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Small Molecules Targeting and Modulating Toxic Tau Oligomeric Strains

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Small Molecules Targeting and Modulating Toxic Tau Oligomeric Strains

by

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Dedication

This work is dedicated to my parents Santo Lo Cascio and Domenica Pagano, Fortunato and all my family. Thank you for your encouragement and unconditional support.

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Small Molecules Targeting and Modulating Toxic Tau Oligomeric Strains

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Abstract: Alzheimer's disease (AD) is one of over 18 different disorders known as tauopathies, characterized by the pathological aggregation and accumulation of tau, a microtubule-associated protein. Tau aggregates are heterogeneous and can be divided into two major groups: large metastable neurofibrillary tangles (NFTs) and oligomers. Recently, it has been shown that tau oligomers are highly toxic *in vitro* and efficient seeds for the propagation of pathology as compared to NFTs. While the toxicity of recombinant tau oligomers has been studied extensively, within the same aggregation state, tau exhibits conformational differences, termed tau oligomeric strains. Due to the dynamic nature of these strains, little is currently known about the mechanisms underlying their formation and characteristics. Therefore, modulating their aggregation states and conformations through the use of small molecules could be a powerful therapeutic strategy that targets toxicity regardless of other factors involved in the formation of tau oligomeric strains. Herein, I used biochemical and biophysical *in vitro* techniques to characterize preformed tau oligomers and brain-derived tau oligomers (BDTOs) in the presence and absence of small molecules, including Azure C (AC) and newly synthesized compounds such as heparin like oligosaccharides and curcumin derivatives. Interestingly, AC, heparin like oligosaccharides, and curcumin analogs are able to bind and modulate tau oligomers aggregation pathways resulting in the formation of tau structures with decreased toxicity as assessed in human neuroblastoma SH-SY5Y cell line and primary cortical neuron cultures. These results provide novel insights into tau aggregation and may lead to the discovery of new compounds effective against one or more tau strains. Identification of such active compounds may lay the groundwork for developing novel therapeutic agents as well as advancing the diagnostic field for the detection of toxic tau oligomers and differential diagnosis for tauopathies.

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LIST OF ABBREVIATIONS

AC: Azure C

AD: Alzheimer's disease

AFM: Atomic Force Microscopy

APP: Amyloid Precursor protein

Aβ: Amyloid Beta

AβO: Amyloid Beta oligomers

Aβ56: 56 kDa Aβ oligomers

BBB: Brain Blood Barrier

BDTOs: Brain-Derived Tau Oligomers

Cal: Calebin-A analogs

CBP: CREB –binding protein

Cdk2: Cyclin-dependent kinase2

Cdk5: Cyclin-dependent kinase5

CH: Heterocyclic Curcumin analogs

CL: Curcumin-like derivatives

CNS: Central Nervous System

CREB: cAMP response element-binding protein

DLB: Dementia with Lewy bodies

EGCG: (-) – Epigallocatechin Gallate

ELISA: Enzyme Linked Immunosorbent Assay

FAD: familial Alzheimer's disease

FPLC: Fast Protein Liquid Chromatography

FTD: Frontotemporal dementia

FTDP-17: Frontotemporal dementia with Parkinsonism linked to chromosome 17

GSK3β: Glycogen Synthase Kinase 3β

HDAC6: histone deacetylase 6

HemiC: Hemi-curcuminoids

HPLC: High-Performance Liquid Chromatography

Htau: Human Tau

LMTX: Tau aggregation inhibitor

MAPT: Microtubule-Associated Protein Tau

MARK: Microtubule Affinity Regulating Kinases

MB: Methylene Blue

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NFTs: Neurofrilliray Tangles

O-GlcNAcylation: O-linked β-N-acetyl glucosamination

PBS: Phosphate buffered saline

PHF: Paired helical filaments

PNS: Periferic nervous system

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PP2B: Protein phosphatase 2B

PP2C: Protein phosphatase 2C

PRD: Proline-rich domain

PSA:Puromycin-sensitive aminopeptidase

PSP: Progressive Supranuclear Palsy

RS: Resveratrol

RT: Room Temperature

SDS-PAGE: Sodium Dodecyl Sulphate – PoliAcrylamide Gel Electrophoresis

TAI: Tau aggregation inhibitor

TauO: Tau oligomers

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline, 0.1% Tween 20

TDP-43: TAR DNA-binding protein 43

ThT: Thioflavin T

TOMA: Tau Oligomer Monoclonal Antibody

α-syn: α synuclein

CHAPTER 1. LITERATURE REVIEW

INTRODUCTION

Age-related neurodegenerative disorders are one of the leading causes of death and disability in the elderly population. These diseases are characterized by synaptic dysfunction and progressive neuronal damage as well as cell death. The clinical manifestations depend on the afflicted brain region as well as the number and type of cells damaged. This leads to motor, behavioral and cognitive dysfunctions, along with dementia and psychological disorders with severely debilitating outcomes including the disruption of daily activities. Millions of people worldwide are affected by dementia and it is estimated to reach about 152 million people by 2050 (https://www.alz.co.uk/research/world-report-2018). Alzheimer's disease (AD) is the most common form of dementia and the sixth leading cause of death in the United States. Most AD cases are sporadic, with multiple risk factors, including aging, environmental stress, and diet, which are suggested to play critical pathogenic roles. The remaining AD cases, which account for 5-10% of total cases, are rare but inherited from one generation to the next and are referred to as familial AD (FAD) (Ringman and Coppola 2013). Other age-related neurodegenerative diseases that present symptoms of cognitive decline and dementia are Frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB) as well as diseases clinically classified as primary motor disorders such as Progressive supranuclear palsy (PSP) and Parkinson's disease (PD) (Guo, Noble et al. 2017).

NEURODEGENERATION AND PROTEINOPATHIES

The study of the etiology of neurodegenerative diseases has taken into account many pathological mechanisms involved in these disorders. A common feature of many neurodegenerative diseases is the pathological aggregation and accumulation of abnormal or misfolded proteins in the brain, which are believed to be the major cause of synaptic loss and neuronal death observed in these disorders (Taylor, Hardy et al. 2002). Under physiological conditions, common cellular proteins cannot fold correctly, therefore affecting their ability to carry out cellular and physiological functions. Although "chaperone" molecules recognize and fold abnormal proteins (Ellis 2007, Maiti, Manna et al. 2014), the presence of proteostasis maintenance mechanisms would take care of the proteins that undergo misfolding and adapt conformational changes. The two major systems involved in proteostasis maintenance are the lysosomal autophagy and the ubiquitin-proteasome pathways (Nedelsky, Todd et al. 2008). Lysosomes act to degrade protein aggregates, while the proteasome would degrade ubiquitin-tagged proteins recognized by heat shock proteins (Ciechanover and Kwon 2015). Nevertheless, these mechanisms can be compromised in many neurodegenerative diseases therefore failing to maintain proteostasis, and resulting in misfolding and aggregation of abnormal proteins as well as formation of insoluble and fibrillar amyloid inclusions (Sweeney, Park et al. 2017). Many neurodegenerative diseases including AD, PD, PSP and several others are considered to be proteopathies with one or more different proteins involved in each disorder (Taylor, Hardy et al. 2002, Maiti, Manna et al. 2014).

ALZHEIMER'S DISEASE

Alzheimer's disease is the most prevalent progressive neurodegenerative disease

associated with age and the most common form of dementia discovered in the early 900s (Hardy and Allsop 1991). AD is characterized clinically by progressive loss of memory, language problems, social withdrawal, deterioration of executive functions and eventually death (Citron 2002, Tarawneh and Holtzman 2012). Histopathologically, as Alzheimer's progresses, the brain shrinks dramatically and it is characterized by serious cortex damage, with progressive degeneration of limbic and cortical brain structures, mainly in the temporal lobe (Tarawneh and Holtzman 2012). This atrophy also affects the cortical association areas and the hippocampus, which is critical for the formation of new memories (Jahn 2013). Together with cortical degeneration, it is also possible to observe an enlargement of ventricles and a functional alteration of Wernicke's and Broca's areas (Mesulam, Thompson et al. 2015). The major neuropathological features of AD are synaptic and neuronal degeneration and the presence of amyloid plaques and neurofibrillary tangles (NFTs). The major protein component of the plaques is the amyloid β-peptide (Aβ), which is a 39-42 amino acid peptide that originates from a much larger transmembrane protein, the amyloid precursor protein (APP) (Selkoe 1994), whereas NFTs are composed of hyperphosphorylated forms of the microtubule-binding protein, tau (**Figure 1**).

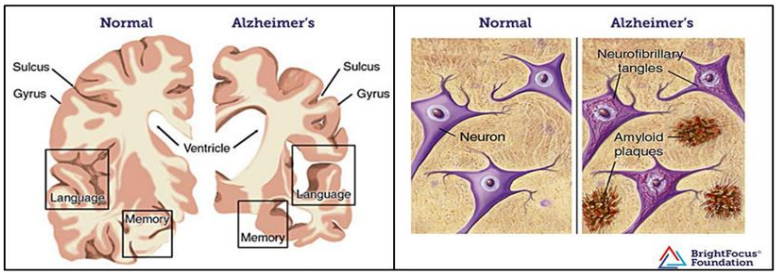


Figure 1.1 Brain cross-sections of normal and AD brains and schematic showing amyloid plaques and neurofibrillary tangles.

These two insoluble protein aggregates are believed to play critical roles in the neurodegenerative process. However, the exact molecular mechanisms by which they cause neurodegeneration has not been established yet. However, it is widely accepted that altered APP expression or proteolytic processing, or changes in Aβ stability and aggregation are involved in AD. These in turn result in a chronic imbalance between Aβ production and clearance. Therefore, Aβ is released and can be accumulated extra- as well as intra-cellularly (Dickson 2004). Various therapeutic strategies have been proposed to reduce amyloid load in AD patients. It has been shown that a chronic reduction in Aβ leads to a reduction in AD pathology as well as improvements in cognitive performance in animal models of the disease and, potentially, in AD patients (Hock, Konietzko et al. 2003). Despite a strong body of evidence supporting an important role of tau in AD (Ballatore, Lee et al. 2007, Haroutunian, Davies et al. 2007, Iqbal, Liu et al. 2009), the amyloid hypothesis (Hardy and Allsop 1991, Hardy and Selkoe 2002) proposes that Aβ is the sole cause of AD and that tau aggregation is one of many downstream events that are triggered by Aβ aggregation and deposition. However, the disappointing outcome of amyloid-reducing pharmacological agents, particularly clinical trials of anti-Aβ immunotherapy (Carlsson 2008) , has revitalized research on the role of tau in AD.

TAU IN NEURODEGENERATION

Tau aggregation plays an important role in many other neurodegenerative diseases. Indeed, neurofibrillary tangles are not exclusive inclusions of AD, as these lesions are also characteristic of other pathologies (Querfurth and LaFerla 2010), collectively referred to as tauopathies including PSP, Pick's disease, PD, FTD and several others (Hutton, Lendon et al. 1998, Irwin 2016). Although, the neuropathological hallmark of this large group of

diseases is the accumulation and deposition of abnormal aggregates of tau in the brain, they are diversified and comprise filamentous neuronal, or neuronal as well as glial tau inclusions, which are found in association with focal neurodegeneration. Interestingly, evidence from post-mortem brains revealed that the pattern of the accumulation of amyloid inclusions, size and appearance differ significantly between individual AD brains and associate poorly with the disease severity. Contrarily, tau pathology develops at specific sites and follows a characteristic pattern depending on the brain regions and cell types affected. Since amyloid pathology, in the absence of NFTs, poorly correlates with cognitive impairment or appreciable neurodegeneration, tau pathology appears to play a causal role in AD. Moreover, NFTs in AD brains more accurately describes the progression of AD pathology and post-mortem brain histopathology can be used to stage AD (Braak and Braak 1991, Braak and Braak 1996, Alafuzoff, Arzberger et al. 2008). Furthermore, mutations in the tau gene, MAPT, cause familial Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), providing evidence of a direct linkage between tau dysfunction and neurodegenerative diseases (Clark, Poorkaj et al. 1998, Hutton, Lendon et al. 1998, Goedert and Spillantini 2000, Pittman, Fung et al. 2006). Moreover, amyloid plaques are not found in individuals with Frontotemporal lobar degeneration-tau, FTLD-Tau. This finding suggested that abnormal aggregation of tau is essential for the neurotoxicity, cognitive and behavioral impairments characteristic of AD and related pathologies. Additionally, aged Htau mice, expressing non-mutant human tau isoforms in the absence of mouse tau, develop NFTs and extensive cell death (Andorfer, Acker et al. 2005). Moreover, mice that conditionally express a mutant human tau gene displayed a progressive accumulation of NFTs in neurons as well as behavioral deficits and neuronal

loss; interestingly, suppressing the mutant tau gene expression restored memory and halted neuronal loss (Santacruz, Lewis et al. 2005). Furthermore, cultured hippocampal neurons from tau knockout mice show to be resistant to β-amyloid-induced cell death, providing direct evidence of tau's role in Aβ-related neurodegeneration in AD (Rapoport, Dawson et al. 2002). Indeed, neurons expressing mouse or human tau proteins showed to be degenerated in the presence of Aβ as assessed by morphological analysis, while tau-depleted neurons displayed no degeneration in the presence of Aβ. Furthermore, decreased endogenous tau improves Aβ-induced deficits in an AD mouse model; mice with normal tau levels showed age-related memory loss, deposition of amyloid plaques as well as behavioral deficits, while mice with decreased tau levels showed a typical pattern of amyloid deposition without memory or behavioral impairments (Ashe 2007, Roberson, Scearce-Levie et al. 2007). Furthermore, decreasing Aβ levels alone by immunotherapy in 3xTg-AD mouse model, which contains both plaques as well as NFTs, did not improve cognitive deficits (Oddo, Vasilevko et al. 2006).

Hence, this evidence and observations using both post-mortem brains as well as animal models suggest that tau aggregation plays a crucial role in AD and related diseases. Therefore, understanding the physiological and pathological function and role of tau is a challenge to identify new therapeutic targets and approaches (Wang and Mandelkow 2016).

MAPT GENE

Human tau is encoded by a single gene, *MAPT*, which is located on the long arm of chromosome 17 (17q21) (**Figure 1.2**).

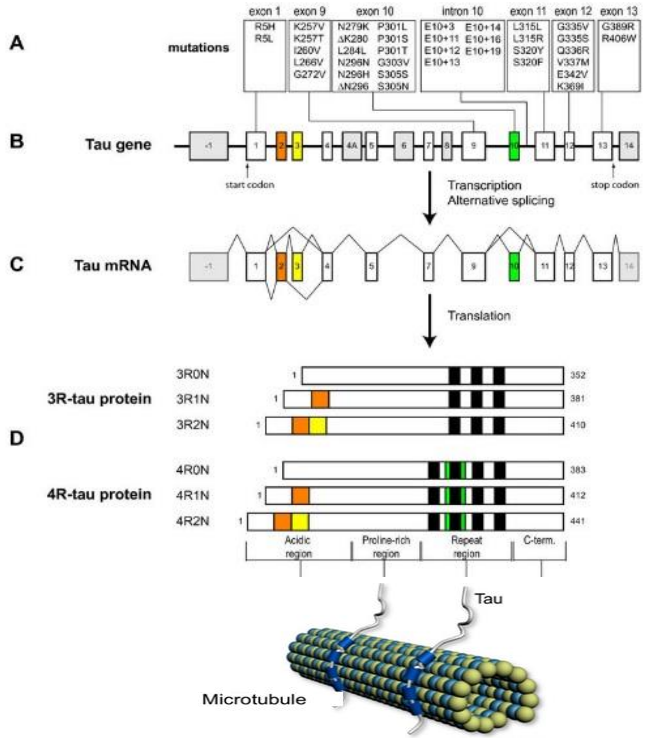


Figure 1.2 Schematic representation of the human tau gene, mRNA and protein isoforms.
The human tau gene is located on chromosome 17q21 and contains 16 exons (**B**). White boxes represent constitutive exons and the grey or coloured boxes represent alternatively spliced exons. Identified mutations in exons 1–13, and intron 10, of the tau gene are shown using the numbering of the 441-amino acid isoform of tau (**A**). Exon -1 is part of the promoter and is transcribed but not translated, as is the case for exon 14 (**C**). Exons 4A, 6 and 8 are not transcribed in human. Exons 2, 3 and 10 are alternatively spliced, as demonstrated by the different lines linking these exons (**C**), generating a total of 6 different mRNAs which are translated into six different tau isoforms (**D**). These isoforms differ by the absence or presence of one or two N-terminal inserts encoded by exon 2 (orange box) and 3 (yellow box), as well as the presence of either three or four repeat regions coded by exons 9, 10, 11 and 12 (black boxes) in the C-terminus. The second repeat, encoded by exon 10, is highlighted in green. (Gendron and Petrucelli 2009)

This gene coding for tau protein is abundantly expressed in the central (CNS) and peripheral (PNS) nervous systems at the axonal level of mature and growing neurons and,

in lower amounts, in oligodendrocytes and astrocytes. Tau has a ubiquitous expression in immature neurons, whereas in mature neurons it is found primarily in the axonal compartment (Hirokawa, Funakoshi et al. 1996). The correct location of tau in axons is important because its presence in the somatodentritic compartment has been seen to be attributable to one of the first signs of neurodegeneration (Braak, Alafuzoff et al. 2006).

The *MAPT* gene is comprised of 16 exons with two non-coding, 0 and 14, and 14 coding or partially coding exons (Andreadis, Brown et al. 1992). In the human CNS, alternative mRNA splicing of exons 2, 3 and 10 gives rise to six tau isoforms ranging in size from 352 to 441 amino acids. Therefore, alternative splicing of exon 10 determines the production of either three (3R) or four (4R) microtubule-binding repeats (Goedert, Spillantini et al. 1989, Spillantini and Goedert 2013). Each repeat comprises 30-31 amino acid sequences and each one is separated from the other by a 13-14 amino acids insert (Lee, Cowan et al. 1988). The ratio between 3R and 4R tau isoforms is approximately 1 in the normal adult brain, thus equal amounts are present in the cerebral cortex of healthy brains (Hong, Zhukareva et al. 1998, Guo, Noble et al. 2017). Tau expression varies in different brain regions; cerebellum has less 0N3R tau isoform compared to other brain regions and globus pallidus show an increased amount of 4R tau isoforms (McMillan, Korvatska et al. 2008, Majounie, Cross et al. 2013). Alternative splicing of *MAPT* exons 2 and 3 results in three isoforms with zero (0N), one (1N) or two (2N) insert of 29 amino acids in the amino terminal region of tau, that are believed to be responsible for the interaction with the plasma membrane (Rademakers, Cruts et al. 2004). Alternative splicing of tau is developmentally regulated; thus, all six tau isoforms are expressed in the CNS of the adult human brain while the isoform 0N3R is the only one to promote microtubules assembly more efficiently

compared to the ones with 3R microtubule-binding repeats (Goedert and Jakes 1990, Trinczek, Biernat et al. 1995). The second and third microtubule binding repeats contain two hexapeptide motifs, VQIINK (known as PHF6*), and VQIVYK (known as PHF6), respectively. These two motifs display high β-sheet propensity and are able to self-assemble without external stimuli (von Bergen, Friedhoff et al. 2000). The fourth and last domain is the carboxyl-terminus (amino acids 370-441) which is common to all six human CNS tau isoforms (Chen, Kanai et al. 1992, Gendron and Petrucelli 2009). The function of this domain or of the proteins that bind to this domain is not well established yet. Nevertheless, some studies have been suggesting that modifications in this domain may affect other tau regions thus influencing both the interaction with and phosphorylation by other proteins (Reynolds, Garwood et al. 2008).

POST-TRANSLATIONAL MODIFICATIONS

During normal development, the microtubule-associated protein tau undergoes many post-translational modifications including hyperphosphorylation, glycosylation, acetylation, ubiquitination, glycation, nitration, and truncation. However, in pathological conditions these modifications may lead to tau self-assembly and aggregation.

Hyperphosphorylation

The most important and disease relevant tau post-translational modification is the hyperphosphorylation, which is regulated during development and can alter tau's biological functions. Tau phosphorylation is high in the fetal human brain and decreases with age because of the phosphatase activation. Phosphorylation can involve at least 85 different sites, including 45 serine, 35 threonine, and 5 tyrosine residues (Hanger, Anderton et al.

