter clinical trials was AADvac1 (Axon peptide 108 conjugated to KLH, Axon neurosciences, Bratislava, Slovak Republic). It is a synthetic peptide derived from amino acids 294 to 305 of tau sequence coupled to keyhole limpet hemocyanin (KLH) through an N-terminal cysteine and administered through Alhydrogel alum adjuvant. Initially the safety of administering the vaccine was evaluated. AADvac1 was designed to target misfolded tau in AD and its tolerability and efficacy in participants has been evaluated in a phase 1 clinical trial conducted in 3 centers in Austria on patients with mild to moderate AD [178]. A subsequent phase 2 clinical trial with 185 participants is underway and the primary outcome of the results will be a test of the safety of the drug to the participants and to see if it elicits a hyperallergic response from the immune system [179].

The vaccine ACI-35 is a 16 residue peptide that includes the phosphorylated residues S396 and S404, and provides active immunization [179, 180]. It elicits a rapid immune response in P301L mice with a mild reduction of hyperphosphorylated tau pathology. Increased IgG titers and improved motor function were observed in vaccinated P301L mice model [180]. Currently, a phase I clinical trial is running for this active vaccine to assess safety profile along with secondary outcomes which includes biomarkers, functional and clinical changes.

Example of passive immunotherapy is administering compound RG7345. It is a human monoclonal antibody targeting specific tau phosphorylated epitope at site S422 which is predominant in neuronal dendrites [181, 182]. It demonstrated reduced tau pathology and intracellular clearance of tau antibody complexes in a triple transgenic mice model of AD and is undergoing a phase 1 clinical trial [181].

Recently, another new humanized tau monoclonal antibody called armanezumab has been developed which is specific to the N terminal region of pathological tau. It was tested against full length recombinant tau as well tau isolated from the brains of AD patients. When armanezumab was used on SH-SY5Y neuroblastoma cells, it demonstrated its ability to inhibit tau toxicity. It also reduced phosphorylated tau and total tau content in tau (P301S)/Tg mice [183]. Currently, no clinical trials have been performed with armazenumab, and therefore its efficacy to cross the blood brain barrier and protect the cells against tau toxicity in human brain cannot be evaluated. However, the discovery of such antibodies are opening up avenues for more research with tau directed immunotherapy.

4.2.2 Microtubule stabilizing agents

Among other tau based anti-AD drugs, multiple microtubule stabilizing agents have been tested. The purpose of developing microtubule stabilizing agents was to create a drug which could stabilize microtubules in the absence of tau protein. Dissociation of tau from microtubules result in axonal transport impairment and synaptic dysfunction. Some compounds such as paclitaxel, epothilone D or TPI 287 were used in transgenic AD mice models. In a preventive study, epothilone D was administered to young PS19 tau transgenic mice which initially lacked tau pathology. With age, while the untreated mice developed axonal microtubule loss, dystrophy and

spatial learning deficits, epothilone D treatment reduced these changes [184]. In another study, administration of epothilone D in PS19 mice model reversed behavioral and cognitive deficits and cleared tau pathology in the mice [185]. However, due to the absence of results from the majority of the drugs targeting microtubule stabilization, along with the presence of toxic side effects the studies were halted for AD [186].

Another microtubule stabilizing agent named davunetide, which is an eight amino acid peptide derived from activity-dependent neuroprotective protein (ADNP), has demonstrated its potential to decrease Aβ and hyperphosphorylated tau levels in ADNP knockout mouse models [187]. In spite of the encouraging results obtained through a variety of tests, phase II and III results demonstrated the inefficiency of davunetide with pure tauopathy PSP, which halted its development.

4.2.3 Tau protein kinase inhibitors

Post translational modification, especially phosphorylation, of tau is a common event even in normal brain. However, in AD patients, abnormal phosphorylation of tau is observed [21]. This abnormal phosphorylation in AD patients results in a disturbance of the balance between kinases and phosphatases in the brain. Abnormal phosphorylation is one cause of the disassociation of tau from microtubules [13]. Efforts have been made to develop kinase inhibitors which can balance the number of kinases and phosphatases present in the AD brain. A GSK-3β inhibitor called tideglusib was in clinical trials for AD and progressive supranuclear palsy [173]. In a Phase IIa trial, it was orally administered to 30 patients with a dosage of 400-1000 mg/day for 20 weeks. Positive outcomes such as better cognitive ability was observed. Four out of 5 groups of patients with mild to moderate AD had significantly better response on Mini Mental State Examination (MMSE) [188]. Unfortunately, in a phase IIb clinical trial, conducted to assess safety and efficacy

of tideglusib in mild to moderate AD patients, no statistically significant different outcomes were observed in AD patient receiving tideglusib with respect to the AD patient group placed on placebo [189]. Moreover, diarrhea and asymptomatic transaminase elevations were seen as side effects. There are no current approved trials undergoing with tideglusib. Another alternative approach for targeting tau phosphorylation could be activation of phosphatases which could reduce phosphorylation of tau and restore the balance of kinases and phosphatases in the AD brain. One of the candidates for phosphatases is PP2A, but clinical trials has not been started yet with them [41, 62].

4.2.4 Tau aggregation inhibitors (TAI)

In order to learn about tau aggregation kinetics and how viable tau aggregation inhibitors are, it is necessary to induce tau polymerization *in vitro*.

4.2.4.1. *In vitro* tau polymerization

In vitro tau polymerization can be obtained by addition of inducer molecules such as polyanionic compounds. For example, glucosaminoglycan heparin [78], polyglutamate [175, 190[190] and RNA [191], and fatty acids or fatty acid like molecules such as arachidonic acid [192], docosahexaenoic acid [193] and alkyl sulfonate detergents [194] have been used to induce tau aggregation. The mechanism by which these compounds facilitate polymerization has not yet been fully elucidated. It is hypothesized that negative charge on the polyanionic inducer molecules might be a reason why aggregation of tau molecules take place, as the relatively positively charged carboxy terminal of tau can bind to the negatively charged anionic molecules. The negative charges on the polyanionic inducers and tau binding to the inducer molecules can result in an “induced fit” conformation to the microtubule binding regions of tau similar to the model proposed for tau binding to microtubules [195]. Higher levels of arachidonic acid are observed in brains of AD

patients [5]. It has been observed that fatty acid inducer compounds such as arachidonic acid induce tau aggregation [194]. Although the tau fibrillization that takes places under the influence of arachidonic acid resembles straight filaments, they mimic the characteristics of PHFs commonly found in AD [196].

Native tau does not readily form secondary or tertiary structure. Therefore, the change of conformation of soluble tau into insoluble highly ordered aggregates of tau filaments has been a mystery for scientists for a long time. Thus, the ability to be able to form aggregates of tau *in vitro* have allowed scientists to learn more about the pathological forms of tau found in AD and other tauopathies. Also, as tau aggregation occurs rapidly *in vitro* with the help of inducers, it can be used to study the efficiency of various tau aggregation inhibitors. The knowledge gained from these experiments can be used in the formulation of therapeutics and testing them in order to evaluate their efficacy before conducting *in vivo* studies.

4.2.4.2. Tau aggregation inhibitor-based therapies

Several tau-directed approaches for treating AD have been developed. However, in cell-based and *in vitro* assays, small molecular weight compounds such as tau aggregation inhibitors (TAIs) have shown the ability to inhibit aggregation of tau proteins and some have already been tested in humans. Several classes of tau aggregation inhibitors have been isolated from plants, fungi, or have been chemically synthesized. Chemical compounds such as polyphenols [197, 198], porphyrins [198], phenothiazines [198], benzothiazoles, azaphilones [199], and anthraquinones [200] are a few of the many classes of compounds which have shown a tendency to prevent tau aggregation *in vitro* studies. All these compounds share the common structural motif of containing at least one aromatic ring structure. TAIs can be broadly categorized into two groups – covalent TAIs and non-covalent TAIs.

Covalent TAIs function in one of two ways. They can either covalently modify tau, or they can lead to the formation of bonds between different tau molecules, or between different sites on the same molecule. In either case, these TAIs lead to tau molecules that are unable to aggregate [186, 201]. Natural products such as oleocanthal [202], oleuopeinaglycone [203], and epigallocatechin gallate (EGCG) [204], are some well-known and studied covalent TAIs. Oleocanthal reacts with epsilon groups of lysine residues including the residues present in the MTBR to form imines [202, 205].

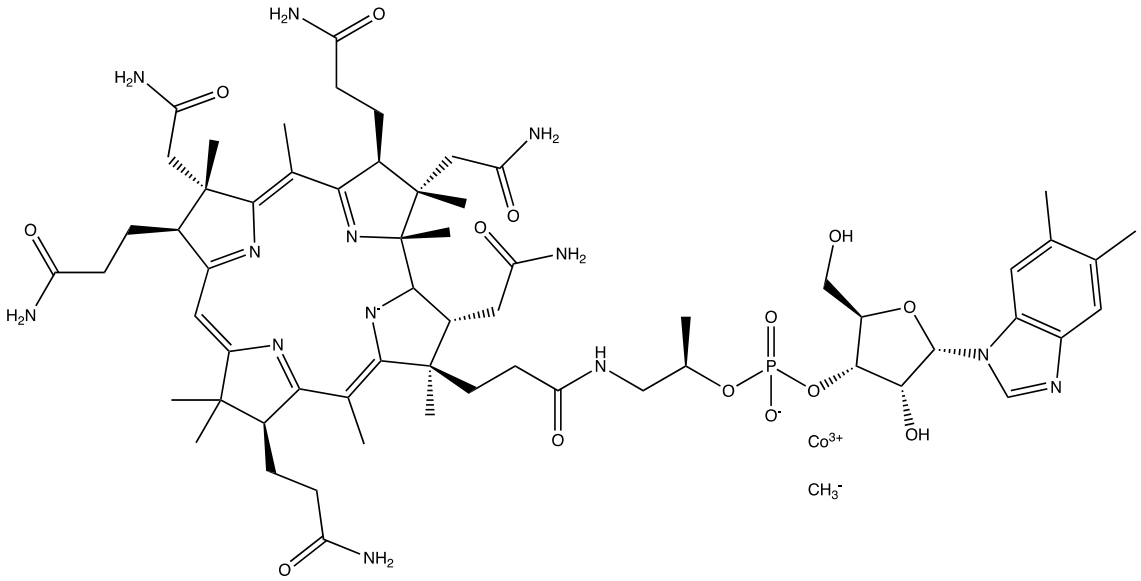


Figure 5: Methylcobalamin from Vitamin B12

Vitamin B12 is also categorized as a tau polymerization inhibitor. Vitamin B12 deficiency is linked to inactivation of PP2A and therefore, subsequent phosphorylation and aggregation of tau protein [206]. It has been hypothesized that vitamin B12 binds to tau protein apart from playing a role in activating PP2A [207-209]. The addition of methylcobalamin (Figure 5), the active form of vitamin B12, to monomeric tau resulted in quenching of tyrosine fluorescence (tyrosine is the only fluorophore in tau protein, the ring structure of tyrosine enables the electrons to reach higher resonating states upon excitation with light) at 304 nm and also at 360 and 405 nm [206]. Such

spectroscopic changes are observed due to the interaction of methylcobalamin with the thiol groups and formation of thiolactocobalamine, which might suggest that there are intermolecular interactions with cysteine residues of tau [206]. To validate the hypothesis that there are cysteine interactions of tau, the authors used cysteine blocked tau with methylcobalamin and observed no spectroscopic changes at 360 and 405 nm. This data reinforced their hypothesis that vitamin B12 binds to the protein tau via interaction with the cysteine residues. From these experiments, it could be concluded that tau binds to vitamin B12 via interaction at the cysteine residues to form a tau/vitamin B12 complex that hinders the formation of stacking of tau monomers and formation of fibrils [206]. However, all these experiments were performed *in vitro* and hence it is yet unknown whether vitamin B12 will show the same efficiency against tau aggregates *in vivo*.

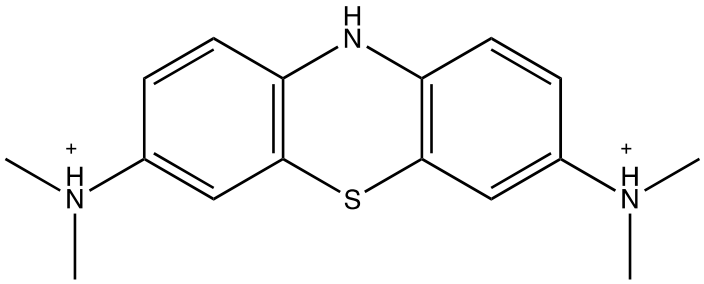


Figure 6: Structure of LMTX

In recent years, methylene blue (MB) or methylthionium chloride has been repurposed as medical treatment for tau pathologies [210]. In 1996, Wischik et al. reported phenothiazines such as methylene blue reverse the proteolytic stability of the protease-resistant tau aggregates by inhibiting the tau-tau interaction at the hexapeptide motifs of the MTBR domain [211]. MTC is a tricyclic phenothiazine derivative which exists in equilibrium between reduced LMT (leuco-mrthylthionium chloride) and oxidized form (MT+) [212]. MTC has been used previously to treat malaria, methemoglobinemia and depression [213]. Due to its long history of prior clinical use, it was used in tau aggregation inhibitor studies. MTC efficiently crosses the blood brain barrier and selectively penetrates neurons – particularly the hippocampal cells after systemic administration.

In various studies, MTC has shown to interfere with the tau-tau binding that is required to form aggregates. However, there are fewer results reported for efficacy in tau-tau aggregation in *in vivo* studies. In a recent study, 6 weeks of oral treatment with MTC did not show reversal of the NFT pathology in the transgenic AD mouse model rTg4510, which develops a robust tangle pathology [214]. But even though scientists failed to obtain positive results in cases where MTC was administered in the presence of preformed filaments, MTC reduced tau levels without affecting the insoluble tau levels in JNPL3 (P301L) transgenic pretangle mice [215]. This showed that MTC can reduce the soluble tau levels when insoluble aggregates have not yet formed and thus has potential for further studies [186]. Reduced forms of MTC are functionally stable in the human brain. However, it is not easy to obtain the reduced form of MTC. A stable reduced version of MTC (leucomethylthioninium with a suitable counter ion, LMTX or Trx0237) (Figure 6) has been developed which has better tolerability and absorption than MTC. Trx0237 is the successor of Rember™ which is a methylthionium chloride (MTC) derivative compound. [171] LMTX or Trx0237™ is currently undergoing clinical trial phase 3 [216, 217].

The second class of TAIs, the noncovalent TAIs, interact with tau species non-covalently through multiple mechanisms and with multiple different structures [210, 218]. However, the mechanism by which most of the TAIs work has not yet been elucidated. The cross β spines share a common structural feature which is termed a "steric zipper" where side chains from the two β sheets form a tightly interlocking dehydrated interface such that the resulting β sheet bilayer forms a fundamental building block of fibrillary aggregates [219]. It has been hypothesized that non-covalent TAIs may block the formation of steric zipper structures (which play a role in the formation of the β sheets) or that they can influence reconfiguration of the tau molecule.

Apart from MTC derivatives, which have gained attention over the past few years, there are other small molecules which are naturally or synthetically derived and could be developed as commercially available potential tau aggregation inhibitors. Several synthetic and natural products with the common structural feature of containing at least one aromatic ring have recently gained attention due to their ability to inhibit tau aggregates induced by heparin or by arachidonic acid *in vitro*.

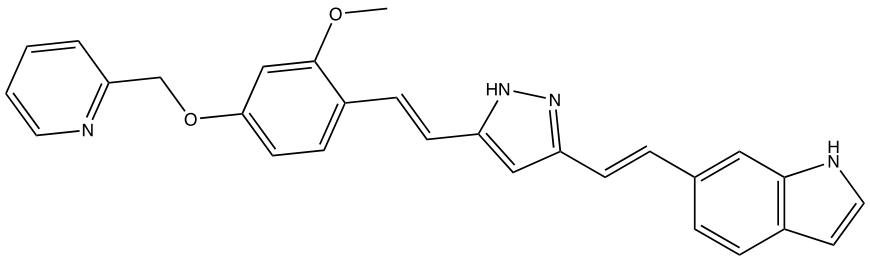


Figure 7: Structure of PE859, a curcumin derivative

One such TAI is PE859 (a curcumin derivative) which was observed to inhibit heparin induced full length tau and 3R tau oligomers *in vitro* (Figure 7) [220]. The IC_{50} (the concentration of compound required to provide 50% inhibition of tau aggregation) for full length tau and 3-repeat tau with PE859 is 2.23 μM and 0.81 μM respectively [220]. This suggests that smaller doses of PE859 can induce disassembly as well as inhibit the formation of tau aggregates as lower doses can get easily absorbed by the immune system and it can be expected that it will not induce any unexpected unwanted immune response which might have adverse side effects. Upon evaluation of rotarod performance of JNPL3 mice placed on placebo along with JNPL3 mice on oral administration of PE859, it was observed that the JNPL3 mice dosed with PE859 had less decline in motor coordination compared to untreated mice [220]. Therefore, PE859 might have played a role in delaying onset and progression of the motor dysfunction. With further investigation, it was also found that mice receiving a dosage of PE859 had lower accumulation of sarkosyl insoluble

tau as well as sarkosyl soluble tau [220]. This result suggested that PE859 showed significant decrease of tau aggregates and resulted in improvement of motor function [220]. Orally administered PE859 was absorbed in blood and 80% of PE859 in blood was transferred to brain, suggesting that PE859 can cross the blood brain barrier and hence can be modified into a drug for CNS disease [220]. However, high doses of PE859 such as 40 mg/kg had to be administered to the mice. Such high doses might elicit an unknown response from the human immune system as the immune system could treat such high amounts of drugs as antigens and degrade them even before they reach the brain. PE859 has not yet been tested on human subjects and therefore, the possibility of development of this small molecule into a drug is yet to be studied.

Studies conducted by Comejo et. al., have observed that rosmarinic acid (Figure 7) prevents tau fibrillization by preventing the assembly of the β sheet structure during the formation of tau aggregates [221]. *Rosmarinus officianalis L*. belongs to the *Lamiaceae* family and has been used as an anti-inflammatory and antimicrobial agent [222, 223]. Five compounds were isolated from *R. officianalis* and all of them were demonstrated to inhibit tau aggregates, but one of the five compounds, named compound 5, was observed to be the most efficient of the compounds. It was also observed that formation of tau aggregates was inhibited due to the interaction of compound 5 with the β sheets [221]. Although, this study demonstrated the ability of derivatives from rosmarinic acid to inhibit tau aggregation, the interaction of compounds with the microtubules and its effect on the stabilization of microtubule by tau has not been evaluated by the authors. Therefore, even though compounds from rosmarinic acid are exciting as TAIs, the possibility of this compound to be modified as a potential drug candidate remains to be seen.