2009). Adult human brain contains 2-3 moles of phosphate per mole of tau (Iqbal, Liu et al. 2010). This seems to be the optimal condition for the interaction of tau with tubulin and the consequent microtubules assembly (Lindwall and Cole 1984). However, under pathological conditions, tau phosphorylation is increased resulting in decreased tau affinity for microtubules following cytoskeleton destabilisation, particularly in neurons. It is still unknown which of the many identified tau phosphorylation sites are essential for disease pathogenesis and which ones may become phosphorylated only after the formation of tau pathology. However, tau phosphorylation in the proline-rich region disrupts its microtubule assembly activity inducing a subtle increase in the propensity of tau to self-aggregate, while phosphorylation in the C-terminus region significantly promotes tau self-aggregation (Liu, Li et al. 2007). Moreover, tau phosphorylation not only detaches tau from microtubules but can also induce tau missorting from axons into the somatodendritic compartment, compromising axonal microtubule integrity and inducing synaptic dysfunction (Hoover, Reed et al. 2010). In addition, tau phosphorylation alters its association with interacting partners including the plasma membrane, DNA and Fyn, thus negatively affecting tau function in a range of signalling pathways. Numerous tau kinases have been found such as Glycogen Synthase Kinase 3β (GSK3β), which is highly expressed in neurons and plays an important role both in physiological and pathological conditions (Hanger, Hughes et al. 1992). Other tau kinases include the microtubule-associated regulatory kinase (MARK) (Drewes, Trinczek et al. 1995), cyclin-dependent kinase 2 and 5 (cdk2, cdk5) (Baumann, Mandelkow et al. 1993).

Among the phosphatases involved in tau dephosphorylation, protein phosphatase 2A (PP2A) appears to be the principal tau phosphatase *in vivo* (Goedert, Jakes et al. 1995);

PP1, PP2B and PP2C are also capable of dephosphorylating tau *in vitro* (Buee, Bussiere et al. 2000, Johnson and Stoothoff 2004). Inhibition of tau kinases including GSK3β as well as activation of tau phosphatase such as PP2A (Wang, Grundke-Iqbal et al. 2007), have shown to be beneficial. However, unintended adverse consequences for other proteins and harmful side effects are important unresolved concerns for their use as potential therapeutic strategies (Frost, Meechoovet et al. 2011, Smith, Medda et al. 2012, Mennenga, Gerson et al. 2015).

O-GlcNAcylation

In addition to phosphorylation, tau is also altered by a number of other post-translational modifications. Modulation of O-linked β-N-acetyl glucosamination (*O*-GlcNAcylation) may alter both tau phosphorylation status as well as its aggregation, thus making it a viable target (Fischer 2008, Diwu 2013, Yuzwa, Cheung et al. 2014). Glycated tau has been shown to be abnormally elevated in AD brains as compared to control brains and associated with high toxicity (Yan SD 1995, Ko, Ko et al. 1999, Chen, Wei et al. 2009). Tau N-glycosylation occurs in the hyperphosphorylated form, while the unmodified form can be *O*-glycosylated. O-GlcNAcylation implies the addition of a sugar to Serine/Threonine amino acid residues modifying both nuclear and cytoplasmic proteins with dynamics similar to phosphorylation. In tauopathies, due to impaired intracellular transport and/or glucose metabolism, tau O-GlcNAcylation involves abnormal hyperphosphorylation of the protein(Liu, Iqbal et al. 2004). In addition, *O*-GlcNAcylation has been reported to suppress and slow down tau aggregation, thus the reduction in tau *O*-Glc-NAcylation, observed in AD, brains might contribute to the increased phosphorylation and aggregation of tau protein (Liu, Iqbal et al. 2004).

Acetylation

Acetylation of tau is emerging as an important post-translational modification relevant to both its physiological and pathological functions. Tau acetylation is mediated by cAMP-response element binding protein (CREB)-binding protein (CBP). Similar to phosphorylation, acetylation is associated with site-specific effects on tau that may be either toxic or protective, making its targeting complex. Inhibition of histone deacetylase 6 (HDAC6), an acetylation modifier, have shown to be both beneficial and detrimental as a therapeutic approach against tau aggregation (Ricobaraza A 2009, Fass, Reis et al. 2013, Xiong, Zhao et al. 2013, Cook, Carlomagno et al. 2014, Noack, Leyk et al. 2014).

Nitration

AD patients have increased tau nitration that occurs at 4 sites: Tyrosines 18, 29, 197, and 394. Nitration of these residues have been shown to significantly decrease the binding to the microtubules and, depending on the nitration sites, can either promote or inhibit tau aggregation. This modification may depend on the accumulation of oxidants and represent cerebral oxidative damage (Wang and Liu 2008).

Truncation

Aberrant fragmentation of tau is also associated with increased formation of tau aggregates (Khlistunova, Biernat et al. 2006, Pickhardt, Larbig et al. 2007, Pickhardt M 2007), making it as an important potential mechanism for toxicity in disease. Truncation, occurs at several site-specific tau cleavages, including Glu391 or Asp421, that have also been detected in AD brains and well correlated with the disease progression. Furthermore, mostly of these truncated forms of tau are found in PHF, suggesting that tau truncation may

contribute and enhance tau aggregation in AD brains (Gamblin, Chen et al. 2003, Avila, Lucas et al. 2004). Further research is needed to better understand the upstream modulators of tau aggregation and the efficacy and potential risks of targeting them in disease.

TAU AGGREGATION

In its native functional state, tau is an unfolded monomeric protein playing an important role in stabilizing microtubules as well as in axonal transport. However, in the diseased state, tau is hyperphosphorylated and detached from microtubules due to its decreased affinity, thus resulting in self-aggregation through the two hexapeptide motifs in the repeat domains (Ballatore, Lee et al. 2007, Wang, Xia et al. 2013). Unfolded proteins tend to be in highly disorganized states and would become stable through aggregation (**Figure 1.3**).

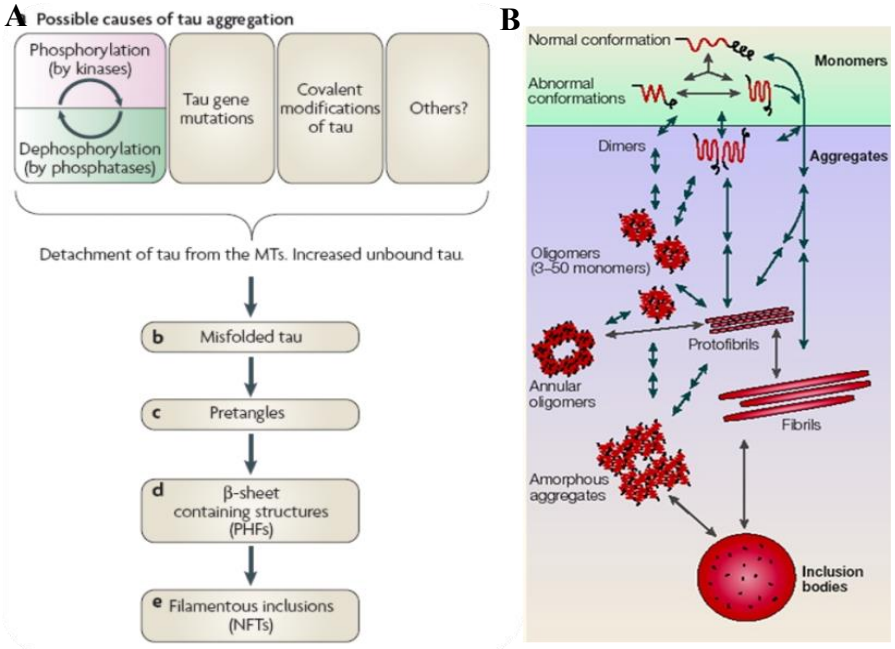


Figure 1.3 Schematic representation of the different stages of the formation of pathological tau aggregates.
A. Abnormal disengagement of tau from the MTs and a concomitant increase in the cytosolic concentration of tau are likely to be the key events that lead to tau-mediated neurodegeneration. Direct causes of abnormal disengagement of tau from the MTs include an imbalance of tau kinases and/or phosphatases, mutations of the tau gene, covalent modification of tau causing and/or promoting misfolding, and possibly other causes such as other post-translational modifications (Ballatore, Lee et al. 2007).
B. Conformational change of the monomer, perhaps with several possible abnormal conformations, initiates the aggregation process. Aggregation begins as soon as there is an association of two or more abnormal proteins or parts of proteins (Ross and Poirier 2005).

Insoluble and intracellular tau aggregates

Once tau detaches from the microtubule, it acquires highly ordered β-sheet structures as it assembles into insoluble, hyperphosphorylated PHF as well as less frequent straight filaments that constitute NFTs in AD and related tauopathies. Hence, tau hyperphosphorylation is thought to be an early event in the cascade leading from soluble to insoluble tau protein. However evidence demonstrating that hyperphosphorylation is sufficient for filament formation is still lacking. Hyperphosphorylation may promote aggregation of tau protein into abnormal filaments due to the negative charge imparted by phosphorylation, which neutralizes the basic charges of tau, thus facilitating intermolecular interaction and aggregation (Alonso, Zaidi et al. 2001). An alternative explanation is that hyperphosphorylation detaches tau from microtubules, thus increasing the pool of unbound tau. Moreover, unbound and hyperphosphorylated tau may compete with microtubules for binding to normal tau and other microtubule associated proteins, thereby sequestering them and enhancing disassembly of microtubules (Alonso, Zaidi et al. 2001). As compared to microtubule-bound tau, unbound tau may be more degradation-resistant and more likely to aggregate. Reduced proteolysis of hyperphosphorylated tau may also increase the pool of soluble tau available for formation of PHF. Thus, abnormal phosphorylation of tau may result in an increase in the total cellular pool of tau, and may change its solubility, thus negatively regulating stability of microtubules (Litersky and Johnson 1992, Litersky, Scott et al. 1993, Litersky and Johnson 1995).

Soluble and extracellular tau aggregates

A growing body of evidence suggests that large metastable tau aggregates, including NFTs, are not causally linked to AD symptoms. Cell death and synaptic lesions

occur independently of NFTs formation in animal models (Andorfer, Acker et al. 2005, Santacruz, Lewis et al. 2005, Berger, Roder et al. 2007, Yoshiyama, Higuchi et al. 2007, Brunden, Trojanowski et al. 2008, Polydoro, Acker et al. 2009, Lasagna-Reeves, Castillo-Carranza et al. 2011, Spires-Jones, Kopeikina et al. 2011, Cowan, Quraishe et al. 2012). Furthermore, NFTs-containing neurons can survive for years in both human and mouse brain (Morsch, Simon et al. 1999, de Calignon, Fox et al. 2010). Synaptic dysfunction and neuronal loss precede or are independent of NFTs formation (Gomez-Isla, Hollister et al. 1997, Terry 2000, Maeda, Sahara et al. 2006, van de Nes, Nafe et al. 2008, Patterson, Remmers et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012), suggesting that other soluble tau oligomeric species exert effects during the early stage of AD and other tauopathies (Gerson and Kayed 2013, Gerson, Castillo-Carranza et al. 2014). Hence, the correlation between NFTs in the brains of AD patients with the disease progression remains contentious (Tabaton, Cammarata et al. 1989, Braak and Braak 1991, Arriagada, Growdon et al. 1992, Bird, Nochlin et al. 1999, Morsch, Simon et al. 1999, Delacourte and Buee 2000, Cash, Aliev et al. 2003, Bretteville and Planel 2008, Congdon and Duff 2008, Hernandez and Avila 2008).

Furthermore, recent studies from biochemical, cell-based and transgenic mouse models, suggest that pre-filament forms of tau may be the most toxic and disease propagating form of tau aggregates (Marx 2007, Brunden, Trojanowski et al. 2008). As seen by other amyloid oligomers, tau oligomers have also been shown to exert their neurotoxic effects when applied extracellularly to cultured neuronal cells and induce an increase in the levels of intracellular calcium (Demuro, Mina et al. 2005, Gomez-Ramos, Diaz-Hernandez et al. 2006, Gomez-Ramos, Diaz-Hernandez et al. 2008). In addition,

evidence using mouse models suggests that tau oligomers play a critical role in initiating the neurodegeneration process that leads to both cognitive and behavioral impairments. Indeed, these phenotypes are concurrent with the accumulation of soluble tau aggregates and not associated with intracellular insoluble tau aggregates (Brunden, Trojanowski et al. 2008). In addition, studies on aged Htau mice expressing non-mutant human tau suggested that cell death does not correlate with NFTs formation (Andorfer, Acker et al. 2005); Furthermore, the P301S mutant human tau transgenic mouse model (P301S Tg) developed hippocampal synapse loss as well as impaired synaptic function and microglia activation before the formation of the fibrillar tau aggregates (Yoshiyama, Higuchi et al. 2007). In addition, tau oligomers were biochemically characterized in the JNPL3 transgenic mice that express human tau with the P301L mutation, and in the transgenic rTg4510 mice that overexpress human tau, carrying as well the P301L mutation. Interestingly, the accumulation of tau oligomers better associated with either neuronal loss or behavioral deficits as compared to NFTs. Taken together, these findings and observations suggest that the accumulation of tau oligomers, behavioral impairments, and neuronal loss precede tangles formation (Sahara, Lewis et al. 2002, Spires, Orne et al. 2006, Berger, Roder et al. 2007). Furthermore, tau oligomers, isolated from post-mortem human brains and biochemically characterized, showed a well correlation between the disease progression and their accumulation in the brains of AD patients. In addition, it has been reported increased levels of tau oligomers in the frontal cortex, occurring at a very early clinical stage of the disease (Braak stage I), when the clinical manifestation of AD symptoms and NFTs are not yet present (Maeda, Sahara et al. 2006, Maeda, Sahara et al. 2007). Therefore, all the above findings and observations suggest that the small, hydrophobic, soluble and

dynamic tau aggregates are believed to be highly toxic *in vitro* and the cause of synaptic as well as mitochondrial dysfunction *in vivo*. Moreover, they are present intra- and extracellularly and are elevated in disease brains, playing a crucial role in neuronal cytopathology (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012).

Tau Strains

The concept of prion-like induction and spreading of pathogenic proteins has been proposed for many neurodegenerative diseases (Polymenidou and Cleveland 2011, Munch and Bertolotti 2012, Walker Lc 2013). Recently, researchers started to consider tau as well as other amyloid proteins, including fibrils of Aβ (Heilbronner G 2013, Lu JX 2013) and α-synuclein (α-syn) (Guo, Covell et al. 2013) as "prion-like" in their characteristics due to their ability to template the misfolding and the aggregation of native protein leading to the formation of distinct conformations that are known as strains. Therefore, within the same aggregation state, tau exhibits conformational differences that could exert diverse downstream effects (Frost and Diamond 2010, Sanders, Kaufman et al. 2014).

Prion-like spread of tau

One of the greatest challenges and point of interest in neurodegenerative tauopathies is determining the mechanism behind the stable propagation of distinct misfolded and pathological tau and the stereotypic spread of tau pathology from initial brain regions throughout the brain in a trans-synaptic pattern as disease progresses (Liu, Drouet et al. 2012). Indeed, *in vivo* studies show that tau conditionally expressed in the

entorhinal cortex and injected tau aggregates can spread throughout the brain along neuroanatomically connected brain areas (Clavaguera, Bolmont et al. 2009, de Calignon, Polydoro et al. 2012, Liu, Drouet et al. 2012) (**Figure 1.4**).

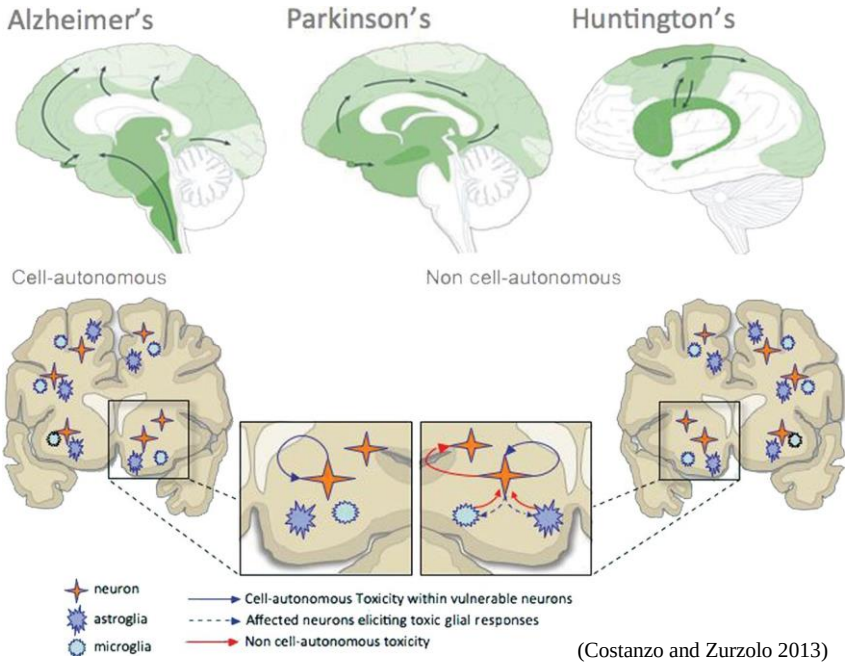


Figure 1.4 Schematic describing the prion-like spread of protein aggregates.

Intracerebral injections of brain extract from the transgenic P301S mice, that exhibits filamentous tau aggregates, induce tau aggregate formation and spreading. Indeed, tau pathology spreads from the injection site to anatomically connected brain regions 15 months post-injection in the transgenic mouse model ALZ17, which express the longest human tau isoform without exhibiting filamentous tau aggregates, and 12 months post-injection in wild-type mice (Clavaguera, Bolmont et al. 2009). Based on these and several others studies, it has been suggested that tau proteins spread in a prion-like mechanisms (Clavaguera, Bolmont et al. 2009, Brundin, Melki et al. 2010, Frost and Diamond 2010). However, the mechanism by through the toxic tau aggregates are moving between cells is

still unclear and more investigations are needed to better understand how tau is released in the extracellular space to be then internalized into neighboring or anatomically connected cells and sub-sequential templated aggregation within those cells. In addition, none of these studies specifically investigate how tau oligomers mechanistically induce seeding and propagation of tau pathology. More evidence has demonstrated that tau can be released by extracellular vesicles including exosomes (Saman, Kim et al. 2012) and ectosomes (Dujardin, Begard et al. 2014).

Understanding how tau strains seed pathological forms of the protein that propagates to different brain regions is critical to devising a solution to either slow or prevent the disease progression.

EXOSOME AND ECTOSOME EXTRACELLULAR RELEASE

Unconventional cellular pathways via vesicles such as exosome and ectosome have been proposed as a mechanism of tau release. Exosomes are small membranous vesicles, ranging from 30 to 100 nm diameter, secreted naturally into the extracellular space upon fusion of multivesicular bodies with the plasma membrane. Recent evidences suggest these extracellular vesicles of endosomal origin may assist in spreading aggregated tau species among neurons pathology. Indeed, tau associated with exosomes has been identified in CSF samples of AD patients (Saman, Kim et al. 2012) and peripheral exosomes isolated from AD brains were able to seed tau aggregation in the brain of normal mice (Winston, Goetzl et al. 2016). In addition, it has also been proposed that microglia-associated exosomes may play a role in the propagation of tau pathology. Abnormal tau aggregates are taken up by microglia and sub-sequentially released via exosomes that are then internalized by cortical neurons leading to tau propagation (Asai, Ikezu et al. 2015).

Additionally, ectosomes seem to be also involved in the spreading of tau. Ectosomes are extracellular vesicles ranging from 50 to 1000 nanometers that are released directly by budding from the plasma membrane (Dujardin, Begard et al. 2014).

Mechanism of tau internalization

Currently, there are very few insights into how tau aggregates are internalized. Internalization mechanisms also include actin-dependent, proteoglycan mediated macropinococytosis (Zeineddine and Yerbury 2015) and receptor-mediated endocytosis including caveolae- and clathrin-mediated endocytosis (Peters, Mironov et al. 2003, Cirrito, Kang et al. 2008) that may play critical roles in the uptake of smaller oligomeric tau, which has not been previously characterized.