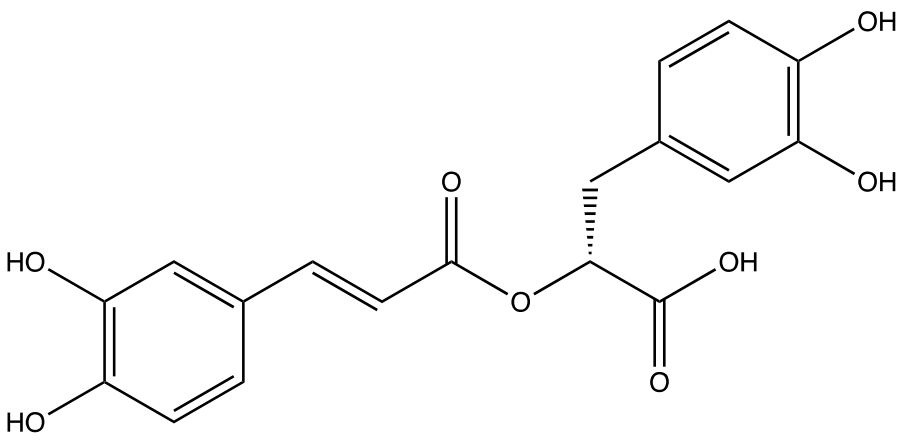


Figure 8: Structure of rosmarinic acid from which compound 5 was isolated.

Fulvic acid (Figure 8) has a variety of nutraceutical properties and is one of the most interesting naturally-occurring phytochemicals with its extremely high antioxidant properties and apparent neuroprotective effect [224-226]. Fulvic acid when used against tau aggregates induced by heparin showed inhibition in formation of tau aggregates as well as it is able to disassemble preformed tau fibrils [224]. Therefore, fulvic acid is a promising compound and subsequent studies have to be performed in order to learn about the efficacy of fulvic acid against tau aggregation *in vivo*.

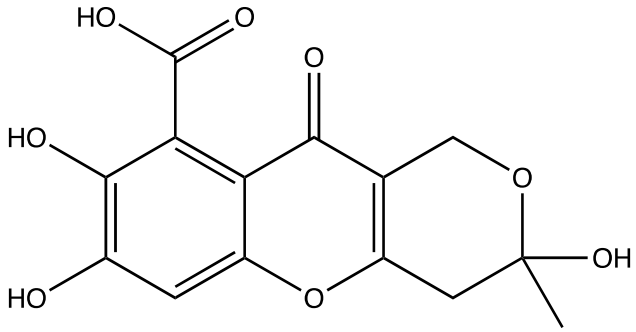


Figure 9: Structure of fulvic acid

Compounds from the family of anthraquinones such as emodin (Figure 9), daunorubicin, and Adriamycin, inhibit PHF formation and disassemble tau aggregates induced by heparin, *in vitro* at IC_{50} and DC_{50} values in the low micromolar range. IC_{50} and DC_{50} values in this range suggest that smaller concentration of these compounds could inhibit formation of tau aggregates

as well as disintegrate pre-formed aggregates, respectively. These compounds were observed not to interfere with the stabilization of microtubules by tau either [227]. Fungi are known to produce natural products which have been a rich source of compounds with medicinal properties. In nature, fungi such as *Aspergillus nidulans* produce secondary metabolites that are enriched with anti-amyloid properties to compete with biofilms (major building blocks of which are amyloid aggregates) produced by bacteria [228]. Although, amyloid proteins differ in sequence, the amyloid fold has common structural features that extend across diverse proteins. Thus, it can be hypothesized that fungal compounds that have anti-amyloid activity can be used to develop compounds which can inhibit tau aggregation. Paranjape et al. conducted studies with secondary metabolites isolated from *A. nidulans* which were screened for compounds with ring structures for their ability to inhibit tau aggregation induced by arachidonic acid *in vitro* [229]. Among the compounds tested, 2, ω-dihydroxyemodin, asperthecin, and asperbenzaldehyde were found to be the most efficient inhibitors of tau aggregation [229]. 2,ω-dihydroxyemodin and asperthecin have similar structures but asperbenzaldehyde represented a new class of TAI (Figure 10). Additionally, asperbenzaldehyde can be converted to azaphilone compounds, some of which have strong lipoxygenase inhibitor activity [199, 229]. Eleven azaphilone compounds were generated by semi-synthetic diversification of asperbenzaldehyde and screened for tau aggregation inhibition activity [184]. A subset of these compounds, namely aza-8, aza-9 (Figure 10), aza-12 and aza-13 could inhibit the formation of tau aggregates and could disassemble pre-formed tau aggregates [199]. Additionally, 4 of these compounds have lipoxygenase activity which could reduce fatty acid metabolites of arachidonic acid and docosahexaenoic acid that are elevated in AD [230]. Therefore, these compounds exhibited the ability to inhibit the formation of tau aggregates as well as reduce preformed tau aggregates and could partially preserve tau's ability to bind to tubulin and

promote microtubule assembly, and therefore they could be used to develop a novel scaffold for TAI molecules [199].

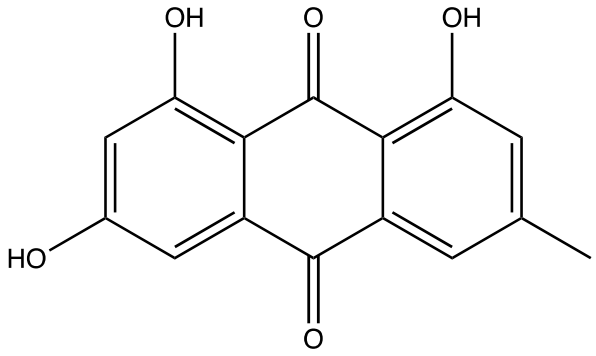
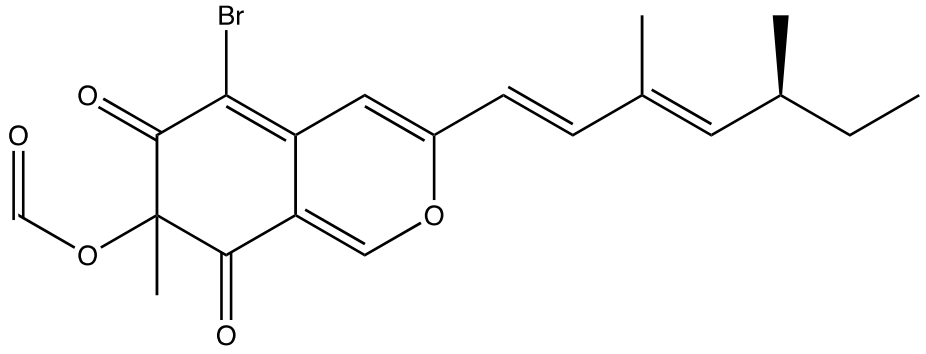


Figure 10: Structure of emodin

Aza 9



Asperbenzaldehyde

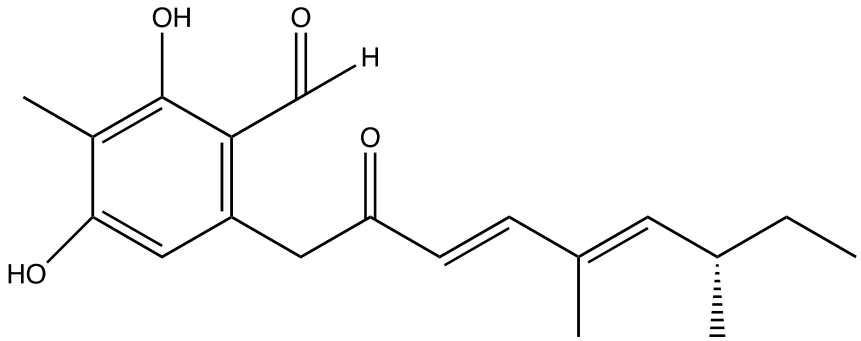


Figure 11: Structures of asperbenzaldehyde and aza-9 which is derived from its precursor asperbenzaldehyde.

4.2.5. Oligonucleotide treatments for tau

Oligonucleotide therapy has been developed over a long period of time and drugs for two neuromuscular diseases has been recently approved by the FDA. In oligonucleotide therapy, anti-sense oligomers that mimic the targeted mRNA are synthesized which will bind to the mRNA encoding a target protein. It destroys the mRNA encoding for the protein and therefore, there is an overall reduction in the content of the target protein [231].

Recently, oligonucleotide treatments for tau have been proposed. Scientists from Washington University School in St. Louis have used a drug named as Tau^{ASO12} on PS-19 mice

models which overexpresses P301S tau. They delivered Tau^{ASO12} via a tiny osmotic pump which funneled the oligonucleotide directly into the mice brain. They stopped the treatment after a month and then they checked the brain tissues after 2 months. They found less tau pre-mRNA in the mice models and in some cases, it also reversed the neurological damage [231]. Therefore, developing antisense oligonucleotides reducing tau content in the brain is a promising technique which can be used for the future development of tau directed therapies.

Chapter 5. Conclusion

The Aβ cascade hypothesis has played a critical role in the types of drugs that have been developed for AD for many years. It is still believed by some that if Aβ deposits could be removed from the brain, AD pathology could be prevented. However, with the failure of several anti-Aβ drugs in clinical trials along with data suggesting that tau aggregation can independantly result in many neurodegenerative disorders, more emphasis is being placed on the development of tau-directed therapies for AD and other tauopathies [232]. Tau belongs to the class of structural microtubule associated proteins, the major function of which is stabilizing and promoting microtubule assembly [2]. However, after post-translational modifications, such as abnormal phosphorylation or truncation, the protein forms toxic oligomers which reduces its propensity to bind microtubules [42]. This results in the formation of NFTs and eventually neurodegeneration. Tau based therapies have gained a lot of attention over the past few years due to their efficiency in some *in vivo* models and this has given some hope that tau-based therapies can be used to treat AD.