MACROPINOCYTOSIS

Macropinocytosis is an internalization mechanism that involves actin polymerization, ruffling the membrane so that it folds back on itself to internalize lipids, extracellular fluid, and receptors (Sarrazin, Lamanna et al. 2011, Zeineddine and Yerbury 2015). Heparan sulfate proteoglycans, HSPGs, are receptors that trigger macropinocytosis and have been implicated in the internalization of tau fibrils and trimers uptake (Holmes, DeVos et al. 2013, Mirbaha, Holmes et al. 2015, Lewis and Dickson 2016). Indeed, pathological tau aggregates bind to HSPGs on the cell surface of the neuron, thus stimulating micropinocytosis and cellular uptake of tau that act as seed for the trans-cellular propagation. Macropinocytosis has been the favoured mechanism for the aggregates internalization due to the size of the internalized vesicle, which is larger than other forms of internalization.

CLATHRIN-MEDIATED ENDOCYTOSIS

Clathrin-mediated endocytosis refers to a mechanism of internalization whereby a ligand is endocytosed with its receptor through an interaction of the receptor with clathrin and adaptors (Godlee and Kaksonen 2013, Robinson 2015). Clathrin assembles into 200 nm vesicles (Traub 2009). Different clathrin molecules interact to form pentagons and hexagons creating a basket around the vesicle. Dynamin is required to separate the vesicle from the membrane (Robinson 2015). Recent evidence has shown that aggregated tau uptake is dynamin dependent and distinct from micropinocytosis that was reported to be the major route for tau aggregates internalization for non-neuronal cells (Holmes, DeVos et al. 2013). There is evidence that the ligand binding to the receptor can initiate clathrin assembly, but this may be cargo dependent, as low density lipoprotein receptor overexpression increased clathrin-mediated endocytosis while transferrin did not (Godlee and Kaksonen 2013). Polymorphisms in the clathrin adaptor protein have been associated with the presence of PHF-tau and increased risk for developing AD (Ando, Brion et al. 2013).

CAVEOLAE-MEDIATED ENDOCYTOSIS

Caveolae are membrane invaginations resulting from the assembly of caveolins, cavins, and other proteins (Cheng and Nichols 2016). Caveolae have been suggested to play a role in mechanical stress protection and sensing due to their ability to flatten under tension (Sinha, Köster et al. 2011, Cheng and Nichols 2016). In addition, caveolae-mediated endocytosis is suggested to be stimulated by receptors or proteins that interact with glycosylphosphatidylinositol and has been implicated in the regulation of lipid composition (Peters, Mironov et al. 2003, Cheng and Nichols 2016). Prion proteins have

been shown to be internalized by caveolae-mediated endocytosis (Peters, Mironov et al. 2003). However, other amyloid proteins have not been investigated. The vesicles that result from caveolae-mediated endocytosis are 50-100 nm in diameter (Richter, Floetenmeyer et al. 2008). Hence, caveolae may play a role in oligomer internalization, due to the smaller size of oligomers.

THERAPEUTICS TARGETING TAU AGGREGATES

The large body of evidence supporting the key role of tau in neurodegenerative diseases suggests the importance of tau as a potential target for the development of successful disease-modifying therapeutics (Ballatore, Lee et al. 2007, Haroutunian, Davies et al. 2007). Unfortunately, the ability of aggregating proteins to spread and multiply makes treatment difficult and highlights the need to diagnose these disorders earlier and more effectively in order to begin treatment prior to the initiation of the massive spread of pathology (Holtzman, John et al. 2011, Singh, Srivastav et al. 2016). While tau is an intracellularly expressed protein, the recent evidence for the presence of extracellular tau aggregates and their importance in the spread suggests that extracellular treatments may be equally important in disease prevention. Targeting extracellular tau aggregates in later disease stages may be of even greater importance to halt the extension of damage. Moreover, environmental conditions in the extracellular space may increase the aggregation potential of tau (Ottaviano, Handy et al. 2008, Di Stasio and De Cristofaro 2010, Bekard, Asimakis et al. 2011, Gerson and Kayed 2016). Thus, strategies targeting the extracellular aggregates responsible for the spread of disease are one of the most promising techniques against tauopathies (**Figure 1.5**).

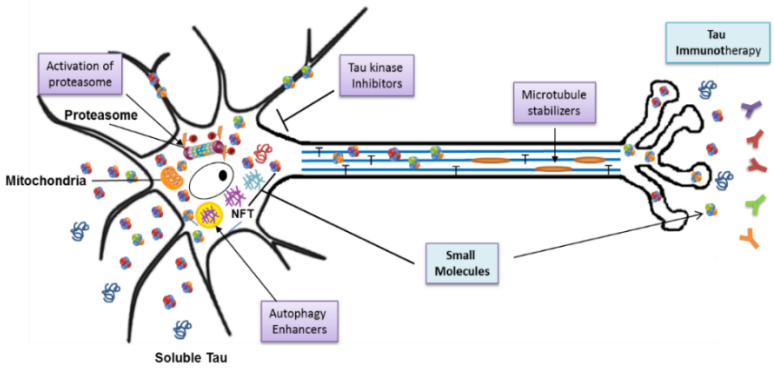


Figure 1.5 Schematic representing therapeutic approaches targeting tau.

Therapeutic approaches targeting tau include: interference with the splicing machinery in order to decrease the four-repeat tau isoforms; activation of autophagic/lysosomal and proteasomal pathways; reduction of tau hyperphosphorylation using inhibitors of tau kinases; pharmacological stabilization of microtubule networks; tau-directed immunotherapy and inhibition of tau aggregation by small molecules.

INHIBITION OF TAU HYPERPHOSPHORYLATION

The inhibition of tau hyperphosphorylation as therapeutic approach to slow down AD pathology was first introduced in 1998 (Gong and Iqbal 2008). A kinase inhibitor was found to be able to reduce tau hyperphosphorylation as well as the formation of soluble toxic tau aggregated and to prevent motor deficits in mice expressing mutant human tau (Iqbal and Grundke-Iqbal 1998). However, a major disadvantage of targeting kinases is the inhibition of the normal physiological functions of these common enzymes and the consequent side effects.

ACTIVATION OF PROTEASOME AND AUTOPHAGOSOME PATHWAYS

Ubiquitin-proteasome and autophagic-lysosomal are the two major pathways used by the cells for turning over dysfunctional proteins. Tau was found to be sensitive to calpain proteolysis (Johnson, Jope et al. 1989) and, recently, a genetic screen recognized the puromycin-sensitive aminopeptidase (PSA) as a potent modifier of tau pathology due to its ability to degrade either recombinant and PHF tau isolated from AD brain (Sengupta, Horowitz et al. 2006).

STABILIZATION OF MICROTUBULES

Microtubule-binding drugs could be beneficial in treating tauopathies by functionally substituting and compensating the loss-of-function of the microtubule-binding protein, tau (Ballatore, Brunden et al. 2012, Quraishe, Sealey et al. 2016). Indeed, tau once abnormally hyperphosphorylated or aggregated is no longer bound and is not able to stabilize the microtubules (Zhang, Maiti et al. 2005). Paclitaxel, which is an approved and well-characterized chemotherapeutic drug that binds and stabilize microtubules, was tested in transgenic mice and showed to restore axonal transport and ameliorating motor impairment (Zhang, Maiti et al. 2005).

TAU CLEARANCE BY IMMUNOTHERAPY

Immunotherapy approaches designed to specifically reduce the most toxic protein aggregates are a promising treatments for neurodegenerative diseases (Castillo-Carranza, Guerrero-Muñoz et al. 2014, Valera, Spencer et al. 2016) (Kontsekova, Zilka et al. 2014, Panza, Solfrizzi et al. 2016). Immunotherapy involves active and passive immunization. In active immunization, the antigen of interest is isolated and administered to activate the immune system, thus to create its own antibodies against the toxin, while in passive immunization, antibodies are developed and administered to patients to compete the antigen of interest (Schneider and Mandelkow 2008, Baxter 2014) (Rajamohamedsait, Rasool et al. 2017). Although, both strategies hold merit, initial human trials of active immunotherapy against Aβ caused severe encephalitis, forcing clinical trials to be halted early and suggesting that similar strategies for tau protein should be approached with caution and careful evaluation of potential autoimmune effects (Dodart, Bales et al. 2002, Orgogozo, Gilman et al. 2003, Masliah, Rockenstein et al. 2011, Mandler, Valera et al.

2014). Indeed, pre-clinical studies have found that active tau immunization induces dangerous levels of inflammation (Rosenmann, Grigoriadis et al. 2006, Rozenstein-Tsalkovich, Grigoriadis et al. 2013). Therefore, passive immunotherapy as alternative is considered harmless and more controllable approach (Castillo-Carranza DL 2013, Gerson, Castillo-Carranza et al. 2014, Pedersen and Sigurdsson 2015, Wisniewski and Goñi 2015) (Moreth, Mavoungou et al. 2013).

Passive immunotherapy targeting tau in the triple transgenic AD mouse model, 3xTg-AD, expressing mutated APP and tau, show to decrease tau pathology by lowering hyperphosphorylated tau and improving cognitive deficits, without decreasing levels of toxic Aβ (Dai CL 2015). As promising as these results are, conflicting studies showing negative effects in AD models with the lowering of total tau have been seen (Dawson, Cantillana et al. 2010).

As tau oligomers are likely the toxic form of tau in disease and may be responsible for the spread of pathology from one brain region to another (Wittmann, Wszolek et al. 2001, Berger, Roder et al. 2007, Lasagna-Reeves, Castillo-Carranza et al. 2011, Sydow, Van der Jeugd et al. 2011, Lasagna-Reeves 2012, Gerson and Kayed 2013), the efficacy of immunization has been evaluated using tau oligomer-specific antibody in two different tau transgenic mouse models and, interestingly, it was found to significantly reduce behavioral deficits without affecting tau monomer or NFTs levels (Castillo-Carranza, Sengupta et al. 2014).

However, previous studies that have showed massively harmful effects by targeting total tau in an APP overexpressing mouse, highlighted the importance of testing tau immunotherapy in additional animal models and not only in tau transgenic mice (Mably,

Kanmert et al. 2015). Crucially, recent findings suggested that targeting tau oligomers in Tg2576 mice overexpressing mutated APP resulted in protection against memory deficits without evidence of side effects or inflammation (Castillo-Carranza, Guerrero-Muñoz et al. 2015). Treatment with tau oligomer-specific antibody lowered both levels of tau oligomers and toxic Aβ aggregate, Aβ*56 (Castillo-Carranza, Guerrero-Muñoz et al. 2015), which are believed to be present early in AD and correlates with tau toxicity and may play a role in synaptic dysfunction (Sokolow, Henkins et al. 2012, Handoko, Grant et al. 2013, Lesne 2013). Furthermore, the ability of a tau oligomer-specific antibody to mediate also Aβ toxicity suggests that passive immunotherapy against oligomeric tau may exert more efficient therapeutic effects in mixed pathology diseases (Eisele, Monteiro et al. 2015) rather than targeting proteins that aggregate upstream of tau alone, such as Aβ and α-syn (Castillo-Carranza, Guerrero-Muñoz et al. 2015, Apicco, Ash et al. 2018). It has also been previously shown that oligomers specifically, but not fibrils, are capable of cross-seeding between different amyloidogenic proteins and that tau and α-syn may co-aggregate in disease (Lasagna-Reeves, Castillo-Carranza et al. 2010, Guerrero-Muñoz, Castillo-Carranza et al. 2014, Sengupta, Guerrero-Munoz et al. 2015, Castillo-Carranza, Guerrero-Munoz et al. 2018). Therefore, depleting tau oligomers may disrupt amyloid structures formed from multiple proteins (Castillo-Carranza, Guerrero-Muñoz et al. 2015, Dai, Tung et al. 2017).

INHIBITION OF TAU AGGREGATION BY SMALL MOLECULES

An alternative and potential approach to the above therapeutic strategies is the use of small molecules that can affect tau aggregation pathways and, consequently, its toxicity

(Paranjape, Riley et al. 2015, Pickhardt, Neumann et al. 2015, Panza, Solfrizzi et al. 2016, Gerson, Cascio et al. 2017). Small molecule compounds can easily cross the blood-brain barrier (BBB) due to their low molecular weight (Banks 2009, Mikitsh and Chacko 2014). Furthermore, they can be modified chemically to increase their binding affinity as well as the solubility and bioavailability. In addition, small molecule inhibitors can be developed to target any molecules regardless of their cellular location since they can pass through the targeting both extracellular and intracellular tau oligomeric species (Narlawar, Pickhardt et al. 2008, Dolai, Shi et al. 2011, Lee, Loo et al. 2013, Mikitsh and Chacko 2014).

Beneficial therapeutic effects of small molecules can include modulation of amyloidogenic protein production (Rezai-Zadeh, Arendash et al. 2008, Lee, Lee et al. 2009), modulation of tau oligomeric species by reversing misfolding, binding intermediates, inhibition of the formation of toxic amyloid oligomers or stimulation of the formation of non-toxic oligomers (Wu, Lei et al. 2006, Ehrnhoefer, Bieschke et al. 2008, Ahmad, Ahmad et al. 2011, Liu, Dong et al. 2011) or stable non-toxic tau fibrils (Cisek, Cooper et al. 2014, Eisele, Monteiro et al. 2015), anti-inflammatory effects (Hatcher, Planalp et al. 2008) as well as antioxidant properties (Hatcher, Planalp et al. 2008, Herczenik and Gebbink 2008, Choi, Lee et al. 2012), among others (Waltner-Law, Wang et al. 2002, Wolfram, Wang et al. 2006). In the last years, tau aggregation inhibitors have been a focus of great interest as potential disease-modifying drugs. The search for non-toxic inhibitors of tau aggregation capable of crossing the BBB was performed using a high throughput screen, which resulted in the identification of more than 139 hits (Pickhardt, von Bergen et al. 2005, Larbig, Pickhardt et al. 2007).

Several small molecules have been demonstrated to affect and interact with tau through the disruption of π-stacking such as polyphenols including natural occurring compounds such as Curcumin, (-) – Epigallocatechin Gallate (EGCG) and Resveratrol, which is extracted from grape seeds and showed attenuation of tau pathology in AD animal models (Pickhardt, von Bergen et al. 2005, Kim, Nguyen et al. 2007, Ladiwala, Lin et al. 2010, Wang, Santa-Maria et al. 2010, Hoppe, Coradini et al. 2013, Patil, Tran et al. 2013, Porquet, Casadesús et al. 2013, Varamini, Sikalidis et al. 2013, Cisek, Cooper et al. 2014, Du, Xie et al. 2014, Huang, Tang et al. 2014, Lee, Shin et al. 2014, Pickhardt, Neumann et al. 2015, Wobst, Sharma et al. 2015). In addition, a number of synthetic small molecules have also been designed to inhibit tau aggregation and toxic outcomes (Boutajangout, Sigurdsson et al. 2011, Calcul, Zhang et al. 2012). Many synthetic small molecules have also been found to inhibit tau aggregation including anthraquinones (e.g. Daunorubicin) and phenothiazines (e.g. Methylene Blue, MB) (Wischik, Edwards et al. 1996, Pickhardt, von Bergen et al. 2005, Bulic, Pickhardt et al. 2010, Schirmer, Adler et al. 2011). *In vivo* studies showed that methylene blue decreases tau pathology and toxic effects in mice and C. Elegans (Fatouros, Pir et al. 2012, Hosokawa, Arai et al. 2012); However, some conflicting results have also been seen (van Bebber, Paquet et al. 2010), which may be due to its pleiotropic nature (Stack, Jainuddin et al. 2014). Methylene blue has been shown also to inhibit the aggregation not only of tau but also of other amyloidogenic proteins including TDP-43, α-syn and Aβ (Necula, Breydo et al. 2007).

Therefore, small molecules may represent a viable treatment for a number of neurodegenerative disorders associated with aggregated tau and other amyloid proteins. However, further investigation is needed in order to confirm that these approaches do not

inhibit fibril formation at the cost of stabilizing the toxic oligomer, as seen in many cases (Schafer, Cisek et al. 2013). Accelerating the tau fibrillization process is a potential effective alternative approach, used previously also in the Aβ field (Cheng, Scearce-Levie et al. 2007). Additionally, combination approaches using both aggregation inhibitors and passive immunotherapy targeting toxic proteins for degradation may be more effective than either approach alone.

CHAPTER 2. AZURE C BINDS AND MODULATES TOXIC TAU OLIGOMER

INTRODUCTION

Synthetic small molecules have been found to inhibit tau aggregation including phenothiazines (e.g. Methylene Blue, MB) (Wischik, Edwards et al. 1996, Pickhardt, von Bergen et al. 2005, Bulic, Pickhardt et al. 2010, Schirmer, Adler et al. 2011). MB is the first tau aggregation inhibitor (TAI) found and is also known as methylthionium chloride. Phase III clinical trials of its reduced form, LMTX, that shows increased absorption compared to MB, are still ongoing (Wischik, Edwards et al. 1996, Wischik, Harrington et al. 2014). It has been previously shown that MB and its mono- and di-N-demethylated derivatives, Azure A and Azure B, respectively, inhibit tau aggregation directly through a reduction/oxidation mechanism of tau cysteine residues (Akoury, Pickhardt et al. 2013, Crowe, James et al. 2013). MB has also been shown to affect tau aggregation through inhibiting the molecular chaperone hsp70 (Martin, Baker et al. 2016).

Another dye, belonging to the family of the phenothiazine as well as methylene blue, is Azure C (AC). It has been previously shown that AC modulates hsp70 ATPase activity, consequently leading to the clearance of tau (Jinwal, Miyata et al. 2009). AC has also been shown to interact with and inhibit Aβ42 oligomerization without inhibiting Aβ42 fibrilization (Necula, Breydo et al. 2007).

In the present study, we investigated and evaluated the direct interaction of AC in targeting and modulating oligomeric tau aggregation pathways.

METHODS

Preparation of Tau Oligomers

Recombinant tau protein (tau-441 (2N4R) MW 45.9 kDa) was expressed and purified as described (Margittai and Langen 2004, Margittai and Langen 2006). The tau pellet was treated with 8M urea followed by overnight dialysis against 1X phosphate-buffered saline (PBS) pH 7.4. Tau concentration was measured using bicinchoninic acid protein assay (Micro BCA kit, Pierce) and normalized to 1 mg/ml by adding 1X PBS. Aliquots of tau monomer in PBS were stored at -20°C Each 300 µl of tau stock (0.3 mg) was added to 700 µl of 1X PBS and incubated for 1 hour on an orbital shaker at room temperature. After shaking, the resulting tau oligomers were purified by fast protein liquid chromatography (FPLC, Superdex 200HR 10/30 column, Amersham Biosciences).

Preparation of Tau Oligomers in presence of Small Molecules

A volume of 100 µl of tau oligomers (1µg/µl) was incubated with Azure C (final concentrations 0.05 — 10µM). AC (Sigma CAS 5321-57-7) and Resveratrol (Sigma CAS 501-36-0) were dissolved in ETOH 75%/DMSO (5:1) at a final concentration of 50 mM and diluted in 1X PBS or ddH_2O for incubation or toxicity assay. Tau oligomers in the presence of the small molecules and controls were incubated on an orbital shaker, without stirring, for 16 hours under oligomerization conditions.

Western Blotting

An amount of 3 µg of each sample were resolved on a pre-cast NuPAGE 4-12% Bis-Tris Gels for SDS-PAGE (Invitrogen) and transferred to nitrocellulose membranes. Then membranes were blocked with 10% nonfat milk in Tris-buffered saline with very low tween 0.01% (TBS-T) overnight at 4°C. Next day, membranes were probed with T22

(1:250) for tau oligomers and Tau 5 (1:10000) for total tau, diluted in 5% nonfat milk for 1 hour at RT. Membranes were then incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) and anti-mouse (1:10000) secondary antibodies to detect, T22 and Tau 5, respectively. ECL plus (GE Healthcare) was used for signal detection.

Reducing condition: Tau oligomers were reduced using 1mM DTT for 30 min at 37°C (Crowe, James et al. 2013). Densitometric analysis of each band was quantified using Image J and analyzed by Student's T-test or two-way ANOVA.