Of the different kinds of tau–directed therapeutics developed over the years, using immunotherapy is a difficult way to reverse the neurodegenerative pathology. Monoclonal antibodies are very specific for the protein species. However, it is not known which phosphorylated

protein must be eliminated to stop the onset or progression of AD. In the case of active immunotherapy, there is a higher risk of side effects as the antibody is exogenously delivered. The antibody might be considered as a foreign particle and subsequently degraded. In passive immunotherapies, the human immune system is stimulated to produce the antibodies. This kind of therapy will be effective as long as the antibodies are present in the patient's system. Since this is not a permanent technique, patients have to be injected with vaccines routinely for the rest of their lives. For a recently developed immunotherapy agent, the sample size used during the first clinical trial of AADvac1 was 30 patients and two patients immediately withdrew from the trial due to the side effects of the medication. The sample size was too small to evaluate the efficacy of the vaccine. Moreover, in the initial trial with the vaccine, one dose was given to the patients and it was not enough to evaluate the immune response elicited by the vaccine. Apart from taking into consideration all the above stated facts, the failure of immunotherapy targeting Aβ plaques (as targeting the plaques could not reverse the AD pathology) have shown that even though immunotherapy might seem effective in *in vitro* and *in vivo* studies, it might fail in clinical trials. Hence, even though AADvac1 and ACI-35 seem to be efficient *in vitro* and *in vivo* studies with mice models, its effectiveness in the human brain is still unclear [179].

Similarly, with microtubule stabilizing agents, they can be used to stabilize the microtubules, but the tau aggregates might continue to form. If not properly controlled, microtubule stabilizing agents can hinder mitosis of the cell, resulting in cellular death. Also, as mentioned previously, abnormally phosphorylated tau and aggregated tau can form toxic species that might result in neurodegeneration over the course of time, even with microtubule stabilization.

Protein phosphorylation is one of the major post translational modifications which determines the functionality of many proteins. Phosphorylation of certain residues are essential for

tau to bind to the microtubules. Tau protein kinase inhibitors and phosphatases can be used to regain the phosphatase and kinase balance in the brain and reduce the phosphorylation of tau which leads to the formation of the toxic forms of tau. But kinase inhibitors have to be very specific for the phosphorylated tau they should target. Inhibition of phosphorylation of tau which is necessary for tau to bind to the microtubules will be an undesirable effect. Also, phosphorylation occurs on many proteins. Therefore, kinase inhibitors might have a detrimental effect if they inhibit phosphorylation of proteins other than tau. Phosphatases cause the reduction of hyperphosphorylated tau and thus reduce the formation of NFT-like structures and memory impairment in animal tau models [233]. Phosphatases have a therapeutic potential to treat AD but compounds stimulating phosphatase activity have not yet undergone clinical trials and it is difficult to predict its outcome at the current stage.

Oligonucleotide treatment for tau targets is directed to reduce or completely remove the total tau content in the brain. Because tau binds to tubulins and stabilizes and promotes microtubule assembly, the reduction of tau or complete removal of tau protein from the brain might result in difficulties in the assembly of microtubules which might give rise to unwanted side effects. Delivery of oligonucleotides to the human body is very difficult as they might be treated as foreign particles by the immune system and result in its immediate degradation. Hence, the oligonucleotide strategy has not yet been well established and thus it's viability in *in vivo* models as well human subjects has not been determined yet.

The most promising therapy for AD is tau aggregation inhibition. As has been discussed in the previous section, tau aggregation results in the formation of PHFs and eventually NFTs. Not only that, tau aggregates are found to be toxic in nature. They can induce apoptosis of cells and can even result in paralysis when expressed in mice models or when tau aggregates are introduced

exogenously in mice models. With the success of certain tau aggregation inhibitors *in vitro*, it has become one of the most researched subjects for treating AD and other tauopathies. TAIs have proven to be a valuable asset in treating tauopathies since they can disassemble as well as inhibit tau aggregate formation *in vitro* as well as in animal models of tauopathies. Most of the TAIs found to be efficient with tau aggregates are derived from natural products. Hence, synthesis and production of the TAIs will not be a herculean task if the TAIs can function as is required to control the AD pathology. Different TAIs can target different portions of the tau aggregates. For example: oleocanthal inhibits fibrillization by blocking tau protein in its natively unfolded conformation [202]. LMTs modify tau protein via oxidation of cysteine residues [234]. Other compounds can block the formation of the steric zipper structures which are common in peptides forming the β sheets [235].

Treating AD and other tauopathies is a major challenge in clinical practice. Therefore, scientists and physicians are being encouraged to combine different drugs together to treat Aβ plaques as well NFTs. When memantine was used with acetylcholinesterase inhibitors, it did not prove to be beneficial for the patient [177]. Rather, the side effects were more severe when combined drugs were given to the AD patients. One combination therapy consisting of memantine and donepezil however showed some evidence of synergistic combination effects of symptomatic therapy with combination drugs [236]. When developing new drugs which inhibit tau aggregation in AD, any drug that not only inhibits the formation of toxic tau oligomers but also is able to disassemble pre-existing tau aggregates (since the patient can get diagnosed with AD after NFTs have already started forming), does not interfere with tau's function of stabilizing microtubule assembly and is able to cross the blood brain barrier will be an ideal choice.

However, the role of tau in AD, along with its association with Aβ and its tendency to form aggregates, has to be studied more extensively to better understand and develop more drugs and therapeutics. Research with TAIs has opened up a new field for exploration for scientists as these drugs, if found to be efficient in human subjects, can not only be used to treat AD but also many other tauopathies.

Chapter 6. References

1. Joshi, H.C., et al., Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. Nature, 1992. 356(6364): p. 80-3.
2. Mandelkow, E. and E.M. Mandelkow, Microtubules and microtubule-associated proteins. Curr Opin Cell Biol, 1995. 7(1): p. 72-81.
3. Roberts, A.J., et al., Functions and mechanics of dynein motor proteins. Nat Rev Mol Cell Biol, 2013. 14(11): p. 713-26.
4. Akhmanova, A. and M.O. Steinmetz, Microtubule +TIPs at a glance. J Cell Sci, 2010. 123(Pt 20): p. 3415-9.
5. Schuyler, S.C. and D. Pellman, Microtubule "plus-end-tracking proteins": The end is just the beginning. Cell, 2001. 105(4): p. 421-4.
6. Perez, F., et al., CLIP-170 highlights growing microtubule ends in vivo. Cell, 1999. 96(4): p. 517-27.
7. Mimori-Kiyosue, Y., N. Shiina, and S. Tsukita, The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules. Curr Biol, 2000. 10(14): p. 865-8.
8. Oakley, B.R., V. Paolillo, and Y. Zheng, gamma-Tubulin complexes in microtubule nucleation and beyond. Mol Biol Cell, 2015. 26(17): p. 2957-62.
9. Lin, T.C., A. Neuner, and E. Schiebel, Targeting of gamma-tubulin complexes to microtubule organizing centers: conservation and divergence. Trends Cell Biol, 2015. 25(5): p. 296-307.
10. Kollman, J.M., et al., Microtubule nucleation by gamma-tubulin complexes. Nat Rev Mol Cell Biol, 2011. 12(11): p. 709-21.
11. Teixido-Travesa, N., et al., The gammaTuRC revisited: a comparative analysis of interphase and mitotic human gammaTuRC redefines the set of core components and identifies the novel subunit GCP8. Mol Biol Cell, 2010. 21(22): p. 3963-72.
12. Zheng, Y., et al., Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature, 1995. 378(6557): p. 578-83.
13. Weingarten, M.D., et al., A protein factor essential for microtubule assembly. Proc Natl Acad Sci U S A, 1975. 72(5): p. 1858-62.
14. Neve, R.L., et al., Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. Brain Res, 1986. 387(3): p. 271-80.
15. Goedert, M., et al., Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron, 1989. 3(4): p. 519-26.
16. Andreadis, A., J.A. Broderick, and K.S. Kosik, Relative exon affinities and suboptimal splice site signals lead to non-equivalence of two cassette exons. Nucleic Acids Res, 1995. 23(17): p. 3585-93.
17. Goedert, M., et al., Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. EMBO J, 1989. 8(2): p. 393-9.
18. Delobel, P., et al., Analysis of tau phosphorylation and truncation in a mouse model of human tauopathy. Am J Pathol, 2008. 172(1): p. 123-31.