Direct ELISA

ELISA assay was conducted as previously described(Lasagna-Reeves, Castillo-Carranza et al. 2010). Briefly, 96 well plates (Nunc immobilizer, amino modules, Thermo Fisher Scientific Waltham, MA) were previously coated with 1.5 µl of tau oligomers in the presence or absence of Azure C using 50 µl of 1X PBS, pH 7.4, as coating buffer. After washing three times with TBS-T, plates were blocked for 1 hour at 37°C with 120 µl of 10% non-fat milk in TBS-T. Plates were then washed three times with TBS-T, and probed with 100 µl of primary antibodies for 1 hour at 37°C, T22 (diluted 1:250 in 5% non-fat milk in TBS-T) and Tau 5 (diluted 1:10000 in 5% non-fat milk in TBS-T). Plates were then washed three times with TBS-T, and incubated with 100 µl of horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, WI), diluted 1:10000 in 5% non-fat milk in TBS-T, for 1 hour at 37°C. Plates were washed three times with TBS-T and developed with 3, 3, 5, 5-tetramethylbenzidine (TMB-1component substrate, KPL, Gaithersburg, MD). The reaction was stopped using 100 µl of 1M HCl and absorbance was read at 450 nm using POLARstar OMEGA plate reader. All experiments were performed in triplicate.

Bis ANS and Thioflavin T (ThT) Fluorescence

Samples were prepared by adding 2 µl of protein (0.3-0.5 µg/µl) and 248 µl of 10 µM bis-ANS (4,4' dianilino- 1,1' binaphthyl-5, 5' disulfonic acid, dipotassium salt), prepared in 100 mM glycine-NaOH buffer (pH 7.4), in a clear bottom 96-well black plate. Each experiment was performed in triplicate. The bis-ANS fluorescence intensity was measured at an emission wavelength of 520 nm upon excitation at 380 nm. For the ThT assay, samples were prepared using 2 µl of protein (0.3-0.5 µg/µl) and 248 µl of 5 µM ThT, dissolved in 50 mM glycine-NaOH buffer (pH 8.5). Each experiment was performed in triplicate. ThT fluorescence intensity was recorded at an emission wavelength of 490 nm upon excitation at 440 nm using a POLARstar OMEGA plate reader (BMG Labtechnologies). Fluorescence spectra of the following solutions were measured as negative controls for both dyes (bis-ANS and ThT): dye alone, dye + vehicle. In addition, fluorescence spectra of dye + AC, and dye + RS were measured to avoid any false positive readings due to the intrinsic fluorescent properties of AC and RS. Each reading was corrected for the corresponding background fluorescence.

Atomic Force Microscopy

Tau oligomers were characterized by AFM as previously described(Lasagna-Reeves, Castillo-Carranza et al. 2010). Briefly, samples were prepared by adding 10 µl tau oligomers in the absence or presence of AC on freshly-cleaved mica and were allowed to adsorb to the surface. Mica were then washed three times with distilled water to remove unbound protein and impurities followed by air-drying. Samples were then imaged with Multimode 8 AFM machine (Veeco, CA) using a non-contact tapping method (ScanAsyst-Air).

Dot Blot

Dot blot assay to detect tau oligomers in the absence or presence of small molecules was performed as previously described(Lasagna-Reeves, Castillo-Carranza et al. 2010), to detect tau oligomers in the absence and presence of small molecules. Briefly, 1.5 μl of each end-product reaction was applied onto nitrocellulose membranes and then blocked with 10% nonfat milk in TBS-T overnight at 4°C. Next day, membranes were probed with T22 (1:250) for immunoreactivity with tau oligomers and Tau 5 (1:10000) for total tau, diluted in 5% nonfat milk for 1 hour at RT. Membranes were then washed three time with TBS-T and incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) and anti-mouse (1:10000) secondary antibodies to detect, T22 and Tau 5, respectively. Blots were then washed three times in TBS-T and ECL plus (GE Healthcare) was used for signal detection.

Densitometric analysis of each band was quantified using Image J and analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test, performed using GraphPad Prism 6.01.

Filter Trap Assay

Filter Trap assay was performed using Bio-Dot® SF Microfiltration Apparatus (Bio-Rad), following established protocols (Wanker, Scherzinger et al. 1999, Winklhofer, Hartl et al. 2001, Eenjes, Dragich et al. 2016). Briefly, 1 μg of each end-product reaction was applied onto nitrocellulose membranes, previously pre-wetted with TBS-T, through the use of a vacuum based bio-slot apparatus. Membranes were then blocked with 10% nonfat milk in TBS-T overnight at 4°C. Next day, membranes were probed with the oligomer-specific tau antibody, T22 (1:250) and total tau antibody, Tau 5 (1:10000) diluted in 5% nonfat milk

for 1 hour at RT. Membranes were then washed three time with TBS-T and incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) and anti-mouse (1:10000) secondary antibodies to detect, T22 and Tau 5, respectively. Membranes were washed three time in TBS-T and ECL plus (GE Healthcare) was used for signal detection.

Densitometric analysis of each band was quantified using Image J and analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test, performed using GraphPad Prism 6.01.

Cell Toxicity Assay - MTT

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco`s modified Eagle's medium (DMEM) and grown to confluence in 96-well plates. Cells (≈10,000 cells /well) were treated both with 2.0 µM tau oligomers and 2.0 µM tau oligomers pre-incubated with 5µM of Azure C (AC). Cells viability was corrected by the vehicle background. All measurements were performed in triplicate. The cytotoxic effect was determined using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for assessing cell viability following manufacturer's instructions. Optical density (OD) was measured at 490 nm with POLARstar OMEGA plate reader (BMG Labtechnologies). Cell viability was calculated as the percentage of the OD value of treated cells compared with untreated controls, according to the following equation: Viability= (OD SAMPLE/OD CONTROL) x 100. Statistical analysis was based on one-way analysis of variance (ANOVA), performed using GraphPad Prism 6.01.

Immunofluorescence

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco`s modified Eagle's medium (DMEM) and grown to confluence using poli-L-lysine coated coverslip in 24-well plates. Cells (≈20,000 cells /well) were treated for 1 hour with 0.5 µM tau oligomers and 0.5 µM tau oligomers incubated with 5µM of Azure C (AC). Cells were fixed in chilled methanol followed by permeabilization in 0.5% Triton-X 100 diluted in 1X PBS for 10 min. After washing in 1X PBS for 10 min, cells were blocked in goat serum for 1 hour and incubated in Tau 5 (1:500) overnight. The next day, cells were washed three times with 1X PBS and then incubated with goat anti-mouse IgM Alexa-568 (1:700, Invitrogen) for 1 hour. After washing three times with PBS (10 min each), cells were then stained with DAPI (Vector Laboratories) and mounted using Vectashield mounting medium (Fluoromount-4',6-diamidino-2-phenylindole). Cells were imaged with an epifluorescence microscope (Nikon Eclipse 800) using standard Nikon FITC and DAPI filters. Images were analyzed with ImageJ and analyzed by Student's T test, performed using GraphPad Prism 6.01.

Statistical Analysis

All densitometry results are quantified using ImageJ and presented as the mean and standard deviations of all the determinations performed. T22 signal was normalized to the generic tau antibody, Tau 5. Data were analyzed by Student's T test and two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The criterion for statistical significance was $P < 0.05$ using GraphPad Prism software 6.01. Each experiment was performed in triplicate (n=3).

RESULTS AND DISCUSSION

Highly purified tau oligomers (TauO) were incubated with AC at substoichiometric concentrations (final concentrations 0.05-10 μM). Reactions were conducted at room temperature on an orbital shaker, without stirring, for 16 hours under oligomerization conditions as described in the schematic (**Figure 2.1A**) Tau oligomers in the absence of AC were used as control.

Each reaction was assessed using the oligomer-specific antibody, T22, that reacts specifically with tau oligomers and not monomeric or fibrillar tau. T22 immunoreactivity was evaluated by direct enzyme linked immunosorbent assay (ELISA) and dot blot (**Figure 2.1B-D**). The half-maximal activity concentration AC_{50} was determined from dose-response curves (**Figure 2.1B-F**). Incubation of TauO with 5 μM AC resulted in a significant decrease in TauO levels (**Figure 2.1B-D**), confirmed also by filter trap analysis (**Figure 2.1E-F**).

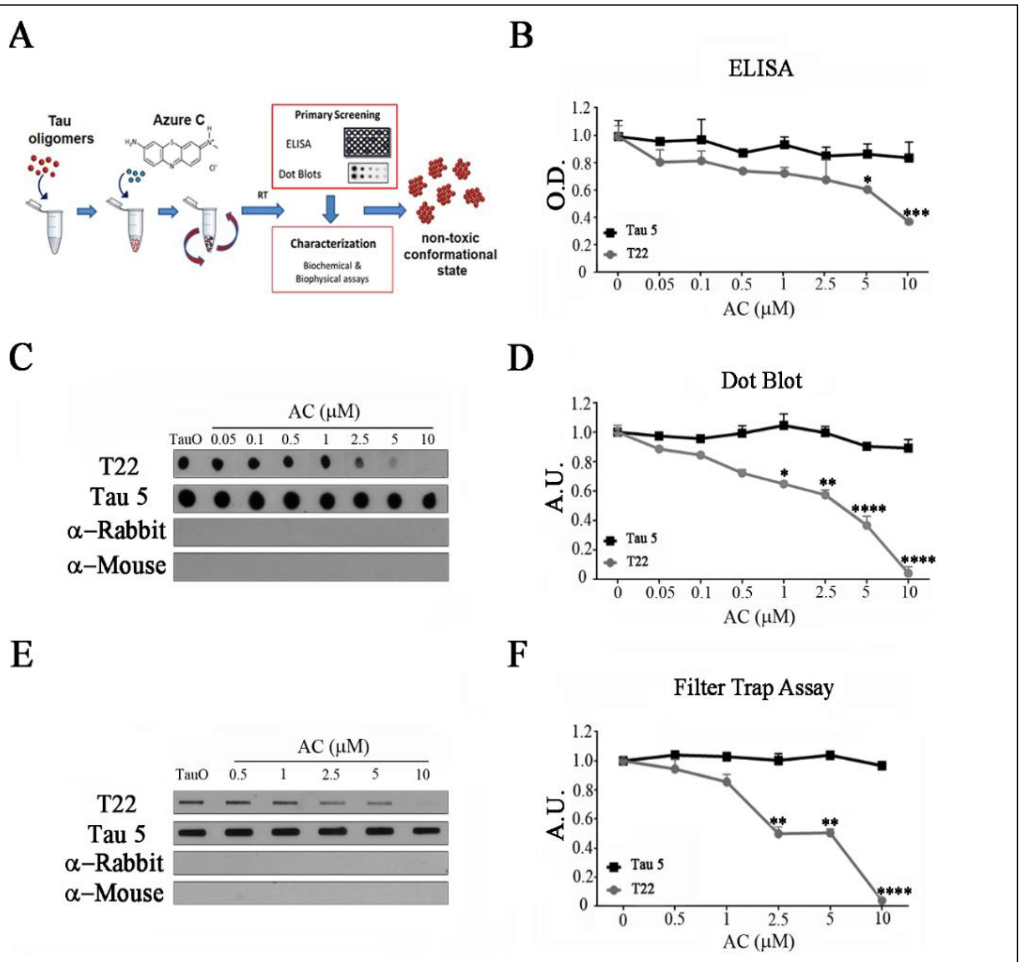


Figure 2.1 Biochemical analyses of oligomeric tau after incubation substoichiometric concentrations of AC.
(**A**) Schematic describing the general approach used to treat preformed tau oligomers with AC. The reactions were conducted at room temperature on an orbital shaker under oligomerization conditions. (**B**) Direct ELISA shows that tau oligomer levels decrease in the presence of micromolar concentration of AC. Tau oligomers decrease significantly in the presence of 5 μM AC. (**C-D**) Dot blot analysis of TauO in the presence of increasing substoichiometric concentrations (0.05-10 μM) of AC probed with T22, Tau 5, or secondary antibodies alone. Data show that incubation with AC decreases tau oligomer levels in a concentration-dependent manner as seen by the reduced T22 immunoreactivity compared to control. Statistics are based on three independent assays. (**E-F**) Filter trap analysis of TauO in the presence of increasing substoichiometric concentrations (0.05-10 μM) of AC probed with T22, Tau 5, or secondary antibodies alone. Tau oligomer levels are significantly decreased with AC (2.5-10 μM) compared to the untreated tau oligomers, while there is no differences in total tau levels. Statistics are based on three independent assays, where each sample was loaded in duplicate. Bars and error bars represent means and standard deviations, respectively (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Based on these results, tau oligomers were incubated in the absence or presence of 5 μM AC under oligomerization conditions for the further experiments. To confirm the effect of AC incubation on TauO, western blot analysis was performed using the anti-oligomeric specific tau antibody, T22, and the total tau antibody, Tau 5 (**Figure 2.2A-C**). The data showed significant reduction of T22 immunoreactivity in the presence of AC compared to the untreated control. Direct ELISA confirmed the significant effects of 5 μM AC on TauO levels at micromolar concentrations (**Figure 2.2D**). Furthermore, MB and its derivatives, Azure A and B, have been shown to act through a reduction/oxidation mechanism (Akoury, Pickhardt et al. 2013). Western blot analysis of tau oligomers treated with AC under reducing conditions revealed that they were identical to the ones probed using non-reducing conditions (**Figure 2.2F**), which indicate that AC does not act through this mechanism. Dialysis was performed for 1, 6, and 24 hours, using spectrum dialysis devices with 1000 Da MW cut off, to remove AC. Western blot analysis of dialyzed samples show no changes as compared to the undialyzed samples dialysis devices with 1000 Da MW cut off to remove AC. Western blot analysis of dialyzed samples show no changes as compared to the undialyzed samples (**Figure 2.2G**).

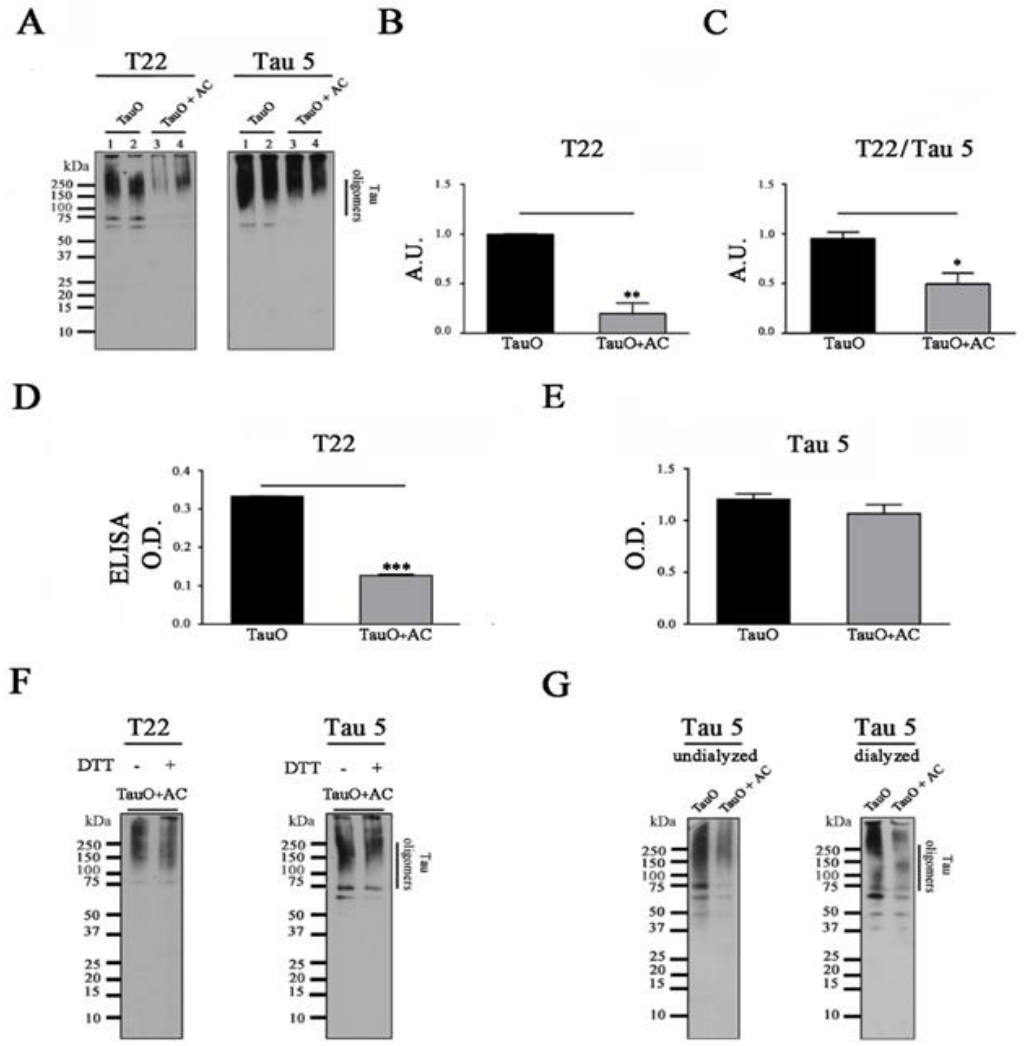


Figure 2.2 Biochemical analyses of oligomeric tau incubated with 5 µM AC.

(**A-C**) Western blot analysis: lanes 1 and 2 tau oligomers (TauO); lanes 3 and 4 tau oligomers incubated with 5 µM AC (TauO+AC) probed with oligomeric and total tau antibodies, T22 and Tau 5, respectively. (B) Results show decreased levels of tau oligomers (75-250 kDa) in the presence of AC, compared to TauO alone control. (C) T22 signal normalized using the generic Tau 5 antibody. (**D-E)** ELISA analysis of oligomeric tau using T22 and Tau 5 antibodies. In the presence of 5 µM AC, tau oligomers levels decreased significantly. **(F)** Western blot analysis of samples under reducing conditions show no differences suggesting that AC effects are independent of tau oxidation/ reduction. **(G)** Western blot analysis show no changes in the samples after 24 hours of dialysis to remove AC. Bars and error bars represent means and standard deviations, respectively (*p<0.05; **p<0.01; ***p<0.001).

Atomic force microscopy (AFM) was performed to characterize the aggregation state of the end product of each reaction and assess their nature.

Tau oligomeric structures images displayed a homogeneous spherical morphology in absence of AC (**Figure 2.3A-B**) while in the presence of AC **(Figure 2.3C-D**), I observed the tendency of tau oligomers to form and assemble into clusters of aggregates. These data are consistent with the 4,4' dianilino- 1,1' binaphthyl-5, 5' disulfonic acid, dipotassium salt (bis-ANS) and Thioflavin T (ThT) fluorescence assays that showed decreased binding of hydrophobic oligomers with bis-ANS as well as the absence of fibril formation with AC incubation, respectively (**Figure 2.3E-F**). Initially, both assays were carried out for 48 hours with measurements being recorded at 4, 8, 16, 24, 36 and 48 hours (**Figure 2.3G-H**). Since no significant changes were observed after 16 hours of incubation, all further experiments were ended at 16 hours. Moreover, to account for any intrinsic AC fluorescence in the bis-ANS and ThT measurements, negative as well as positive controls were used and readings were corrected for the background fluorescence.

Taken together, these results suggest that AC decreases the levels of tau oligomers promoting the formation of clusters of tau aggregates.