19. Hanger, D.P., B.H. Anderton, and W. Noble, Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends Mol Med, 2009. 15(3): p. 112-9.
20. Sergeant, N., et al., Biochemistry of Tau in Alzheimer's disease and related neurological disorders. Expert Rev Proteomics, 2008. 5(2): p. 207-24.
21. Lee, V.M., M. Goedert, and J.Q. Trojanowski, Neurodegenerative tauopathies. Annu Rev Neurosci, 2001. 24: p. 1121-59.
22. Liu, F. and C.X. Gong, Tau exon 10 alternative splicing and tauopathies. Mol Neurodegener, 2008. 3: p. 8.
23. Guo, T., W. Noble, and D.P. Hanger, Roles of tau protein in health and disease. Acta Neuropathol, 2017. 133(5): p. 665-704.
24. Mukrasch, M.D., et al., The "jaws" of the tau-microtubule interaction. J Biol Chem, 2007. 282(16): p. 12230-9.
25. Goedert, M. and R. Jakes, Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. EMBO J, 1990. 9(13): p. 4225-30.
26. Jeganathan, S., et al., The natively unfolded character of tau and its aggregation to Alzheimer-like paired helical filaments. Biochemistry, 2008. 47(40): p. 10526-39.
27. Jeganathan, S., et al., Global hairpin folding of tau in solution. Biochemistry, 2006. 45(7): p. 2283-93.
28. Couchie, D., et al., Primary structure of high molecular weight tau present in the peripheral nervous system. Proc Natl Acad Sci U S A, 1992. 89(10): p. 4378-81.
29. Kadavath, H., et al., The Binding Mode of a Tau Peptide with Tubulin. Angew Chem Int Ed Engl, 2018. 57(12): p. 3246-3250.
30. Kadavath, H., et al., Tau stabilizes microtubules by binding at the interface between tubulin heterodimers. Proc Natl Acad Sci U S A, 2015. 112(24): p. 7501-6.
31. Kolarova, M., et al., Structure and pathology of tau protein in Alzheimer disease. Int J Alzheimers Dis, 2012. 2012: p. 731526.
32. Chen, J., et al., Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. Nature, 1992. 360(6405): p. 674-7.
33. He, H.J., et al., The proline-rich domain of tau plays a role in interactions with actin. BMC Cell Biol, 2009. 10: p. 81.
34. Morris, M., et al., The many faces of tau. Neuron, 2011. 70(3): p. 410-26.
35. Sharma, V.M., et al., Tau impacts on growth-factor-stimulated actin remodeling. J Cell Sci, 2007. 120(Pt 5): p. 748-57.
36. Jenkins, S.M. and G.V. Johnson, Tau complexes with phospholipase C-gamma in situ. Neuroreport, 1998. 9(1): p. 67-71.
37. Hwang, S.C., et al., Activation of phospholipase C-gamma by the concerted action of tau proteins and arachidonic acid. J Biol Chem, 1996. 271(31): p. 18342-9.
38. Sanchez-Mejia, R.O., et al., Phospholipase A2 reduction ameliorates cognitive deficits in a mouse model of Alzheimer's disease. Nat Neurosci, 2008. 11(11): p. 1311-8.
39. Ding, H., P.J. Dolan, and G.V. Johnson, Histone deacetylase 6 interacts with the microtubule-associated protein tau. J Neurochem, 2008. 106(5): p. 2119-30.
40. Perez, M., et al., Tau--an inhibitor of deacetylase HDAC6 function. J Neurochem, 2009. 109(6): p. 1756-66.

41. Cho, J.H. and G.V. Johnson, Primed phosphorylation of tau at Thr231 by glycogen synthase kinase 3beta (GSK3beta) plays a critical role in regulating tau's ability to bind and stabilize microtubules. J Neurochem, 2004. 88(2): p. 349-58.
42. Arendt, T., J.T. Stieler, and M. Holzer, Tau and tauopathies. Brain Res Bull, 2016. 126(Pt 3): p. 238-292.
43. Billingsley, M.L. and R.L. Kincaid, Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. Biochem J, 1997. 323 (Pt 3): p. 577-91.
44. Hasegawa, M., et al., Characterization of mAb AP422, a novel phosphorylation-dependent monoclonal antibody against tau protein. FEBS Lett, 1996. 384(1): p. 25-30.
45. Noble, W., et al., The importance of tau phosphorylation for neurodegenerative diseases. Front Neurol, 2013. 4: p. 83.
46. Ross, C.A. and M.A. Poirier, Protein aggregation and neurodegenerative disease. Nat Med, 2004. 10 Suppl: p. S10-7.
47. Simic, G., et al., Tau Protein Hyperphosphorylation and Aggregation in Alzheimer's Disease and Other Tauopathies, and Possible Neuroprotective Strategies. Biomolecules, 2016. 6(1): p. 6.
48. Abraha, A., et al., C-terminal inhibition of tau assembly in vitro and in Alzheimer's disease. J Cell Sci, 2000. 113 Pt 21: p. 3737-45.
49. Martin, L., et al., Tau protein kinases: involvement in Alzheimer's disease. Ageing Res Rev, 2013. 12(1): p. 289-309.
50. Woodgett, J.R., Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J, 1990. 9(8): p. 2431-8.
51. Leroy, K., et al., Early axonopathy preceding neurofibrillary tangles in mutant tau transgenic mice. Am J Pathol, 2007. 171(3): p. 976-92.
52. Swatton, J.E., et al., Increased MAP kinase activity in Alzheimer's and Down syndrome but not in schizophrenia human brain. Eur J Neurosci, 2004. 19(10): p. 2711-9.
53. Hong, M., et al., Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. J Biol Chem, 1997. 272(40): p. 25326-32.
54. Gomez-Ramos, A., et al., Inhibition of GSK3 dependent tau phosphorylation by metals. Curr Alzheimer Res, 2006. 3(2): p. 123-7.
55. Patzke, H. and L.H. Tsai, Calpain-mediated cleavage of the cyclin-dependent kinase-5 activator p39 to p29. J Biol Chem, 2002. 277(10): p. 8054-60.
56. Shelton, S.B. and G.V. Johnson, Cyclin-dependent kinase-5 in neurodegeneration. J Neurochem, 2004. 88(6): p. 1313-26.
57. Liu, F., et al., Aberrant glycosylation modulates phosphorylation of tau by protein kinase A and dephosphorylation of tau by protein phosphatase 2A and 5. Neuroscience, 2002. 115(3): p. 829-37.
58. Patrick, G.N., et al., Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature, 1999. 402(6762): p. 615-22.
59. Plattner, F., M. Angelo, and K.P. Giese, The roles of cyclin-dependent kinase 5 and glycogen synthase kinase 3 in tau hyperphosphorylation. J Biol Chem, 2006. 281(35): p. 25457-65.
60. Van den Haute, C., et al., Coexpression of human cdk5 and its activator p35 with human protein tau in neurons in brain of triple transgenic mice. Neurobiol Dis, 2001. 8(1): p. 32-44.

61. Hallows, J.L., et al., Decreased cyclin-dependent kinase 5 (cdk5) activity is accompanied by redistribution of cdk5 and cytoskeletal proteins and increased cytoskeletal protein phosphorylation in p35 null mice. J Neurosci, 2003. 23(33): p. 10633-44.
62. Drewes, G., et al., Microtubule-associated protein/microtubule affinity-regulating kinase (p110mark). A novel protein kinase that regulates tau-microtubule interactions and dynamic instability by phosphorylation at the Alzheimer-specific site serine 262. J Biol Chem, 1995. 270(13): p. 7679-88.
63. Biernat, J., et al., Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. Mol Biol Cell, 2002. 13(11): p. 4013-28.
64. Liu, F., et al., Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. Eur J Neurosci, 2005. 22(8): p. 1942-50.
65. Li, M., A. Makkinje, and Z. Damuni, Molecular identification of I1PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. Biochemistry, 1996. 35(22): p. 6998-7002.
66. Tsujio, I., et al., Inhibitors of protein phosphatase-2A from human brain structures, immunocytological localization and activities towards dephosphorylation of the Alzheimer type hyperphosphorylated tau. FEBS Lett, 2005. 579(2): p. 363-72.
67. Tanimukai, H., I. Grundke-Iqbal, and K. Iqbal, Up-regulation of inhibitors of protein phosphatase-2A in Alzheimer's disease. Am J Pathol, 2005. 166(6): p. 1761-71.
68. Arendt, T., et al., Reversible paired helical filament-like phosphorylation of tau is an adaptive process associated with neuronal plasticity in hibernating animals. J Neurosci, 2003. 23(18): p. 6972-81.
69. Barghorn, S. and E. Mandelkow, Toward a unified scheme for the aggregation of tau into Alzheimer paired helical filaments. Biochemistry, 2002. 41(50): p. 14885-96.
70. Meraz-Rios, M.A., et al., Tau oligomers and aggregation in Alzheimer's disease. J Neurochem, 2010. 112(6): p. 1353-67.
71. Mocanu, M.M., et al., The potential for beta-structure in the repeat domain of tau protein determines aggregation, synaptic decay, neuronal loss, and coassembly with endogenous Tau in inducible mouse models of tauopathy. J Neurosci, 2008. 28(3): p. 737-48.
72. Berriman, J., et al., Tau filaments from human brain and from in vitro assembly of recombinant protein show cross-beta structure. Proc Natl Acad Sci U S A, 2003. 100(15): p. 9034-8.
73. von Bergen, M., et al., Assembly of tau protein into Alzheimer paired helical filaments depends on a local sequence motif ((306)VQIVYK(311)) forming beta structure. Proc Natl Acad Sci U S A, 2000. 97(10): p. 5129-34.
74. Luo, Y., et al., Structural Insight into Tau Protein's Paradox of Intrinsically Disordered Behavior, Self-Acetylation Activity, and Aggregation. J Phys Chem Lett, 2014. 5(17): p. 3026-3031.
75. Andronesi, O.C., et al., Characterization of Alzheimer's-like paired helical filaments from the core domain of tau protein using solid-state NMR spectroscopy. J Am Chem Soc, 2008. 130(18): p. 5922-8.
76. Daebel, V., et al., beta-Sheet core of tau paired helical filaments revealed by solid-state NMR. J Am Chem Soc, 2012. 134(34): p. 13982-9.
77. Santa-Maria, I., et al., Neurotoxic dopamine quinone facilitates the assembly of tau into fibrillar polymers. Mol Cell Biochem, 2005. 278(1-2): p. 203-12.