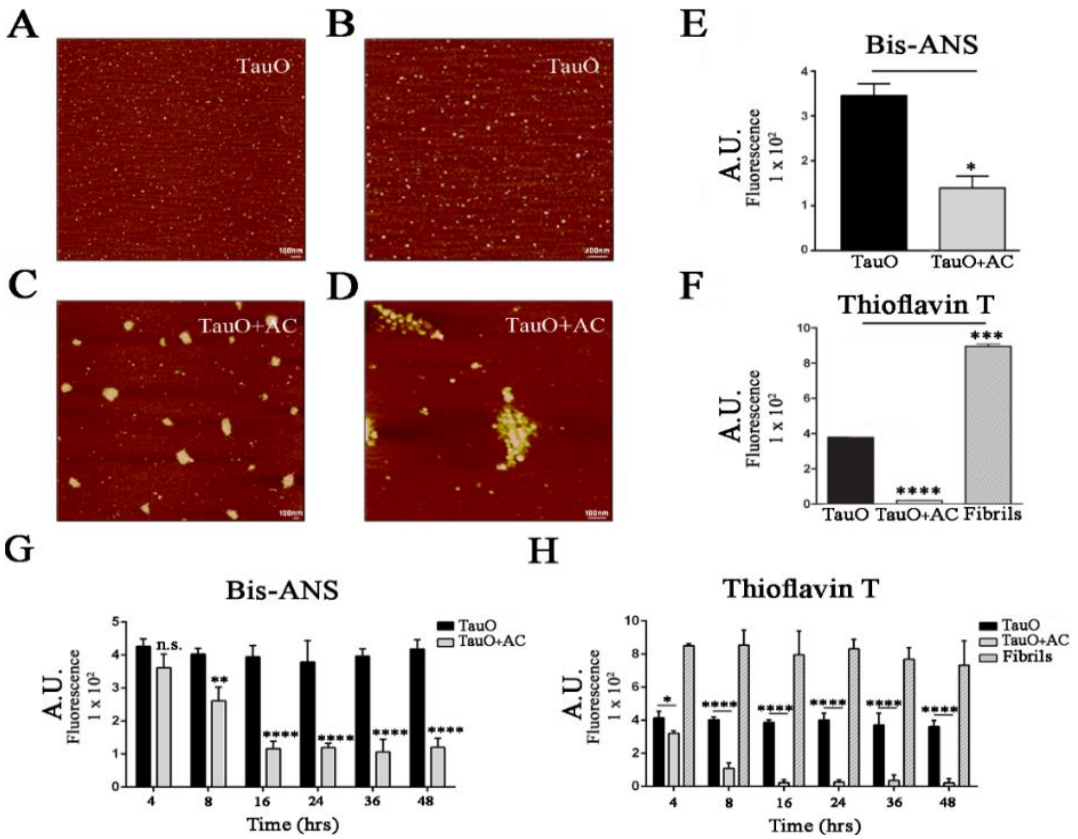


Figure 2.3 Biophysical analyses of oligomeric tau in absence or presence of AC.

(**A-D**) Atomic Force Microscopy images of TauO (A-B) and TauO incubated with 5 μM AC (C-D). AFM images show the ability of AC to form clusters of non-toxic tau aggregates. Scale bars = 100 nm. (**E-F**) Tau oligomers alone or in the presence of 5 μM AC were assessed by bis-ANS and Thioflavin T spectroscopy. Hydrophobic oligomers showed reduced binding with bis-ANS (E) in the presence of AC as compared to oligomers alone. Thioflavin T spectroscopy analysis (F) show no presence of fibrils formation after incubation with AC. (**G-H**) Time course analyses of bis-ANS (G) and Thioflavin T (H). No significant changes were observed after 16 hours of incubation, therefore all experiments were performed at 16 hours incubation. Bars and error bars represent means and standard deviations, respectively (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Next, to evaluate the toxicity of these tau aggregated species resulting from co-incubation of TauO with AC, I used the human neuroblastoma cell line SH-SY5Y.

Our lab has been extensively shown the toxicity of recombinant tau oligomers as well as brain-derived tau oligomers from different tauopathies on cultured SH-SY5Y cells compared to fibrillar and monomeric tau (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2012, Castillo-Carranza, Gerson et al. 2014, Lasagna-Reeves, Sengupta et al. 2014, Gerson, Castillo-Carranza et al. 2016).

Cells were exposed to tau oligomers alone and in the presence of AC (**Figure 2.4**).

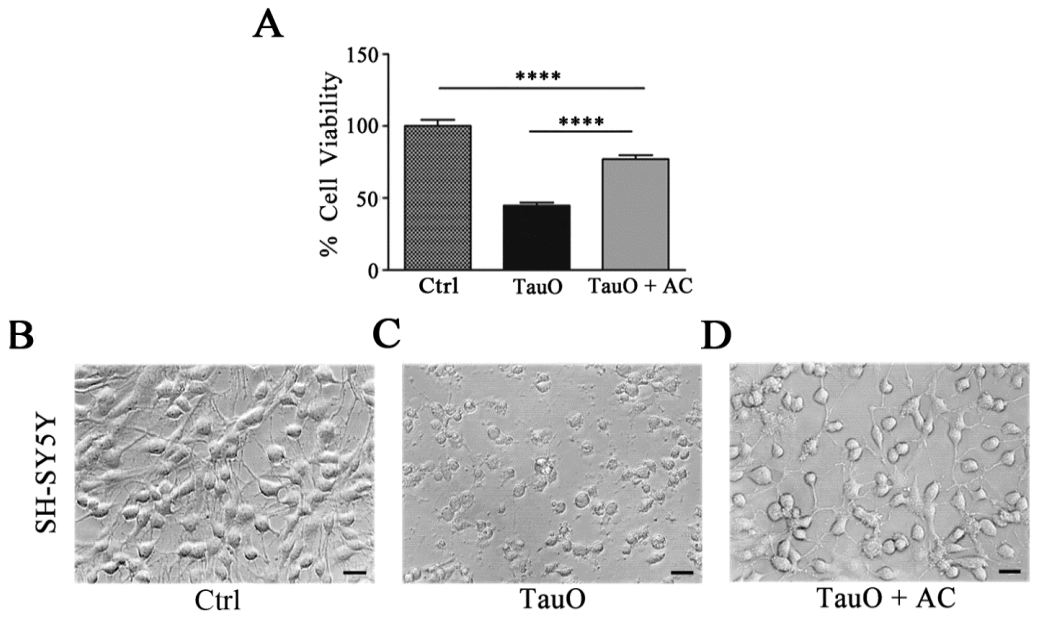


Figure 2.4 SH-SY5Y neuroblastoma cells viability.

(**A**) SH-SY5Y human neuroblastoma cells viability after exposure to 2 µM tau oligomers, or 2 µM tau oligomers with 5 µM AC. Untreated cells were used as control (Ctrl). Treatment of SH-SY5Y cells with TauO+AC had significantly higher cell viability compared to TauO alone. Cell viability was calculated as a percentage of the untreated control. Each treatment was performed in triplicate (n = 3). Bars and error bars represent means and standard deviations, respectively (****$p<0.0001$). (**B-D**) Untreated SH-SY5Y cells (B) were compared to cells treated for 24 hours with TauO alone (C) or TauO in the presence of AC (D) and evaluated for morphological changes. Scale bar =20 µm.

SH-SY5Y cell viability significant decreased after treatment with TauO alone, while the presence of AC (final concentration 5 μM) reduced their toxicity as shown by the higher level of cell viability using MTT assay (**Figure 2.4A**). Cells were also evaluated for morphological differences (**Figure 2.4B-D**), showing cell shrinkage and loss of their processes after treatment with tau oligomers alone, compared to either the untreated control or to cells exposed to tau oligomers in the presence of AC.

Furthermore, epifluorescence images of human SH-SY5Y cells, after treatment with sub-lethal concentration (0.5μM) of tau oligomers in the absence and presence of AC (**Figure 2.5A**), showed a significant reduction of percentage of Tau 5- immunoreactivity as compared to the cells given TauO alone, revealing a consequent reduction of tau oligomers uptake (**Figure 2.5B**). Furthermore, the analysis of integrated density showed a significant increase in cells treated with TauO+AC (white arrows) as compared to the ones given TauO, demonstrating that the incubation with AC promote the formation of larger tau aggregates (**Figure 2.5C**). Altogether, these results suggest that AC-induced aggregates are less prone to be taken up by cells as compared to untreated toxic tau oligomers.

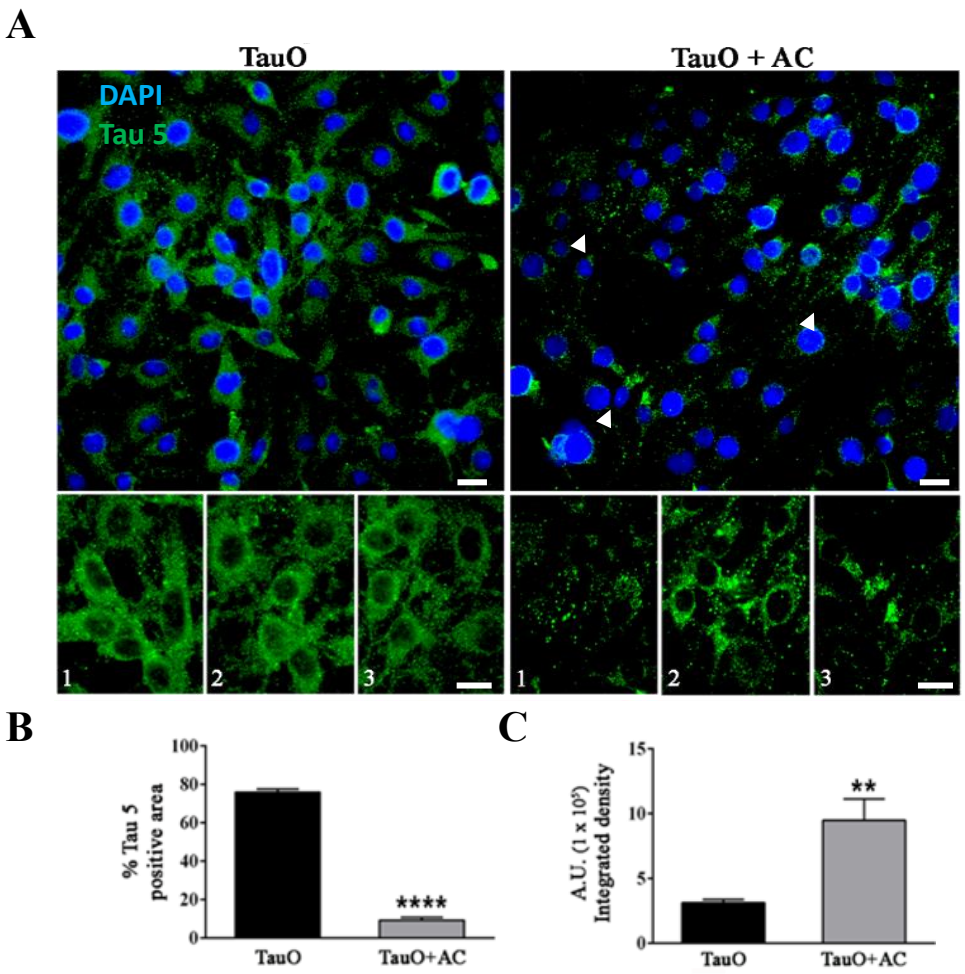


Figure 2.5 Representative epifluorescence images of human SH-SY5Y neuroblastoma cells after treatment with TauO or TauO+AC.

(**A**) SH-SY5Y treated with sub-lethal concentration (0.5µM) of tau oligomers in the absence and presence of AC. TauO or TauO+AC are labeled with Tau 5 and DAPI, shown in green and blue respectively. (**B-C**) Analyses of the percentage of area positive to Tau 5 (**B**) and integrated density (**C**) for each condition are conducted in three different selected regions of interest (1,2 and 3) characterized by same size and comparable number of cells. SH-SY5Y cells treated with tau oligomers in the presence of AC show a significant reduction of percentage of Tau 5-immunoreactivity. Integrated density analysis shows a significant increase in cells treated with TauO +AC (white arrows) as compared to the ones given TauO alone. Bars and error bars represent means and standard deviations, respectively (**p<0.01; ****p<0.0001). Scale bar = 20 µm.

Next, I investigated the selectivity and specificity of AC compared to a naturally occurring polyphenol found in grapes and red wine, resveratrol (RS) (**Figure 2.6**). It has

been previously shown that RS selectively remodels soluble Aβ oligomers as well as fibrillar intermediates and amyloid fibrils converting them into non-toxic aggregates. (Ladiwala, Lin et al. 2010)

Interestingly, I found that AC selectively interacts with toxic tau oligomers compared to RS, which shows to have no effect in modulating toxic tau oligomeric species. Therefore, I evaluated T22 immunoreactivity with TauO alone and after 16 hours incubation with AC or RS by direct ELISA (**Figure 2.6A-B**). Results showed that, unlike AC, RS is not capable of modulating TauO aggregation. This result was confirmed by filter trap and dot blot analyses (**Figure 2.6C-D**), as well as western blot analysis using Tau 5 antibody, which showed reduced tau oligomeric specie levels after AC treatment, but not RS treatment (**Figure 2.6E-F**). Moreover, bis-ANS fluorescence assay revealed that oligomers treated with AC have very low binding with bis-ANS compared to oligomers treated with RS, which were similar to untreated oligomers (**Figure 2.6G**).

In addition, to confirm AC effects on tau oligomers, I tested AC and RS using crude oligomeric preparations containing oligomers, monomers and protofibrils.
Western blot and bis-ANS analyses were similar to those obtained using purified oligomers confirming that AC does not disassemble oligomers into monomeric tau (**Figure 2.6H-J**). Taken together, these results suggest that AC selectively interacts and modulates toxic tau oligomers as compared to RS that shows no effects on preformed toxic tau oligomeric species.

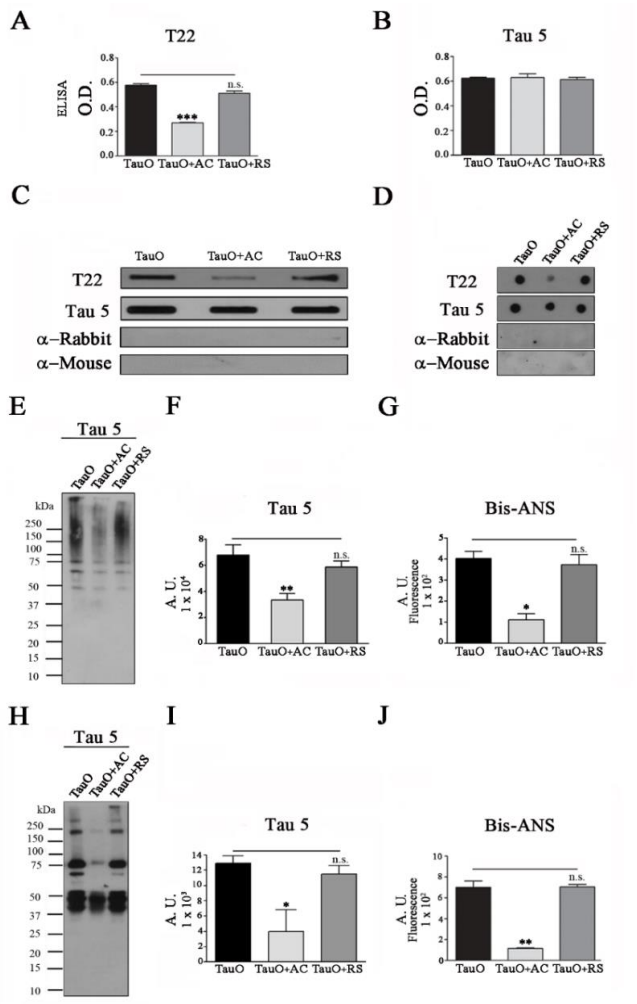


Figure 2.6 Biochemical and biophysical analyses of oligomeric tau alone, pre-incubated with 5 μM AC, or 5 μM of RS.

(**A-B**) ELISA analysis of oligomeric tau using T22 and Tau 5 antibodies. In the presence of AC but not RS, tau oligomers show a significant decrease in T22 immunoreactivity and no changes in Tau 5 immunoreactivity. (**C-D**) Filter Trap assay and dot blot of TauO without and with AC or RS. Tau oligomer levels are decreased in the presence of AC but not RS. (**E-F**) Western blot analysis using total tau antibody, Tau 5, showed reduction of oligomers in samples incubated with AC. Bar graph shows decreased levels of oligomers in the presence of AC, and no differences in the presence of RS compared to the untreated TauO. Statistics are based on three independent assays. (**G**) bis-ANS fluorescence binding assay; only AC treated oligomers have reduced binding to bis-ANS. Collectively, these results suggest that AC but not RS significantly reduced toxic oligomers. (**H-J**) AC and RS were tested using crude oligomeric preparations (containing oligomers and monomers), only AC reduced oligomers similar to what is observed using purified oligomers. Bars and error bars represent means and standard deviations, respectively (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

CONCLUSIONS

With increasing evidence that oligomeric tau species are the most pathogenic structures linked to the onset and progression of neurodegenerative disorders, finding effective interventions and therapeutic approaches are urgently needed. To date, research on tauopathies focused primarily on tau aggregation inhibitors or small molecules with the property of disassembling tau aggregates. The focus should be on finding small molecules that are able to convert toxic tau aggregates to less toxic structures, promoting the formation of a non-toxic conformation or ones that can be more easily degraded by active cellular mechanisms thus preventing the spread of the pathology.

Our data show that AC is able to interact and modulate the aggregation pathway of preformed tau oligomers resulting in the formation of clusters of aggregates, a conformational state that has been shown to be non-toxic. The results presented here lay the foundation for future studies to test the efficacy and beneficial effects of AC using primary cortical neurons cultures and animal models of tauopathies, thus giving reliable insights into AC potential and its mechanism of action. Finally, small molecules, including AC or its derivatives, can be developed as tau PET imaging agents that can be utilized in the clinical setting to detect toxic tau oligomers at the very early stages of AD and related diseases.

CHAPTER 3. BINDING AND NEUROTOXICITY MITIGATION OF TOXIC TAU OLIGOMERS BY HEPARIN LIKE OLIGOSACCHARIDES

INTRODUCTION

Alzheimer's disease (AD) is a progressive degenerative brain disease, which is estimated to affect 5.5 million Americans in 2017 (2017). Although the causes for most AD cases have not been firmly established, the pathology of tau protein is believed to play a causal role in AD and other tauopathies (Ballatore, Lee et al. 2007, Iqbal, Liu et al. 2010, Šimić, Babić Leko et al. 2016). Tau protein in its native and functional state exists as a soluble monomer, which is critical in stabilizing microtubules. However, tau can misfold and aggregate leading to the formation of oligomers and hyperphosphorylated tau aggregates, known as neurofibrillary tangles (NFTs), a hallmark of AD (Braak and Braak 1991, Arriagada, Growdon et al. 1992, Braak and Braak 1996, Serrano-Pozo, Frosch et al. 2011). While NFTs are abundant in the brains of late stage AD patients, some patients show neuronal loss and cognitive deficits prior to the formation of histologically identifiable NFTs (Morsch, Simon et al. 1999, Berger, Roder et al. 2007, Haroutunian, Davies et al. 2007). In animal studies, NFTs have been found not to be associated with neuronal death suggesting that these large insoluble aggregates may not be the key toxic species in AD (Gomez-Isla, Hollister et al. 1997, Yoshiyama, Higuchi et al. 2007, Spires-Jones, Kopeikina et al. 2011, Cowan, Quraishe et al. 2012, Cook, Kang et al. 2015, Kim, Choi et al. 2016).

Recently, the tau oligomers (TauO) hypothesis has been proposed with strong evidence supporting that the soluble, oligomeric tau rather than the NFTs are likely the most toxic species causing disease and efficiently seeding the propagation of the pathology (Brunden,

Trojanowski et al. 2008, Cowan, Quraishe et al. 2012, Cardenas-Aguayo Mdel, Gomez-Virgilio et al. 2014, Guerrero-Munoz, Gerson et al. 2015, Gerson, Mudher et al. 2016). Although, most tau is intracellular, TauO are present in the extracellular space around neurons in human AD patients (Avila 2010, Medina and Avila 2014, Yamada 2017). TauO have been found to propagate through different brain regions, which can be taken up by distal neurons contributing to the spread of neuronal death and associated learning and memory deficits (Kopeikina, Carlson et al. 2011, Lasagna-Reeves 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Fuster-Matanzo, Hernandez et al. 2018). Injection of TauO isolated from the cerebral cortex of AD brains initiated tau pathology in cognitively normal mice, causing synaptic and mitochondrial dysfunction along with memory loss (Lasagna-Reeves 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Castillo-Carranza, Gerson et al. 2014). Even brief exposure to TauO could produce an immediate impairment of long-term potentiation and memory loss (Fá, Puzzo et al. 2016). The tau oligomer hypothesis was further strengthened by the observations that lowering TauO levels using a novel tau oligomer-specific mouse monoclonal antibody protected against behavioral impairments and tau pathology in multiple mouse models without affecting levels of NFTs (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza, Sengupta et al. 2014). Therefore, TauO are potential new therapeutic targets and strategies that can reduce TauO-associated neurotoxicity are highly desired.(Lasagna-Reeves, Castillo-Carranza et al. 2011, Wang and Mandelkow 2016).