78. Perez, M., et al., Polymerization of tau into filaments in the presence of heparin: the minimal sequence required for tau-tau interaction. J Neurochem, 1996. 67(3): p. 1183-90.
79. Bugiani, O., et al., Frontotemporal dementia and corticobasal degeneration in a family with a P301S mutation in tau. J Neuropathol Exp Neurol, 1999. 58(6): p. 667-77.
80. Bulic, B., et al., Tau protein and tau aggregation inhibitors. Neuropharmacology, 2010. 59(4-5): p. 276-89.
81. Sawaya, M.R., et al., Atomic structures of amyloid cross-beta spines reveal varied steric zippers. Nature, 2007. 447(7143): p. 453-7.
82. Kanaan, N.M., et al., Pathogenic forms of tau inhibit kinesin-dependent axonal transport through a mechanism involving activation of axonal phosphotransferases. J Neurosci, 2011. 31(27): p. 9858-68.
83. Mirbaha, H., et al., Tau Trimers Are the Minimal Propagation Unit Spontaneously Internalized to Seed Intracellular Aggregation. J Biol Chem, 2015. 290(24): p. 14893-903.
84. Hu, W., et al., Hyperphosphorylation determines both the spread and the morphology of tau pathology. Alzheimers Dement, 2016. 12(10): p. 1066-1077.
85. Combs, B., K. Voss, and T.C. Gamblin, Pseudohyperphosphorylation has differential effects on polymerization and function of tau isoforms. Biochemistry, 2011. 50(44): p. 9446-56.
86. Iqbal, K., F. Liu, and C.X. Gong, Tau and neurodegenerative disease: the story so far. Nat Rev Neurol, 2016. 12(1): p. 15-27.
87. Guillozet-Bongaarts, A.L., et al., Phosphorylation and cleavage of tau in non-AD tauopathies. Acta Neuropathol, 2007. 113(5): p. 513-20.
88. Gamblin, T.C., et al., Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. Proc Natl Acad Sci U S A, 2003. 100(17): p. 10032-7.
89. Arai, T., et al., Identification of amino-terminally cleaved tau fragments that distinguish progressive supranuclear palsy from corticobasal degeneration. Ann Neurol, 2004. 55(1): p. 72-9.
90. Novak, M., J. Kabat, and C.M. Wischik, Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament. EMBO J, 1993. 12(1): p. 365-70.
91. Novak, M., Truncated tau protein as a new marker for Alzheimer's disease. Acta Virol, 1994. 38(3): p. 173-89.
92. Garcia-Sierra, F., et al., Accumulation of C-terminally truncated tau protein associated with vulnerability of the perforant pathway in early stages of neurofibrillary pathology in Alzheimer's disease. J Chem Neuroanat, 2001. 22(1-2): p. 65-77.
93. Vechterova, L., et al., DC11: a novel monoclonal antibody revealing Alzheimer's disease-specific tau epitope. Neuroreport, 2003. 14(1): p. 87-91.
94. Means, J.C., et al., Caspase-3-Dependent Proteolytic Cleavage of Tau Causes Neurofibrillary Tangles and Results in Cognitive Impairment During Normal Aging. Neurochem Res, 2016. 41(9): p. 2278-88.
95. Berry, R.W., et al., Inhibition of tau polymerization by its carboxy-terminal caspase cleavage fragment. Biochemistry, 2003. 42(27): p. 8325-31.
96. Filipcik, P., et al., First transgenic rat model developing progressive cortical neurofibrillary tangles. Neurobiol Aging, 2012. 33(7): p. 1448-56.

97. Zilka, N., et al., Truncated tau from sporadic Alzheimer's disease suffices to drive neurofibrillary degeneration in vivo. FEBS Lett, 2006. 580(15): p. 3582-8.
98. Corsetti, V., et al., NH2-truncated human tau induces deregulated mitophagy in neurons by aberrant recruitment of Parkin and UCHL-1: implications in Alzheimer's disease. Hum Mol Genet, 2015. 24(11): p. 3058-81.
99. Morishima-Kawashima, M., et al., Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments. Neuron, 1993. 10(6): p. 1151-60.
100. Dorval, V. and P.E. Fraser, Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. J Biol Chem, 2006. 281(15): p. 9919-24.
101. Reynolds, M.R., et al., Tau nitration occurs at tyrosine 29 in the fibrillar lesions of Alzheimer's disease and other tauopathies. J Neurosci, 2006. 26(42): p. 10636-45.
102. Martin, L., X. Latypova, and F. Terro, Post-translational modifications of tau protein: implications for Alzheimer's disease. Neurochem Int, 2011. 58(4): p. 458-71.
103. Winklhofer, K.F., J. Tatzelt, and C. Haass, The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. EMBO J, 2008. 27(2): p. 336-49.
104. Rubinsztein, D.C., Tau toxicity feeds forward in frontotemporal dementia. Nat Med, 2016. 22(1): p. 24-5.
105. Myeku, N., et al., Tau-driven 26S proteasome impairment and cognitive dysfunction can be prevented early in disease by activating cAMP-PKA signaling. Nat Med, 2016. 22(1): p. 46-53.
106. Ozcelik, S., et al., Co-expression of truncated and full-length tau induces severe neurotoxicity. Mol Psychiatry, 2016. 21(12): p. 1790-1798.
107. Bondulich, M.K., et al., Tauopathy induced by low level expression of a human brain-derived tau fragment in mice is rescued by phenylbutyrate. Brain, 2016. 139(Pt 8): p. 2290-306.
108. Nieto, A., et al., Tau-related protein present in paired helical filaments has a decreased tubulin binding capacity as compared with microtubule-associated protein tau. Biochim Biophys Acta, 1991. 1096(3): p. 197-204.
109. Canu, N., et al., Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. J Neurosci, 1998. 18(18): p. 7061-74.
110. Nie, C.L., et al., Amyloid-like aggregates of neuronal tau induced by formaldehyde promote apoptosis of neuronal cells. BMC Neurosci, 2007. 8: p. 9.
111. Ait-Bouziad, N., et al., Discovery and characterization of stable and toxic Tau/phospholipid oligomeric complexes. Nat Commun, 2017. 8(1): p. 1678.
112. Gomez-Ramos, A., et al., Extracellular tau promotes intracellular calcium increase through M1 and M3 muscarinic receptors in neuronal cells. Mol Cell Neurosci, 2008. 37(4): p. 673-81.
113. Flach, K., et al., Tau oligomers impair artificial membrane integrity and cellular viability. J Biol Chem, 2012. 287(52): p. 43223-33.
114. Tian, H., et al., Trimeric tau is toxic to human neuronal cells at low nanomolar concentrations. Int J Cell Biol, 2013. 2013: p. 260787.
115. Kumar, S., et al., Stages and conformations of the Tau repeat domain during aggregation and its effect on neuronal toxicity. J Biol Chem, 2014. 289(29): p. 20318-32.
116. Andorfer, C., et al., Cell-cycle reentry and cell death in transgenic mice expressing nonmutant human tau isoforms. J Neurosci, 2005. 25(22): p. 5446-54.

117. Kidd, M., Paired helical filaments in electron microscopy of Alzheimer's disease. Nature, 1963. 197: p. 192-3.
118. Terry, R.D., The Fine Structure of Neurofibrillary Tangles in Alzheimer's Disease. J Neuropathol Exp Neurol, 1963. 22: p. 629-42.
119. Crowther, R.A. and M. Goedert, Abnormal tau-containing filaments in neurodegenerative diseases. J Struct Biol, 2000. 130(2-3): p. 271-9.
120. Goedert, M., et al., Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. Proc Natl Acad Sci U S A, 1988. 85(11): p. 4051-5.
121. Wischik, C.M., et al., Structural characterization of the core of the paired helical filament of Alzheimer disease. Proc Natl Acad Sci U S A, 1988. 85(13): p. 4884-8.
122. Jones, E.L., et al., The extended tau haplotype and the age of onset of dementia in Down syndrome. Dement Geriatr Cogn Disord, 2008. 26(3): p. 199-202.
123. Poorkaj, P., et al., Tau is a candidate gene for chromosome 17 frontotemporal dementia. Ann Neurol, 1998. 43(6): p. 815-25.
124. Gendron, T.F. and L. Petrucelli, The role of tau in neurodegeneration. Mol Neurodegener, 2009. 4: p. 13.
125. Irwin, D.J., Tauopathies as clinicopathological entities. Parkinsonism Relat Disord, 2016. 22 Suppl 1: p. S29-33.
126. Armstrong, M.J., Progressive Supranuclear Palsy: an Update. Curr Neurol Neurosci Rep, 2018. 18(3): p. 12.
127. Neumann, M., et al., Pick's disease associated with the novel Tau gene mutation K369I. Ann Neurol, 2001. 50(4): p. 503-13.
128. Pasquier, F., V. Deramecourt, and F. Lebert, [From Pick's disease to frontotemporal dementia]. Bull Acad Natl Med, 2012. 196(2): p. 431-42; discussion 442-3.
129. Goedert, M., Tau protein and neurodegeneration. Semin Cell Dev Biol, 2004. 15(1): p. 45-9.
130. alz.org, Cost of Alzheimer's to medicare and medicaid, in Alzheimer's & Dementia: The Journal of the Alzheimer's Association. 2018. p. 13, 325-373.
131. Mega, M.S., et al., The spectrum of behavioral changes in Alzheimer's disease. Neurology, 1996. 46(1): p. 130-5.
132. Mendez, M.F., et al., Seizures in Alzheimer's disease: clinicopathologic study. J Geriatr Psychiatry Neurol, 1994. 7(4): p. 230-3.
133. Piaceri, I., B. Nacmias, and S. Sorbi, Genetics of familial and sporadic Alzheimer's disease. Front Biosci (Elite Ed), 2013. 5: p. 167-77.
134. Bird, T.D., Early-Onset Familial Alzheimer Disease, in GeneReviews((R)), M.P. Adam, et al., Editors. 1993: Seattle (WA).
135. Murphy, M.P. and H. LeVine, 3rd, Alzheimer's disease and the amyloid-beta peptide. J Alzheimers Dis, 2010. 19(1): p. 311-23.
136. Bitan, G., et al., Amyloid beta -protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways. Proc Natl Acad Sci U S A, 2003. 100(1): p. 330-5.
137. Dorszewska, J., et al., Molecular Basis of Familial and Sporadic Alzheimer's Disease. Curr Alzheimer Res, 2016. 13(9): p. 952-63.

138. Farrer, L.A., et al., Apolipoprotein E genotype in patients with Alzheimer's disease: implications for the risk of dementia among relatives. Ann Neurol, 1995. 38(5): p. 797-808.
139. Liu, C.C., et al., Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol, 2013. 9(2): p. 106-18.
140. Wong, C.W., V. Quaranta, and G.G. Glenner, Neuritic plaques and cerebrovascular amyloid in Alzheimer disease are antigenically related. Proc Natl Acad Sci U S A, 1985. 82(24): p. 8729-32.
141. Glenner, G.G. and C.W. Wong, Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. 1984. Biochem Biophys Res Commun, 2012. 425(3): p. 534-9.
142. Bloom, G.S., Amyloid-beta and tau: the trigger and bullet in Alzheimer disease pathogenesis. JAMA Neurol, 2014. 71(4): p. 505-8.
143. Guillozet-Bongaarts, A.L., et al., Tau truncation during neurofibrillary tangle evolution in Alzheimer's disease. Neurobiol Aging, 2005. 26(7): p. 1015-22.
144. Gotz, J., et al., Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. Science, 2001. 293(5534): p. 1491-5.
145. Lewis, J., et al., Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science, 2001. 293(5534): p. 1487-91.
146. Morris, G.P., I.A. Clark, and B. Vissel, Inconsistencies and controversies surrounding the amyloid hypothesis of Alzheimer's disease. Acta Neuropathol Commun, 2014. 2: p. 135.
147. John Carrel, E.n., Another Alzheimer's drug flops in pivotal clinical trial, in Financial loss from Abeta pathology. 2017: Science magazine.
148. Braak, H. and E. Braak, Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging, 1995. 16(3): p. 271-8; discussion 278-84.
149. Braak, H. and E. Braak, Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. Brain Pathol, 1991. 1(3): p. 213-6.
150. Roberson, E.D., et al., Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. Science, 2007. 316(5825): p. 750-4.
151. Leroy, K., et al., Lack of tau proteins rescues neuronal cell death and decreases amyloidogenic processing of APP in APP/PS1 mice. Am J Pathol, 2012. 181(6): p. 1928-40.
152. Phillips, J., et al., Difficulties in disclosing the diagnosis of dementia: a qualitative study in general practice. Br J Gen Pract, 2012. 62(601): p. e546-53.
153. Han, L., et al., Tracking cognitive decline in Alzheimer's disease using the mini-mental state examination: a meta-analysis. Int Psychogeriatr, 2000. 12(2): p. 231-47.
154. Spenciere, B., H. Alves, and H. Charchat-Fichman, Scoring systems for the Clock Drawing Test: A historical review. Dement Neuropsychol, 2017. 11(1): p. 6-14.
155. Fu, H., et al., 3D Visualization of the Temporal and Spatial Spread of Tau Pathology Reveals Extensive Sites of Tau Accumulation Associated with Neuronal Loss and Recognition Memory Deficit in Aged Tau Transgenic Mice. PLoS One, 2016. 11(7): p. e0159463.
156. High-tech scan reveals protein in the brain linked to Alzheimer's disease. A special form of PET scanning offers a more certain diagnosis for some, but at a steep out-of-pocket cost. Harv Mens Health Watch, 2014. 18(7): p. 6.

157. Coughlin, D. and D.J. Irwin, Emerging Diagnostic and Therapeutic Strategies for Tauopathies. Curr Neurol Neurosci Rep, 2017. 17(9): p. 72.
158. Choi, Y., et al., Development of tau PET Imaging Ligands and their Utility in Preclinical and Clinical Studies. Nucl Med Mol Imaging, 2018. 52(1): p. 24-30.
159. Courtier, N., et al., A prognostic tool to predict fatigue in women with early-stage breast cancer undergoing radiotherapy. Breast, 2013. 22(4): p. 504-9.
160. Dani, M., P. Edison, and D.J. Brooks, Imaging biomarkers in tauopathies. Parkinsonism Relat Disord, 2016. 22 Suppl 1: p. S26-8.
161. Klunk, W.E., et al., Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. Ann Neurol, 2004. 55(3): p. 306-19.
162. Xia, C.F., et al., [(18)F]T807, a novel tau positron emission tomography imaging agent for Alzheimer's disease. Alzheimers Dement, 2013. 9(6): p. 666-76.
163. Danysz, W. and C.G. Parsons, Alzheimer's disease, beta-amyloid, glutamate, NMDA receptors and memantine--searching for the connections. Br J Pharmacol, 2012. 167(2): p. 324-52.
164. Matsunaga, S., T. Kishi, and N. Iwata, Combination therapy with cholinesterase inhibitors and memantine for Alzheimer's disease: a systematic review and meta-analysis. Int J Neuropsychopharmacol, 2014. 18(5).
165. McGleenon, B.M., K.B. Dynan, and A.P. Passmore, Acetylcholinesterase inhibitors in Alzheimer's disease. Br J Clin Pharmacol, 1999. 48(4): p. 471-80.
166. Cummings, J., et al., Alzheimer's disease drug development pipeline: 2017. Alzheimers Dement (N Y), 2017. 3(3): p. 367-384.
167. alz.org, FDA approved medications for Alzheimer’s Disease. Alzforum, 2017.
168. Hasselmo, M.E., The role of acetylcholine in learning and memory. Curr Opin Neurobiol, 2006. 16(6): p. 710-5.
169. Khoury, R., J. Rajamanickam, and G.T. Grossberg, An update on the safety of current therapies for Alzheimer's disease: focus on rivastigmine. Ther Adv Drug Saf, 2018. 9(3): p. 171-178.
170. Lewerenz, J. and P. Maher, Chronic Glutamate Toxicity in Neurodegenerative Diseases-What is the Evidence? Front Neurosci, 2015. 9: p. 469.
171. Zhou, Y. and N.C. Danbolt, Glutamate as a neurotransmitter in the healthy brain. J Neural Transm (Vienna), 2014. 121(8): p. 799-817.
172. Riedel, G., B. Platt, and J. Micheau, Glutamate receptor function in learning and memory. Behav Brain Res, 2003. 140(1-2): p. 1-47.
173. Newcomer, J.W., N.B. Farber, and J.W. Olney, NMDA receptor function, memory, and brain aging. Dialogues Clin Neurosci, 2000. 2(3): p. 219-32.
174. Robinson, D.M. and G.M. Keating, Memantine: a review of its use in Alzheimer's disease. Drugs, 2006. 66(11): p. 1515-34.
175. Jones, H.E., et al., The effect of treatment with selective serotonin reuptake inhibitors in comparison to placebo in the progression of dementia: a systematic review and meta-analysis. Age Ageing, 2016. 45(4): p. 448-56.
176. Mendez, M.F., Frontotemporal dementia: therapeutic interventions. Front Neurol Neurosci, 2009. 24: p. 168-78.
177. Sahoo, A.K., et al., Features and outcomes of drugs for combination therapy as multi-targets strategy to combat Alzheimer's disease. J Ethnopharmacol, 2018. 215: p. 42-73.

178. Kontsekova, E., et al., First-in-man tau vaccine targeting structural determinants essential for pathological tau-tau interaction reduces tau oligomerisation and neurofibrillary degeneration in an Alzheimer's disease model. Alzheimers Res Ther, 2014. 6(4): p. 44.
179. Novak, P., et al., Safety and immunogenicity of the tau vaccine AADvac1 in patients with Alzheimer's disease: a randomised, double-blind, placebo-controlled, phase 1 trial. Lancet Neurol, 2017. 16(2): p. 123-134.
180. Theunis, C., et al., Efficacy and safety of a liposome-based vaccine against protein Tau, assessed in tau.P301L mice that model tauopathy. PLoS One, 2013. 8(8): p. e72301.
181. Collin, L., et al., Neuronal uptake of tau/pS422 antibody and reduced progression of tau pathology in a mouse model of Alzheimer's disease. Brain, 2014. 137(Pt 10): p. 2834-46.
182. Buee, L., et al., Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Brain Res Rev, 2000. 33(1): p. 95-130.
183. Agadjanyan, M.G., et al., Humanized monoclonal antibody armanezumab specific to N-terminus of pathological tau: characterization and therapeutic potency. Mol Neurodegener, 2017. 12(1): p. 33.
184. Brunden, K.R., et al., Epothilone D improves microtubule density, axonal integrity, and cognition in a transgenic mouse model of tauopathy. J Neurosci, 2010. 30(41): p. 13861-6.
185. Cao, Y.N., et al., Recent advances in microtubule-stabilizing agents. Eur J Med Chem, 2018. 143: p. 806-828.
186. Panza, F., et al., Tau-Centric Targets and Drugs in Clinical Development for the Treatment of Alzheimer's Disease. Biomed Res Int, 2016. 2016: p. 3245935.
187. Vulih-Shultzman, I., et al., Activity-dependent neuroprotective protein snippet NAP reduces tau hyperphosphorylation and enhances learning in a novel transgenic mouse model. J Pharmacol Exp Ther, 2007. 323(2): p. 438-49.
188. del Ser, T., et al., Treatment of Alzheimer's disease with the GSK-3 inhibitor tideglusib: a pilot study. J Alzheimers Dis, 2013. 33(1): p. 205-15.
189. Lovestone, S., et al., A phase II trial of tideglusib in Alzheimer's disease. J Alzheimers Dis, 2015. 45(1): p. 75-88.
190. Friedhoff, P., et al., Rapid assembly of Alzheimer-like paired helical filaments from microtubule-associated protein tau monitored by fluorescence in solution. Biochemistry, 1998. 37(28): p. 10223-30.
191. Kampers, T., et al., RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments. FEBS Lett, 1996. 399(3): p. 344-9.
192. Wilson, D.M. and L.I. Binder, Free fatty acids stimulate the polymerization of tau and amyloid beta peptides. In vitro evidence for a common effector of pathogenesis in Alzheimer's disease. Am J Pathol, 1997. 150(6): p. 2181-95.
193. Gamblin, T.C., et al., Oxidative regulation of fatty acid-induced tau polymerization. Biochemistry, 2000. 39(46): p. 14203-10.
194. Chirita, C.N., M. Necula, and J. Kuret, Anionic micelles and vesicles induce tau fibrillization in vitro. J Biol Chem, 2003. 278(28): p. 25644-50.
195. Goode, B.L., et al., Structural and functional differences between 3-repeat and 4-repeat tau isoforms. Implications for normal tau function and the onset of neurodegenetative disease. J Biol Chem, 2000. 275(49): p. 38182-9.
196. King, M.E., et al., Ligand-dependent tau filament formation: implications for Alzheimer's disease progression. Biochemistry, 1999. 38(45): p. 14851-9.