Heparan sulfate (HS) and its more sulfated analog heparin are a class of highly negatively charged polysaccharides present on mammalian cells including neuronal cells (Capila and Linhardt 2002, Sarrazin, Lamanna et al. 2011). HS and heparin are composed

of repeating disaccharide subunits with D-glucosamine (GlcN) α-1,4 linked with a uronic acid (either L-iduronic acid (IdoA) or D-glucuronic acid (GlcA)) (Linhardt, Dordick et al. 2007, Dulaney and Huang 2012). The amine moiety, 3-OH and 6-OH of GlcN and 2-OH of the uronic acid of heparin can be sulfated. While heparin has many biological functions, (Capila and Linhardt 2002, Sarrazin, Lamanna et al. 2011) it is not known whether it can interact with TauO. Herein, using structurally well-defined synthetic oligosaccharides, we report for the first time that heparin like oligosaccharides, as small as tetrasaccharides, can bind and interact with the toxic TauO. Furthermore, treatment of cells with heparin like oligosaccharides protects them from TauO-induced toxicity providing an exciting new direction in targeting AD and related tauopathies.

METHODS

Preparation of TauO

Tau oligomers were prepared as previously described in Chapter 2.2.

Preparation of Tau Oligomers in presence of Heparin like Oligosaccharides

TauO (1 µg/µl, 100 µl) were incubated with heparin like oligosaccharides (1:5 molar ratio). Oligosaccharides were dissolved in ddH_2O at a final concentration of 50 mM and diluted in 1X PBS or cell culture medium for incubation or toxicity assays. TauO in the presence of oligosaccharides and controls were incubated without stirring for 16 hours under oligomerization conditions as previously described (Lo Cascio and Kayed 2018).

BLI Binding Assay of Heparin like Oligosaccharides and Tau Oligomers

The heparin oligosaccharides were biotinylated by reaction with sulfo-N-hydroxysuccinimide long-chain biotin (ApexBio Tech LLC). The binding assay was performed on the Octet K2 System (Pall ForteBio). The biotiylated heparin oligosaccharides were absorbed to streptavidin (SA) sensor at a concentration of 50 µM for 2 min. the sensor was then balanced in the assay buffer (PBS containing 0.005% P20) and dipped into tau oligomer solution in the assay buffer at different concentration (4.36, 2.18, 1.09, 0.545, 0.272, 0.136 and 0.0681 µM). After 2 min of association, the sensor was brought back to the previous assay buffer for a 3-min dissociation step. At the end of the assay, the sensor was regenerated in 1 M NaCl to remove the bound tau oligomers. Each measurement was repeated 3 times on the same sensor. The control assay was done with another sensor loaded with saturated biotin solution.

Western Blotting

Western Blot was performed as previously described in Chapter 2.2.

Membranes were probed with T22 (1:250) for tau oligomers and Tau 5 (1:10000) and Tau 13 (1:50.000) for total tau, diluted in 5% nonfat milk for 1 hour at RT. Membranes were then incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) to detect T22 and anti-mouse (1:10000) secondary antibody to detect Tau 5 and Tau 13.

Filter Trap Assay

Filter trap assay was performed as previously described in Chapter 2.2.

Membranes were probed with the oligomer-specific tau antibody, T22 (1:250) and total tau antibodies, Tau 5 (1:10000) and Tau 13 (1:50.000) diluted in 5% nonfat milk for 1 hour at RT. Membranes were then incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) to detect T22 and anti-mouse (1:10000) secondary antibody to detect Tau 5 and Tau 13.

Morphological analysis of TauO by AFM.

Samples were imaged as previously described in Chapter 2.2 (Lasagna-Reeves, Castillo-Carranza et al. 2010, Sengupta, Guerrero-Munoz et al. 2015).

Cell Toxicity assays - LDH

Human neuroblastoma SH-SY5Y cells were cultured and treated for measuring cytotoxicity using LDH release assay (Cytotoxicity Detection KitPLUS -LDH, Roche) following manufacturers' instructions as previously described (Kayed, Head et al. 2003, Lasagna-Reeves, Castillo-Carranza et al. 2010, Sengupta, Guerrero-Munoz et al. 2015). Briefly, cells were maintained in Dulbecco`s modified Eagle's medium (DMEM) and

grown to confluency in 96-well plates. Cells (≈10,000 cells /well) were treated for 24 hours with 2.0 µM TauO or 2.0 µM TauO incubated with 10 µM of heparin like oligosaccharides (25, 27, and 28) followed by assaying with LDH. Optical density (OD) was measured at 490 nm with POLARstar OMEGA microplate reader (BMG Labtech). All measurements were performed in triplicate and corrected by the vehicle background. Statistical analysis was based on one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test performed using GraphPad Prism 6.01.

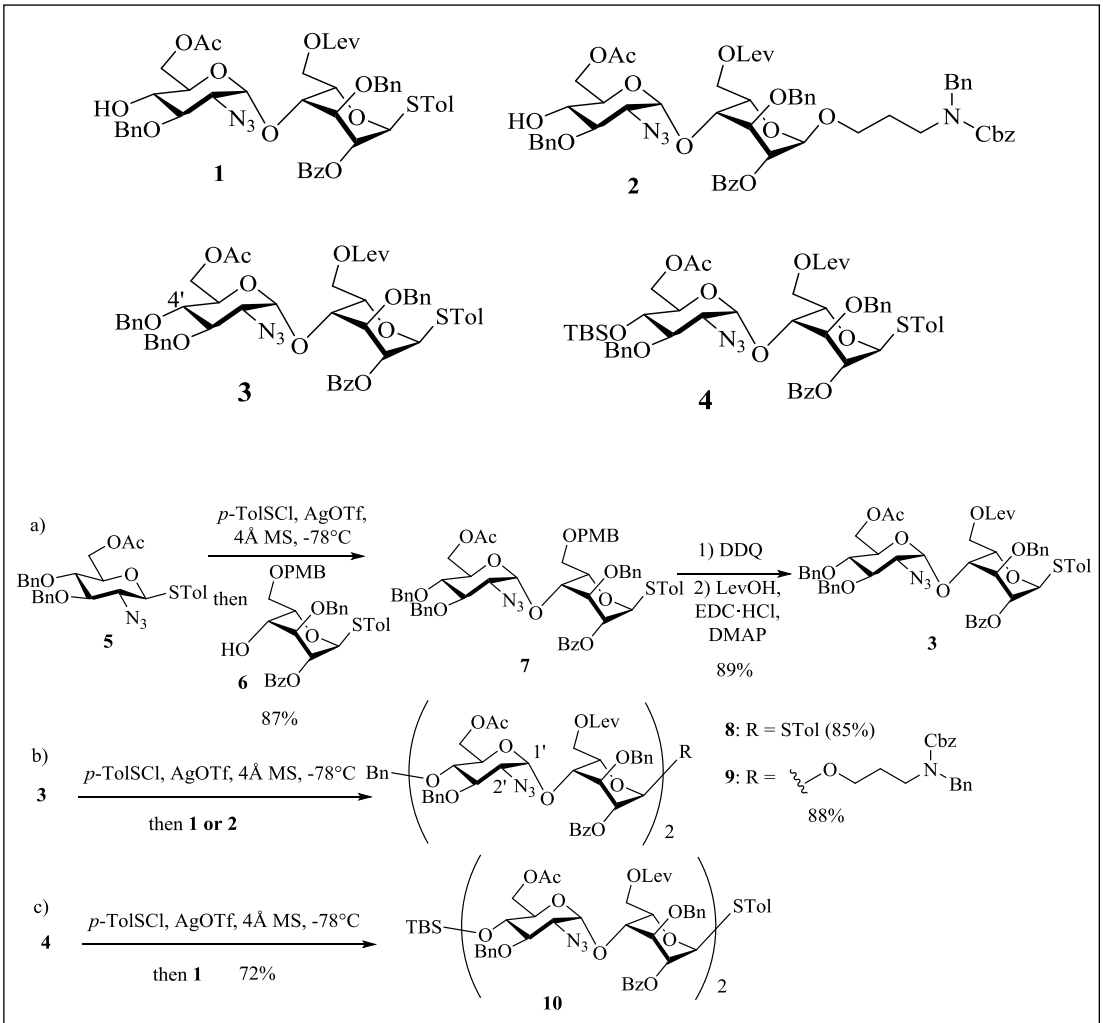
Immunofluorescence

SH-SY5Y cells were maintained in Dulbecco`s modified Eagle's medium (DMEM) and grown to confluence using poly-L-lysine coated coverslip in 24-well plates as described (Castillo-Carranza, Guerrero-Munoz et al. 2018). Cells (≈20,000 cells /well) were treated for 1 hour with 0.5 µM TauO or a mixture of 0.5 µM TauO with 2.5µM of oligosaccharides. After washing off unbound proteins, cells were stained with 5 µg/mL WGA (Wheat Germ Agglutinin) AF 633 for 10 min followed by fixation in chilled methanol. After washing three times with 1X PBS, cells were permeabilized with 0.25% Triton-X 100, diluted in 1X PBS for 10 min. Cells were washed in 1X PBS for 10 min prior to blocking in 5% goat serum for 1 hour and then incubated with Tau 13 antibody (1:1000) overnight. The next day, cells were washed three times with 1X PBS and then incubated with goat anti-mouse IgM Alexa-488 (1:1000, Invitrogen) for 1 hour. After washing three times with PBS (10 min each), cells were then stained with DAPI (Vector Laboratories) and mounted using Vectashield mounting medium (Fluoromount-4',6-diamidino-2-phenylindole). Cells were imaged with confocal microscope Zeiss LSM880 using standard filters for DAPI, GFP and

Texas Red channels. Images were analyzed with ImageJ and statistical analysis was performed by Student's T test, using GraphPad Prism 6.01.

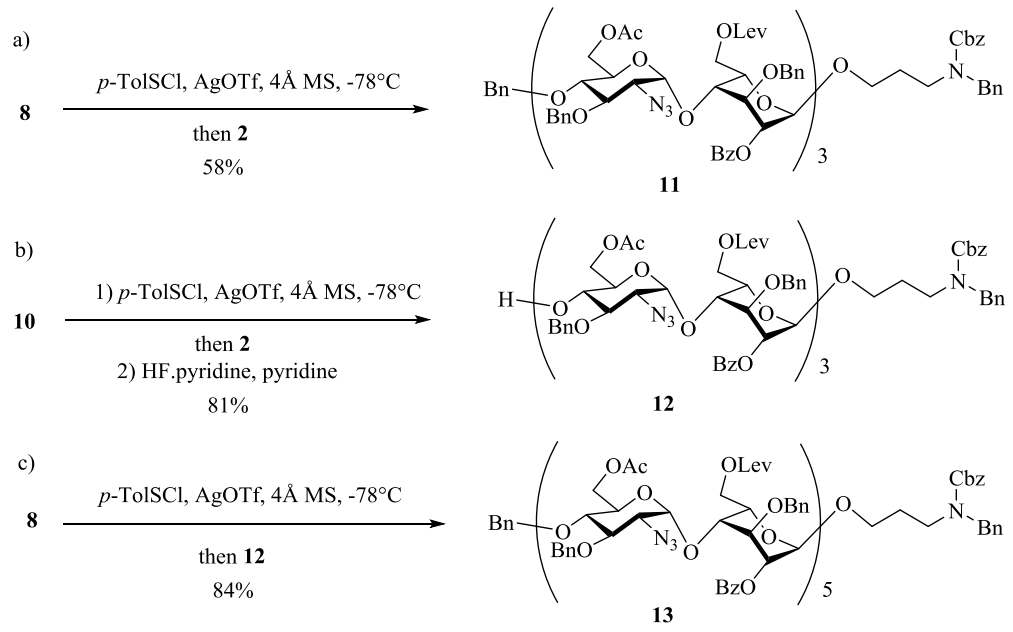
RESULTS AND DISCUSSION

In order to obtain heparin like oligosaccharides, we based our synthetic design on disaccharide modules **1**, **2** and **3**. Disaccharides **1** and **2** were synthesized starting from disaccharide **4** following literature procedures (Dulaney, Xu et al. 2015). For the non-reducing end disaccharide module, while TBS bearing disaccharide **4** could be used, we found it was impossible to remove the TBS group from sulfated oligosaccharides during deprotection (Dulaney, Xu et al. 2015). This consideration prompted us to prepare the 4'-*O*-benzyl (Bn) protected disaccharide **3**. Pre-activation of Bn protected glucosamine donor **5** with *p*-TolSCl and AgOTf (Huang, Huang et al. 2004) followed by the addition of acceptor **6**, gave the α-linked disaccharide **7** in 87% yield (**Scheme 3.1a**). The 1,2-*cis* linkage in the newly formed glycosidic bond was confirmed by NMR analysis ($J_{H1'\text{-}H2'}$ = 3.5 Hz, $J_{H1'\text{-}C1'}$ = 171.0 Hz). Protective group manipulation of **7** yielded the disaccharide module **3**. Reaction of the donor **3** with acceptor **1** generated tetrasaccharide **8** in 85% yield (**Scheme 3.1b**). Alternatively, **3** glycosylated the reducing end disaccharide module **2** giving tetrasaccharide **9** (**Scheme 3.1b**). In a similar manner, TBS bearing tetrasaccharide donor **10** was formed (**Scheme 3.1c**).



Scheme 3.1. Synthesis of tetrasaccharide backbones 8-10.

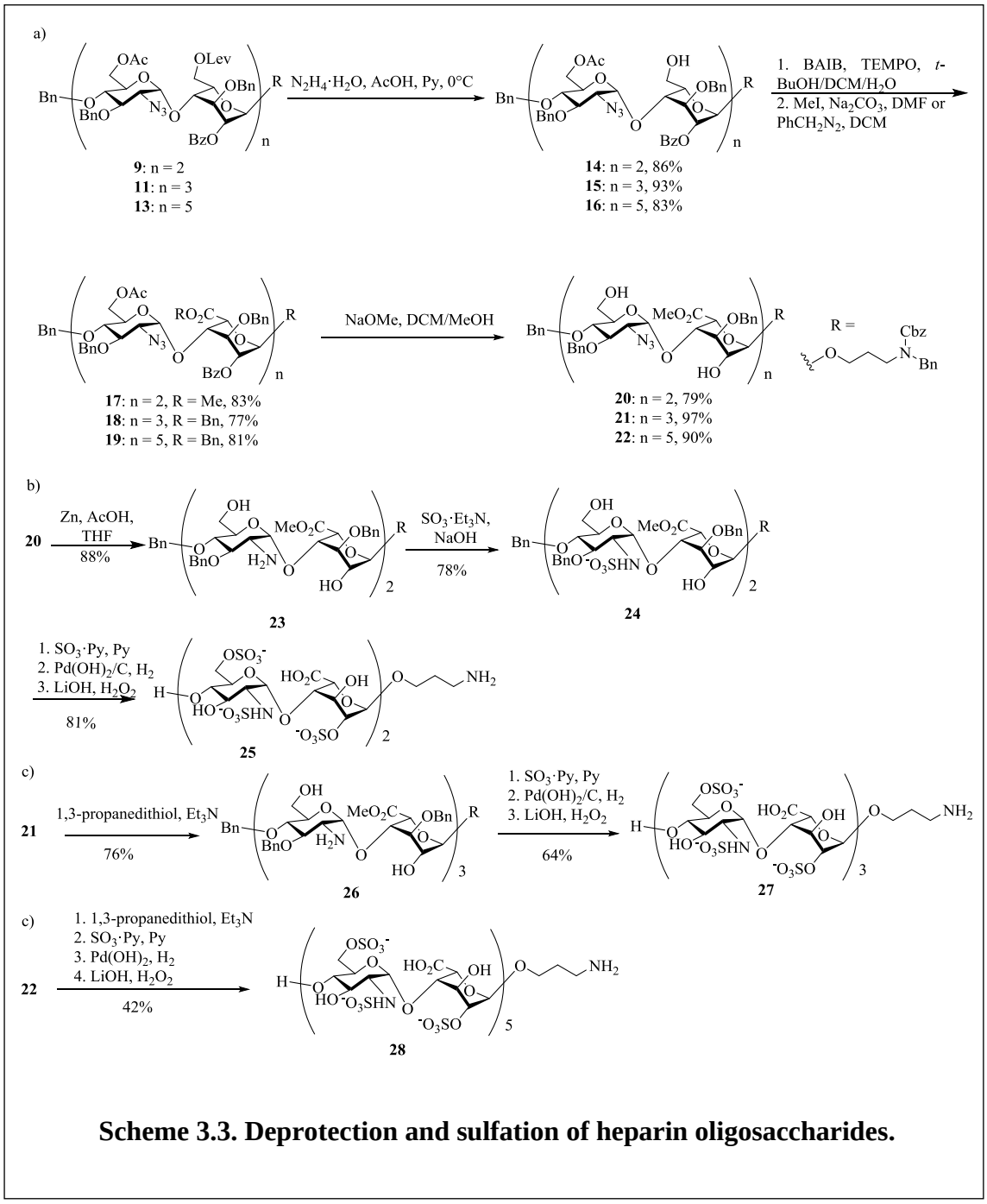
To produce the hexasaccharide backbone, a 4+2 glycosylation was carried out between the tetrasaccharide donor **8** and acceptor **2** leading to hexasaccharide **11** (**Scheme 3.2a**). The 4-*O*-TBS protected tetrasaccharide donor **10** also reacted well with disaccharide **2**. Removal of the TBS group from the glycosylation product produced the hexasaccharide acceptor **12** (**Scheme 3.2b**), which was subsequently glycosylated by tetrasaccharide donor **8**, forming decasaccharide **13** in 84% yield (**Scheme 3.2c**).



Scheme 3.2. Constructions of heparin hexa- and deca-saccharide backbones.

The deprotections and modifications of the backbones were carried out first by removal of 6-*O*-Lev from fully protected tetra-, hexa- and deca-saccharide **9**, **11** and **13** respectively with hydrazine acetate exposing the 6-OH (**Scheme 3.3a**). The conversion of these primary hydroxyl groups to carboxylic acids was mediated by bis(acetoxy)iodobenzene (BAIB) assisted 2, 2, 6, 6-tetramethyl-1-piperidinyloxyl (TEMPO) oxidation.(van den Bos, Codee et al. 2004) Since free carboxylic acids were found to lead to low yields in subsequent sulfation reactions,(Dulaney, Xu et al. 2015) they were protected as either methyl (83% for tetrasaccharide **17** in 2 steps) or benzyl (77% for hexasaccharide **18** and 81% for decasaccharide **19** in 2 steps) esters. Removal of the acyl protecting groups was accomplished by treating oligosaccharides **17**-**19** with sodium methoxide, which gave **20**, **21** and **22** respectively (**Scheme 3.3a**).

The two azido groups in tetrasaccharide **20** were reduced by zinc powder to provide **23** with two free amine groups (**Scheme 3.3b**). Sulfations of free hydroxyls and amines of **23** were performed stepwise. Firstly, **23** was dissolved in methanol with aqueous NaOH solution adjusting the pH to 9.5 in order to deprotonate amine groups. *N*-sulfation was then performed by adding excess $SO_3 \cdot Et_3N$ complex to the mixture to give **24** in 78% yield, which was then subjected to *O*-sulfation with SO_3·pyridine complex in pyridine overnight at 55 °C. Subsequent hydrogenolysis and saponification produced sulfated tetrasaccharide **25**. For heparin hexasaccharide synthesis, **21** was reduced with 1, 3-propanedithiol and triethylamine over 3 days in a yield of 76% (**Scheme 3.3c**). Similar stepwise sulfation as in synthesis of tetrasaccharide **25** was attempted on hexasaccharide **26**, which only led to decomposition of the starting materials. Analysis of the reaction mixture showed the formation of side products due to β-elimination with the oligosaccharide backbone cleaved. Instead, treatment of hexasaccharide **26** with 600 mM SO_3·py complex in pyridine at 55℃ successfully installed both *N*- and *O*-sulfation in one step, which was followed by catalytic hydrogenation and methyl ester hydrolysis, giving the final heparin like hexasaccharide **27** at 64% yield over 3 steps (**Scheme 3.3c**). Analogously, the heparin like decasaccharide **28** was synthesized with an overall yield of 42% from **22** (**Scheme 3.3d**).



Scheme 3.3. Deprotection and sulfation of heparin oligosaccharides.

With the synthetic oligosaccharides in hand, their binding with TauO were analyzed. **25**, **27**, and **28** were biotinylated, immobilized on streptavidin coated biolayer interferometry (BLI) sensors and incubated with various concentrations of TauO. The sensorgrams showed that tetrasaccharide **25** could bind to TauO with a K_D value of 2.79 × 10^{-7} M. Increasing the backbone length of the oligosaccharide to hexa- and deca-saccharides led to enhancements in TauO binding, with K_D values of 1.41 × 10^{-7} M and 3.49 × 10^{-8} M for oligosaccharides **27** and **28,** respectively (**Figure 3.1**).

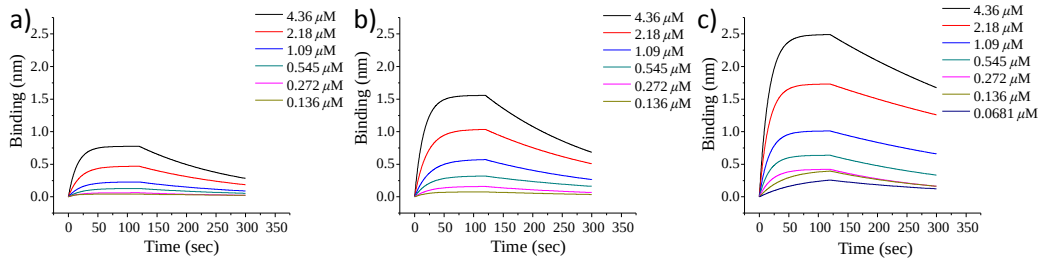


Figure 3.1 Sensograms of heparin like oligosaccharide binding with TauO.

Fit curves of interactions between BLI sensors loaded with a) **25**, b) **27** and c) **28** and TauO at various concentrations were obtained using models from Octet Data Analysis 9.0.0.12. Tetrasaccharide **25** exhibited significant binding to TauO, with longer backbone length leading to stronger binding.

These results suggest that glycans, as short as tetrasaccharide, can already exhibit significant interactions with TauO and longer oligosaccharide backbones enhance the binding leading to stronger binding.

Based on these results, oligomeric tau species were incubated with and without heparin oligosaccharides (25, 27 and 28) at room temperature for 16 hours, under oligomerization conditions (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lo Cascio and Kayed 2018). Tau oligomers in the presence and absence of oligosaccharides were evaluated biochemically to confirm the effects of the newly synthesized glycans on TauO.

Western blot analysis was performed using the anti-oligomeric specific tau antibody, T22, and the total tau antibodies, Tau 5 and Tau 13 (**Figure 3.2A-B**). Western blot analyses showed a significant decrease on tau oligomer levels after incubation with the heparin oligosaccharide compounds as seen by the reduction in T22 immunoreactivity as well as Tau 5 and Tau 13. In addition, filter trap assay confirmed the reduction in tau oligomers levels after treatment with the heparin oligosaccharides (**Figure 3.2C**).

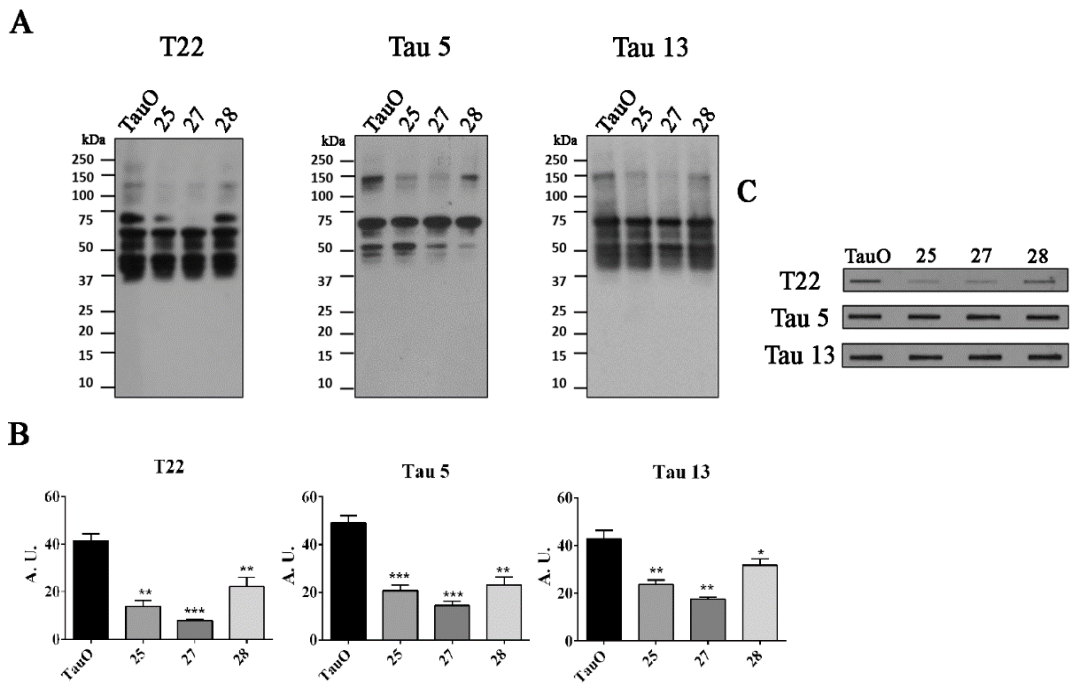


Figure 3.2 Biochemical analyses of Tau oligomers alone or pre-incubated with heparin oligosaccharides.

(**A**, **B**) Western blot analysis of tau oligomers alone (TauO) and incubated with 10 µM of heparin oligosaccharides (25, 27 and 28) probed with the oligomeric tau antibody, T22 and total tau antibodies, Tau 5 and Tau 13. Tau oligomer levels are significantly decreased in the presence of the compounds, compared to the untreated TauO. **(C)** Filter trap analysis probed with T22, Tau 5, or Tau 13. Results show decreased T22 immunoreactivity once tau oligomers are in the presence of 25, 27 and 28 as compared to the untreated tau oligomers, while there is no differences in total tau levels as seen by Tau 5 and Tau 13 immunoreactivity. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: ***$p<0.001$, **$p<0.01$, *$p<0.05$ TauO vs 25, 27, 28. Bars and error bars represent means and standard deviations performed.

In accordance with the binding assay, biochemical characterization of tau oligomers in the presence and absence of heparin oligosaccharides showed that the compounds interact and modulate the aggregation state of toxic tau oligomers resulting in decreased tau oligomer levels as compared to the untreated control, TauO.

Established the binding and the decreased TauO after incubation with the newly synthetized compounds, we evaluated heparin like oligosaccharides' ability to modulate the aggregation state and toxicity of TauO (Lasagna-Reeves, Castillo-Carranza et al. 2010). Atomic force microscopy (AFM) was performed to visualize and characterize the morphology and aggregation state of tau aggregates, which reflects the distinct effects of the compounds on TauO (**Figure 3.3**).

Classically, tau oligomers exist as homogeneous spherical structures (**Figure 3.3a**), while in the presence of heparin like oligosaccharides (**Figure 3.3b-d**), we found that TauO were converted into larger aggregates.

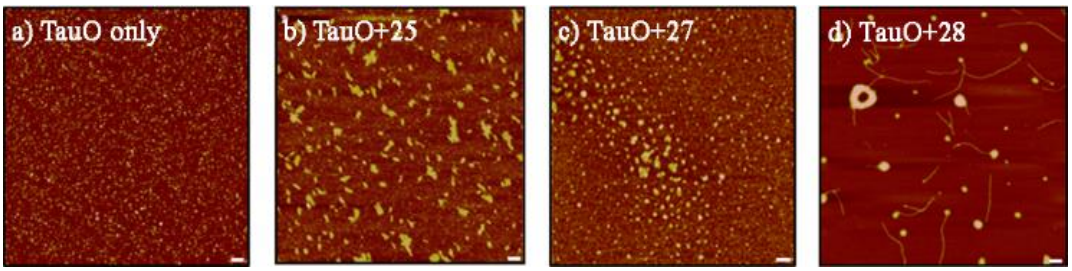


Figure 3.3 Morphology characterization of TauO alone or in the presence of heparin like oligosaccharides.

Atomic Force Microscopy images of TauO without (a) and with oligosaccharides b) **25**, c) **27,** and d) **28** (5X). AFM images show the ability of the glycans to modulate TauO aggregation states converting TauO into much larger aggregates. Scale bars = 100 nm

Next, we also evaluated the ability of the heparin like oligosaccharides to prevent and rescue from TauO-induced cytotoxicity using human neuroblastoma SH-SY5Y. Therefore,

cells were incubated with tau alone or in the presence of heparin like oligosaccharides **25**, **27** and **28,** respectively. The cytotoxicity to SH-SY5Y cells was determined in a lactate dehydrogenase (LDH)-based assay, as the amount of LDH released into the culture media from damaged cells is indicative of cellular cytotoxicity and cytolysis (**Figure 3.4**).

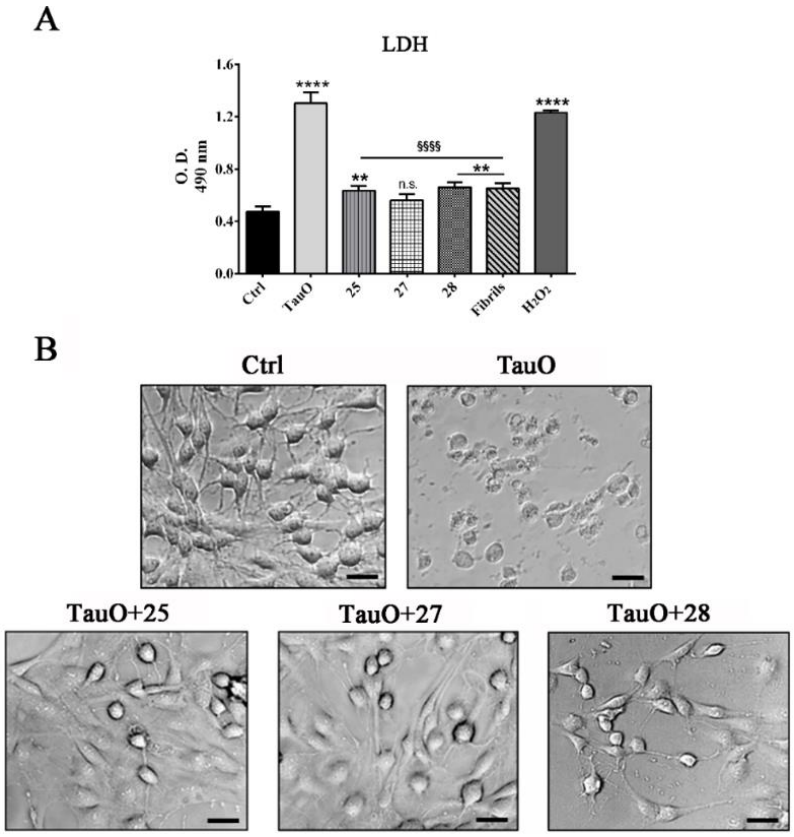


Figure 3.4 Cytotoxicity on SH-SY5Y neuroblastoma after exposure to TauO with and without heparin oligosaccharides' treatment.

(**A**) SH-SY5Y cells cytotoxicity after exposure to 2µM TauO, or 2µM TauO with 10µM of oligosaccharide (25, 27 and 28) and untreated control (Ctrl). Treatment of SH-SY5Y cells with TauO had significantly higher LDH release compared to the untreated control, cells exposed to TauO in the presence of oligosaccharides or cells exposed to high molecular weight tau fibrils. Each experiment was performed in triplicate (n = 3). Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: Ctrl vs TauO, 25, 27, and 28, Fibrils: ****$p<0.0001$, **$p<0.01$; TauO vs 25, 27, and 28: §§§§$p<0.0001$. Bars and error bars represent means and standard deviations performed. (**B**) Untreated SH-SY5Y cells were compared to cells treated for 24 hours with TauO alone or TauO in the presence of oligosaccharides (25, 27, and 28) and evaluated for morphological changes. Scale bar = 20 µm.

Consistent with the idea that TauO being the more toxic tau species, TauO treatment of SH-SY5Y cells led to significantly higher LDH release, while cells incubated with the higher molecular aggregate tau fibrils released much less LDH (**Figure 3.4A**). Excitingly, in the presence of oligosaccharides (10 μM), TauO induced significantly lower levels of LDH release, compared to TauO alone (**Figure 3.4A**). Moreover, cells exposed to each condition were evaluated for morphological differences. In the presence of TauO alone, there was significant cell shrinkage compared to either the control cells (Ctrl) or cells treated with TauO in the presence of heparin like oligosaccharides (**Figure 3.4B**).

Altogether, these results suggest that heparin like oligosaccharides interact with and subsequently convert the toxic TauO into larger aggregates modulating their toxicity.

To further confirm our findings and gain a better understanding of the protective effects of the oligosaccharides, SH-SY5Y cells were treated with sub-lethal concentration of TauO and imaged by confocal microscopy. TauO were observed in large areas of the cytoplasm of cells, indicating extensive cellular internalization of TauO (**Figure 3.5**). Intracellular TauO can instigate mitochondrial damage, induce cytochrome c release and stimulate reactive oxygen species production, which are potential mechanisms for their cytotoxicity. (Usenovic, Niroomand et al. 2015, Shafiei, Guerrero-Munoz et al. 2017) SH-SY5Y cells treated with TauO, co-incubated with oligosaccharide **25, 27** and **28,** show a significant reduction in the percentage of area positive of TauO staining (**Figure 3.5B**). These results suggest that the glycan-induced aggregates are less prone to be taken up by the cells giving an explanation in their reduced cytotoxicity.

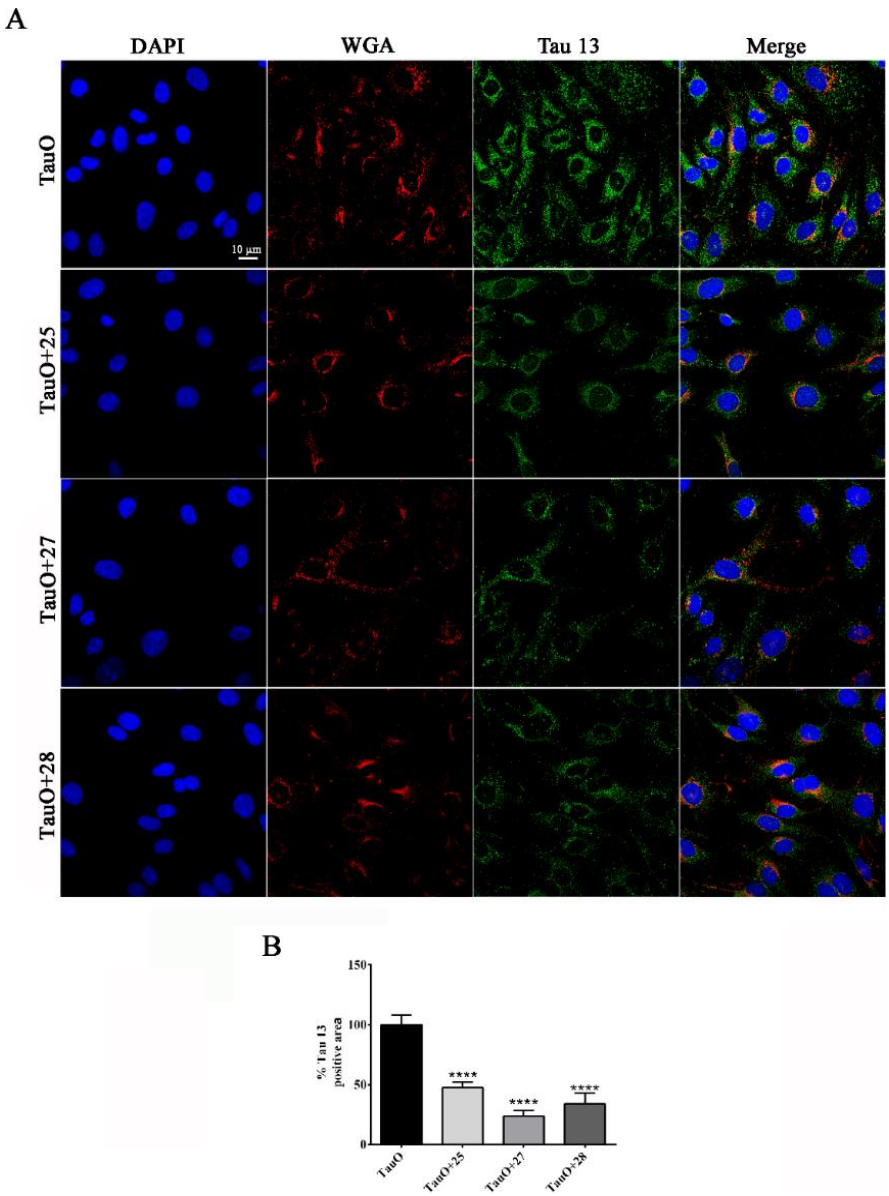


Figure 3.5 Representative confocal images of human SH-SY5Y neuroblastoma cells after treatment with TauO or TauO in the presence of oligosaccharides.

(**A**) SH-SY5Y after 1 hour of treatment with sub-lethal concentration (0.5µM) of TauO in the absence and presence of oligosaccharides (28, 29 and 31). Cells were stained with DAPI (nuclei – blue), WGA (plasma membranes – red) and α-Tau antibody, Tau 13 (green). (**B**) Analysis of the percentage of Tau 13 positive area was performed on selected regions of interest characterized by same size and comparable number of cells. Cells treated with TauO in the presence of heparin oligosaccharides (28, 29, 31) show a significant reduction of % area positive for tau oligomers as compared to the cells exposed to TauO alone suggesting reduction of TauO uptaken by the cells. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: ****$p<0.0001$. Bars and error bars represent means and standard deviations. (Magnification: 63X and scale bar = 10 µm).

Furthermore, representative confocal immunofluorescence images of SH-SY5Y treated with tau oligomers in the presence of oligosaccharides (25, 27 and 28) show a reduction of positive area for tau oligomers in the nuclei as compared to the untreated control, TauO (**Figure 3.6**).

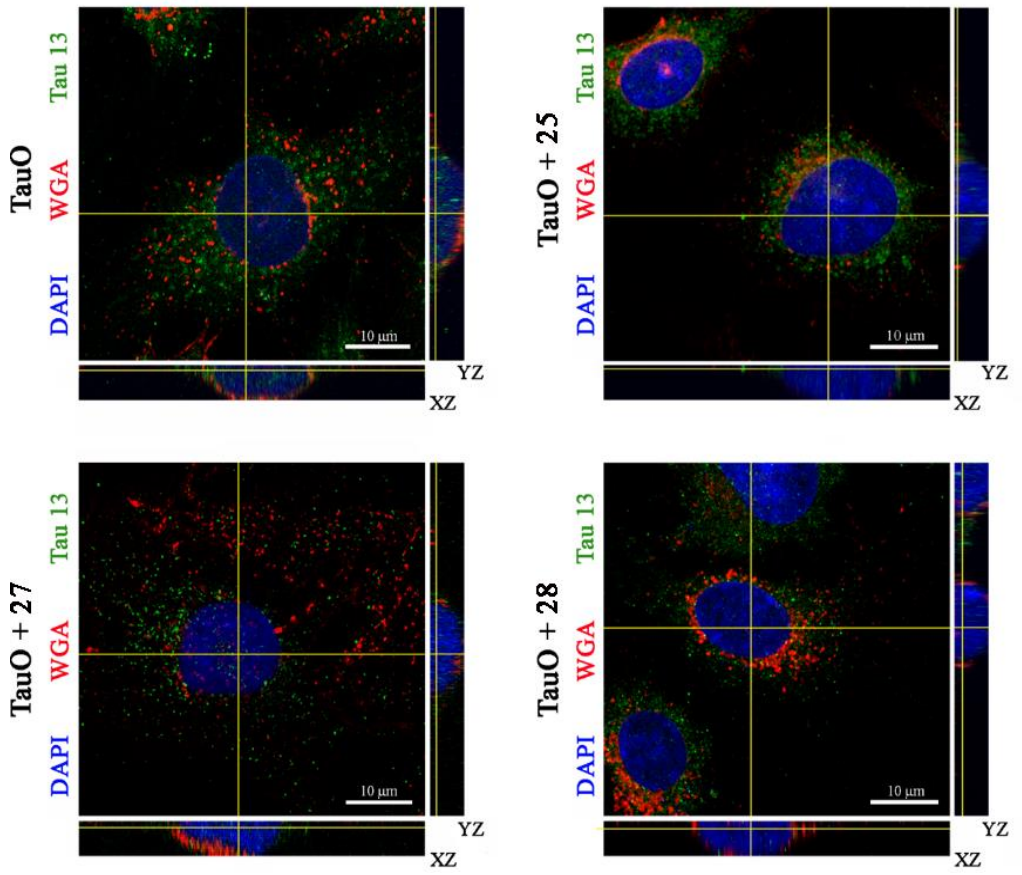


Figure 3.6 Human SH-SY5Y neuroblastoma cells after treatment with TauO and oligosaccharides.