197. Khan, P., et al., Elucidation of Dietary Polyphenolics as Potential Inhibitor of Microtubule Affinity Regulating Kinase 4: In silico and In vitro Studies. Sci Rep, 2017. 7(1): p. 9470.
198. Taniguchi, S., et al., Inhibition of heparin-induced tau filament formation by phenothiazines, polyphenols, and porphyrins. J Biol Chem, 2005. 280(9): p. 7614-23.
199. Paranjape, S.R., et al., Azaphilones inhibit tau aggregation and dissolve tau aggregates in vitro. ACS Chem Neurosci, 2015. 6(5): p. 751-60.
200. Pickhardt, M., et al., Phenylthiazolyl-hydrazide and its derivatives are potent inhibitors of tau aggregation and toxicity in vitro and in cells. Biochemistry, 2007. 46(35): p. 10016-23.
201. Cisek, K., et al., Structure and mechanism of action of tau aggregation inhibitors. Curr Alzheimer Res, 2014. 11(10): p. 918-27.
202. Li, W., et al., Inhibition of tau fibrillization by oleocanthal via reaction with the amino groups of tau. J Neurochem, 2009. 110(4): p. 1339-51.
203. Casamenti, F., et al., Oleuropein Aglycone: A Possible Drug against Degenerative Conditions. In Vivo Evidence of its Effectiveness against Alzheimer's Disease. J Alzheimers Dis, 2015. 45(3): p. 679-88.
204. Wobst, H.J., et al., The green tea polyphenol (-)-epigallocatechin gallate prevents the aggregation of tau protein into toxic oligomers at substoichiometric ratios. FEBS Lett, 2015. 589(1): p. 77-83.
205. Monti, M.C., et al., Modulation of tau protein fibrillization by oleocanthal. J Nat Prod, 2012. 75(9): p. 1584-8.
206. Rafiee, S., et al., Vitamin B12 Inhibits Tau Fibrillization via Binding to Cysteine Residues of Tau. ACS Chem Neurosci, 2017. 8(12): p. 2676-2682.
207. Wei, W., et al., Folate/vitamin-B12 prevents chronic hyperhomocysteinemia-induced tau hyperphosphorylation and memory deficits in aged rats. J Alzheimers Dis, 2011. 27(3): p. 639-50.
208. Kifle, L., D. Ortiz, and T.B. Shea, Deprivation of folate and B12 increases neurodegeneration beyond that accompanying deprivation of either vitamin alone. J Alzheimers Dis, 2009. 16(3): p. 533-40.
209. Moore, E., et al., Cognitive impairment and vitamin B12: a review. Int Psychogeriatr, 2012. 24(4): p. 541-56.
210. Schafer, K.N., et al., Structural determinants of Tau aggregation inhibitor potency. J Biol Chem, 2013. 288(45): p. 32599-611.
211. Wischik, C.M., et al., Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines. Proc Natl Acad Sci U S A, 1996. 93(20): p. 11213-8.
212. Melis, V., et al., Effects of oxidized and reduced forms of methylthioninium in two transgenic mouse tauopathy models. Behav Pharmacol, 2015. 26(4): p. 353-68.
213. Wainwright, M. and L. Amaral, The phenothiazinium chromophore and the evolution of antimalarial drugs. Trop Med Int Health, 2005. 10(6): p. 501-11.
214. Spires-Jones, T.L., et al., Methylene blue does not reverse existing neurofibrillary tangle pathology in the rTg4510 mouse model of tauopathy. Neurosci Lett, 2014. 562: p. 63-8.
215. Congdon, E.E., et al., Methylthioninium chloride (methylene blue) induces autophagy and attenuates tauopathy in vitro and in vivo. Autophagy, 2012. 8(4): p. 609-22.
216. Chen, Q., et al., Tau-Targeted Multifunctional Nanocomposite for Combinational Therapy of Alzheimer's Disease. ACS Nano, 2018.

217. Baddeley, T.C., et al., Complex disposition of methylthioninium redox forms determines efficacy in tau aggregation inhibitor therapy for Alzheimer's disease. J Pharmacol Exp Ther, 2015. 352(1): p. 110-8.
218. Bulic, B., M. Pickhardt, and E. Mandelkow, Progress and developments in tau aggregation inhibitors for Alzheimer disease. J Med Chem, 2013. 56(11): p. 4135-55.
219. Park, J., B. Kahng, and W. Hwang, Thermodynamic selection of steric zipper patterns in the amyloid cross-beta spine. PLoS Comput Biol, 2009. 5(9): p. e1000492.
220. Okuda, M., et al., PE859, a novel tau aggregation inhibitor, reduces aggregated tau and prevents onset and progression of neural dysfunction in vivo. PLoS One, 2015. 10(2): p. e0117511.
221. Cornejo, A., et al., Rosmarinic acid prevents fibrillization and diminishes vibrational modes associated to beta sheet in tau protein linked to Alzheimer's disease. J Enzyme Inhib Med Chem, 2017. 32(1): p. 945-953.
222. Englberger, W., et al., Rosmarinic acid: a new inhibitor of complement C3-convertase with anti-inflammatory activity. Int J Immunopharmacol, 1988. 10(6): p. 729-37.
223. Aruoma, O.I., et al., An evaluation of the antioxidant and antiviral action of extracts of rosemary and Provencal herbs. Food Chem Toxicol, 1996. 34(5): p. 449-56.
224. Cornejo, A., et al., Fulvic acid inhibits aggregation and promotes disassembly of tau fibrils associated with Alzheimer's disease. J Alzheimers Dis, 2011. 27(1): p. 143-53.
225. Novotny, F.J., J.A. Rice, and D.A. Weil, Characterization of fulvic Acid by laser-desorption mass spectrometry. Environ Sci Technol, 1995. 29(9): p. 2464-6.
226. Wang, C., et al., The evidence for the incorporation of fulvic acid into the bone and cartilage of rats. Sci Total Environ, 1996. 191(3): p. 197-202.
227. Pickhardt, M., et al., Anthraquinones inhibit tau aggregation and dissolve Alzheimer's paired helical filaments in vitro and in cells. J Biol Chem, 2005. 280(5): p. 3628-35.
228. Taglialegna, A., I. Lasa, and J. Valle, Amyloid Structures as Biofilm Matrix Scaffolds. J Bacteriol, 2016. 198(19): p. 2579-88.
229. Paranjape, S.R., et al., Inhibition of Tau aggregation by three Aspergillus nidulans secondary metabolites: 2,omega-dihydroxyemodin, asperthecin, and asperbenzaldehyde. Planta Med, 2014. 80(1): p. 77-85.
230. Chu, J. and D. Pratico, Pharmacologic blockade of 5-lipoxygenase improves the amyloidotic phenotype of an Alzheimer's disease transgenic mouse model involvement of gamma-secretase. Am J Pathol, 2011. 178(4): p. 1762-9.
231. DeVos, S.L., et al., Tau reduction prevents neuronal loss and reverses pathological tau deposition and seeding in mice with tauopathy. Sci Transl Med, 2017. 9(374).
232. Kametani, F. and M. Hasegawa, Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease. Front Neurosci, 2018. 12: p. 25.
233. Kins, S., et al., Reduced protein phosphatase 2A activity induces hyperphosphorylation and altered compartmentalization of tau in transgenic mice. J Biol Chem, 2001. 276(41): p. 38193-200.
234. Wischik, C.M., C.R. Harrington, and J.M. Storey, Tau-aggregation inhibitor therapy for Alzheimer's disease. Biochem Pharmacol, 2014. 88(4): p. 529-39.
235. Ahmad, B. and L.J. Lapidus, Curcumin prevents aggregation in alpha-synuclein by increasing reconfiguration rate. J Biol Chem, 2012. 287(12): p. 9193-9.
236. Hendrix, J.A., et al., Challenges, solutions, and recommendations for Alzheimer's disease combination therapy. Alzheimers Dement, 2016. 12(5): p. 623-30.