Representative confocal images of SH-SY5Y after 1 hour of treatment with sub-lethal concentration (0.5µM) of TauO in the absence and presence of oligosaccharides (25, 27 and 28). Cells treated with TauO or TauO + oligosaccharides (25, 27 and 28) and stained with DAPI (nuclei – blue), WGA (plasma membranes – red) and the α-Tau antibody, Tau 13 (green), are represented. Cells treated with TauO in the presence of oligosaccharides (28, 29 and 31) show a reduction of positive area for TauO in the nuclei as compared to the untreated control, TauO. Confocal XZ and YZ projections confirmed that oligosaccharides-induced aggregates are less prone to be internalized by cells as compared to untreated TauO (Magnification: 63X and scale bar = 10 µm).

Indeed, confocal XZ and YZ projections confirmed that oligosaccharides-induced aggregates are less prone to be internalized by cells as compared to untreated TauO. Altogether, our findings suggest that the glycans bind and interact with TauO leading to the formation of larger tau aggregates and inhibiting them from cellular internalization.

CONCLUSIONS

Tau aggregation is a critical mediator of neurodegeneration and has a causal role in AD and other tauopathies (Ballatore, Lee et al. 2007, Iqbal, Liu et al. 2010, Šimić, Babić Leko et al. 2016). Due to the rise in life expectancy, finding an effective prevention and treatment strategy available for tauopathies, becomes increasingly important (Yoshiyama, Lee et al. 2013, Pickhardt, Neumann et al. 2015, Khanna, Kovalevich et al. 2016). Herein, using fully synthetic well-defined glycans, we report the new finding that heparin like oligosaccharide, as short as tetrasaccharide, can bind strongly with the most toxic tau species, i.e., TauO. Their binding affinity can be further enhanced by increasing the length of the oligosaccharide to a decasaccharide.

Our data show that heparin oligosaccharides convert TauO into less toxic high molecular weight species, and mitigate TauO-associated cytotoxicity. In addition, the glycans significantly reduce TauO cellular internalization, which is critical for the progression of the pathology. While heparin is known to bind with high molecular tau aggregates (Sibille, Sillen et al. 2006, Frost, Jacks et al. 2009, Zhu, Fernández et al. 2010, Holmes, DeVos et al. 2013, Jangholi, Ashrafi-Kooshk et al. 2016, Zhao, Huvent et al. 2017), this is the first time that heparin oligosaccharide is shown to interact with TauO.

Importantly, the newly synthesized heparin oligosaccharides could aid in the development of novel therapeutic approaches for AD and related tauopathies.

CHAPTER 4. MODULATING TAU OLIGOMERS AND DISEASE-RELEVANT TAU OLIGOMERIC STRAINS TOXICITY BY NOVEL CURCUMIN DERIVATIVES

INTRODUCTION

Curcumin, a polyphenol extracted from the plant *Curcuma longa*, has several broad biological activities such as antioxidant and anti-inflammatory effects with a low-toxicity profile. Indeed, it plays an important role in the prevention and treatment of many diseases including neurodegenerative disorders (Purkayastha, Berliner et al. 2009, Prasad, Tyagi et al. 2014, Maiti and Dunbar 2018, Rahmani, Alsahli et al. 2018). Curcumin is a high lipophilic molecule with low molecular weight which can easily cross the BBB. Moreover, it is capable of binding and inhibiting the aggregation and deposition of insoluble amyloid aggregates (Yang, Lim et al. 2005, Thapa, Jett et al. 2016). Therefore, it has been shown to alter the misfolding of many amyloid proteins through the disruption of π-stacking due to the presence of conjugated phenol residues (Stefani and Rigacci 2013, Velander, Wu et al. 2017). Curcumin significantly reduces β-amyloid and tau pathology in transgenic AD mouse models (Ma, Zuo et al. 2013, Thapa, Jett et al. 2016). Studies have shown that curcumin is capable of labelling amyloid deposits both *ex vivo* and *in vivo*, disrupting existing plaques and partially restoring distorted neurites in transgenic AD mice (Garcia-Alloza, Borrelli et al. 2007). In addition, curcumin can decrease levels of tau hyperphosphorylation in cells and mice and can also bind to fibrillar tau (Park, Kim et al. 2008). Recently, curcumin was also found to be able to selectively suppress soluble tau dimers in aged Htau mice (Ma, Zuo et al. 2013). In addition, curcumin was also found to improve tau-mediated neuronal dysfunction and neuritic abnormalities in C. Elegans (Miyasaka, Xie et al. 2016).

Therefore, extensive preclinical studies have proposed curcumin as a potential therapeutic approach against AD and related neurodegenerative disease (Shal, Ding et al. 2018). Many human clinical trials have been performed but none of them have been successful and their failures may be due to curcumin's poor solubility in aqueous buffers and low brain bioavailability following oral administration (Ringman, Frautschy et al. 2012). Indeed, curcumin is metabolized very rapidly via glucuronidation, primarily in the liver and intestine, before reaching the systemic circulation and the BBB (Garcea, Jones et al. 2004, Anand, Kunnumakkara et al. 2007, Sharma, Steward et al. 2007, Prasad, Tyagi et al. 2014). Hence, its use as a potential therapeutic for AD and other neurodegenerative diseases has been a challenge. Therefore, alternative formulations and drug delivery systems, including liposomes and nanoparticles, have been formulated to boost its bioavailability (Das, Kasoju et al. 2010, Mohanty and Sahoo 2010, Douglass and Clouatre 2015). Furthermore, curcumin analogs were created to improve its well-established shortcomings (Narlawar, Pickhardt et al. 2008, Dolai, Shi et al. 2011, Lee, Loo et al. 2013, Ahsan, Mishra et al. 2015).

Therefore, based on these previous studies we decided to evaluate first the effect of curcumin on oligomeric tau species using our *in vitro* preparation of tau oligomers. However, to overcome one of the major curcumin drawbacks, its low cerebral bioavailability, which hampers its use as a potential therapeutic agent for AD and related diseases, we established a collaboration with medicinal chemistry experts to synthesize novel curcumin derivatives. The rationale behind the synthesis of the newly synthesized curcumin derivatives is to remove the β-di keto moiety that is assumed to be responsible for curcumin shortcomings (Vyas, Dandawate et al. 2013). The library of our curcumin

derivatives consists of four different classes: Hemi-curcuminoids (HemiC 1-10), Curcumin-like (CL 1-12), Heterocyclic curcumin-like (CH 1-11) and Calebin-A analogs (Cal 1-9) (**Figure 4.1**).

These novel compounds were synthesized to easily cross the BBB, target and modulate tau oligomers aggregation state, neutralizing their toxicity and internalization in an effort to prevent or slow the spread of tau pathology.

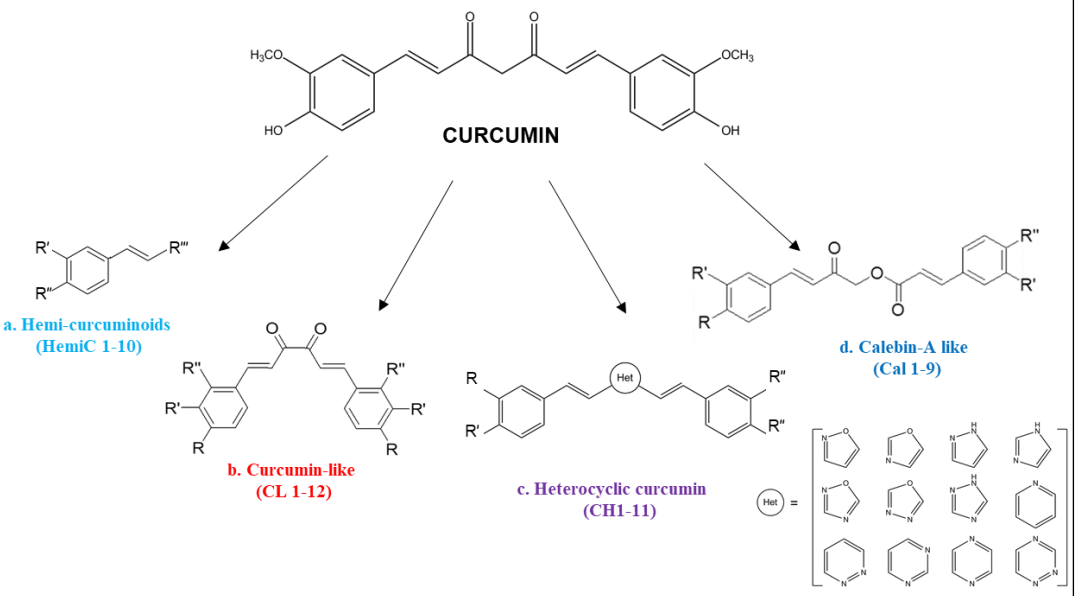


Figure 4.1 Structure of curcumin and newly synthesized curcumin derivatives.

The library of our curcumin derivatives consists of four different classes: Hemi-curcuminoids (a), Curcumin-like (b), Heterocyclic curcumin-like (c), and Calebin-A derivatives (d). The Heterocyclic curcumin-like have been synthesized by following Lipinski's rule of five to obtain active molecules able to easily across the brain blood barrier thus entering readily the brain.

METHODS

Synthesis

All solvent and reagents were used as received, unless otherwise stated. Melting points were determined on a hot-stage apparatus. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at indicated frequencies, residual solvent peak was used as reference. Chromatography was performed by using silica gel (0.040–0.063 mm) and mixtures of ethyl acetate and petroleum ether (fraction boiling in the range of 40–60 °C) in various ratios (v/v). All solvent and reagents were used as received. Compounds **2a, b, e, g** (Wang, Yin et al. 2008), **3a, b, e, g** (Wang, Yin et al. 2008), **2c** [Vander Jagt, D.L.; Deck, L.M.; Abcouwer, S.F.; Bobrovnikova-Marjon, E.; Weber, W.M. US Patent 20060276536], **2d** (Zhu, Mao et al. 2017), **4k** (Battisti, Palumbo Piccionello et al. 2017), **5a** (DiBiase, Lipisko et al. 1979) **5h** (Khurana, Ali et al. 2014), **CL$_{1\text{-}3,5}$** (Sinu, Padmaja et al. 2013), **7** (List, Doehring et al. 2006, Battisti, Palumbo Piccionello et al. 2017), **8** (Rehse and Brehme 1998, Battisti, Palumbo Piccionello et al. 2017), **CH$_4$** (Battisti, Palumbo Piccionello et al. 2017) were prepared as previously reported. Other already known compounds, prepared adapting previously reported methods as indicated below, present melting points and ^{1}H-NMR spectra consistent with those reported in the cited literature.

Preparation of TauO

Tau oligomers were prepared as previously described in Chapter 2.2.

Preparation of Tau Oligomers in presence of Small Molecules

A volume of 100 µl of tau oligomers (1µg/µl) was incubated with Curcumin (1:5; 1:10 molar ratio) and curcumin derivatives (1:5 molar ratio). Compounds were dissolved in ETOH 75%/DMSO (5:1) at a final concentration of 5 mM and diluted in 1X PBS or ddH_2O

for incubation or toxicity assay (final concentration 5 μM). Tau oligomers in the presence of the small molecules and controls were incubated on an orbital shaker, without stirring, for 16 hours under oligomerization conditions as previously described (Lo Cascio and Kayed 2018).

Preparation of Aβ oligomers

Aβ oligomers (AβO) were prepared as previously described (Lasagna-Reeves, Castillo-Carranza et al. 2010) by dissolving 0.3 mg of Aβ pellet in 200 μL of hexafluoroisopropanol (HFIP) and incubating for 10-20 min at room temperature. The resulting solution was added to 700 μL of ddH2O in a siliconized Eppendorf tube with holes placed on top of the cap to allow the slow evaporation of HFIP. The samples were then stirred at 500 rpm using a Teflon-coated micro stir bar for 48 hours at room temperature in the fume hood.

Preparation of Aβ oligomers in the presence of Small Molecules

A volume of 100 μl of Aβ oligomers (0.5μg/μl) was incubated with curcumin derivatives (final concentation 5μM). Compounds were dissolved in ETOH 75%/DMSO (5:1) at a final concentration of 5 mM and diluted in 1X PBS or ddH_2O for incubation or toxicity assay (final concentration 5 μM). Aβ oligomers in the presence of the small molecules and controls were incubated on an orbital shaker, without stirring, for 16 hours under oligomerization conditions as previously described (Lo Cascio and Kayed 2018).

Western Blotting

Western Blot was performed as previously described in Chapter 2.2.

Membranes were probed with T22 (1:250) for tau oligomers and Tau 5 (1:10000) and Tau 13 (1:50.000) for total tau, diluted in 5% nonfat milk for 1 hour at RT. Membranes were

then incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) to detect T22 and anti-mouse (1:10000) secondary antibody to detect Tau 5 and Tau 13.

Dot Blot

Dot Blot was performed as previously described in Chapter 2.2.

Membranes were probed with the oligomer-specific tau antibody, T22 (1:250) and total tau antibodies, Tau 5 (1:10000) and TOMA1(1:200) diluted in 5% nonfat milk for 1 hour at RT. Membranes were then incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) to detect T22 and anti-mouse (1:10000) secondary antibody to detect Tau 5 and TOMA1.

Direct ELISA

Direct ELISA was performed as previously described in Chapter 2.2.

Filter Trap Assay

Filter trap assay was performed as previously described in Chapter 2.2.

Membranes were probed with the oligomer-specific tau antibody, T22 (1:250) and total tau antibodies, Tau 5 (1:10000) and TOMA1(1:200) diluted in 5% nonfat milk for 1 hour at RT. Membranes were then incubated with horseradish peroxidase-conjugated IgG anti-rabbit (1:10000) to detect T22 and anti-mouse (1:10000) secondary antibody to detect Tau 5 and TOMA1.

Cell Toxicity Assay - MTT

MTT assay was performed as previously described in Chapter 2.2.

Morphological analysis of TauO by AFM

AFM was performed as previously described in Chapter 2.2.

Isolation of Brain-derived Tau Oligomers (BDTOs)

Oligomeric tau strains were isolated from brain extract by immunoprecipitation (Lasagna-Reeves 2012, Gerson, Castillo-Carranza et al. 2016). Tosyl-activated magnetic Dynabeads (Dynal Biotech) were coated with 20μg of anti-tau oligomer-specific polyclonal antibody T22, diluted in 0.1 M of borate, pH 9.5 overnight at 37°C. Next, the beads were washed in 0.1% Bovine serum albumin in 0.2 M Tris-HCl, pH 8.5 and then incubated with brain homogenates with rotation at room temperature for 1 hour. Then beads are washed three time in 1X PBS, pH 7.4and eluted using 0.1 M glycine, pH 2.8. Next, pH was adjusted using 1 M Tris-HCl, pH 8.0 and fractions were then centrifuged in a microcon centrifugal filter device, 25 kDa molecular weight cut-off (Millipore) at 14,000 x g for 25min at 4°C. Tau concentration was measured using bicinchoninic acid protein assay (Micro BCA kit, Pierce).

Brain-derived Tau Oligomers in presence of Small Molecules

A volume of 100 μl of BDTOs (0.5μg/μl) was incubated with curcumin derivatives (final concentation 5μM). Compounds were dissolved in ETOH 75%/DMSO (5:1) at a final concentration of 5 mM and diluted in 1X PBS or ddH_2O for incubation or toxicity assay (final concentration 5 μM). Tau oligomers in the presence of the small molecules and controls were incubated on an orbital shaker, without stirring, for 16 hours under oligomerization conditions.

Characterization of Brain-derived Tau Oligomers

Immunoprecipitated tau oligomers were characterized using various biochemical methods as previously described (Lasagna-Reeves 2012, Gerson, Castillo-Carranza et al. 2016). AFM was performed to visualize the morphologies of oligomeric assemblies of isolated proteins. Isolated oligomers (5µL) were injected into an LC-6AD Shimadzu HPLC system fitted with a TSK-GEL G3000 SWXL (30 cm × 7.8 mm) column, Supelco-808541 to determine the size of the isolated oligomers. PBS (pH 7.4) was used as the mobile phase with a flow rate of 0.5 mL/min. A gel filtration standard (Bio-Rad 51-1901) was used for calibrations. Samples (0.8-1 µg) were also tested for their comparative bis-ANS and ThT binding.

Proteinase K digestion

In an Eppendorf tube, molecular grade water, Tris HCl and sodium chloride were added so that the final concentrations for these two buffers became 100 mM and 5 mM, respectively in the entire solution volume. Next tau oligomeric species were added and mixed. Lastly, the PK enzyme was added (final concentration 1µg/ml). Then, the sample tubes were incubated at 37°C for 1 h. The enzymatic reaction was stopped by adding 1 X sample buffer. Samples were then ready to be loaded in the SDS-PAGE gel for electrophoresis or stored at -80°C.

Primary Cortical Neurons

Primary cortical neurons from transgenic mice expressing human full-length tau were prepared and maintained as described previously (Beaudoin, Lee et al. 2012). Briefly, cortical neurons were isolated from embryos at embryonic day 16-18 using Accutase solution (Sigma). Dissociated neurons were plated at a density of 30×10^4 cells/well in 96-

well plates containing high glucose Dulbecco's Modified Eagle Medium (DMEM, Corning) supplemented with 2% B27 (Gibco), 10,000 units/mL penicillin, 10,000 µg/mL streptomycin , and 25 µg/mL Amphotericin B (Gibco). After 2hours, plating medium was removed from cells and replaced with Neurobasal medium (Gibco) plus 2% B27, 0.5 mL L-glutamine (Hyclone), 10,000 units/mL, 10,000 µg/mL streptomycin, and 25 µg/mL Amphotericin B supplement. Cells were grown for 10-12 days in vitro before experiments and 50% of media changes were performed every 3 days. On day 10, neuronal cultures were treated with 0.5µM BDTOs alone and in the presence of Curcumin derivative (at final concentration 5µM) for two hours. The MTT viability assay was performed as previously described in Chapter 2.2.

Immunofluorescence

SH-SY5Y cells were maintained in Dulbecco`s modified Eagle's medium (DMEM) and grown to confluence using poly-L-lysine coated coverslip in 24-well plates as previously described (Castillo-Carranza, Guerrero-Munoz et al. 2018, Sengupta, Montalbano et al. 2018). Cells (≈20,000 cells /well) were treated for 1 hour with 0.5 µM TauO labeled with Alexa Fluor 568 or 0.5 µM TauO labeled with Alexa Fluor 568 pretreated with 5µM of curcumin derivatives. After washing off unbound proteins, cells were stained with 5 µg/mL WGA (Wheat Germ Agglutinin) Alexa Fluor 488 for 10 min followed by fixation in chilled methanol. After washing three times with 1X PBS, cells were permeabilized with 0.25% Triton-X 100, diluted in 1X PBS for 10 min. After washing three times with PBS (10 min each), cells were then stained with DAPI (Vector Laboratories) and mounted using Prolong Gold Antifade mounting media. Slides were then dried in fume hood. Cells were imaged with Keyence BZ-800 Microscope using standard filters for DAPI, GFP and Texas Red

channels and analyses have been conducted using BZ-X Analyzer software. Nikon 100X oil immersion objective was used for capture images that were analyzed by ImageJ and statistical analysis was performed by one-way ANOVA followed by Student's T test, using GraphPad Prism 6.01.

RESULTS AND DISCUSSION

We evaluated first the effect of curcumin on toxic tau aggregates using our *in vitro* preparation of tau oligomers. Therefore, highly purified oligomeric tau species were incubated with and without curcumin (5X and 10X) at RT on an orbital shaker, under oligomerization conditions. Tau oligomers in the absence and presence of curcumin were evaluated biochemically using the oligomer-specific antibody T22 and total tau antibodies, Tau 5 and Tau 13 (**Figure 4.2**).

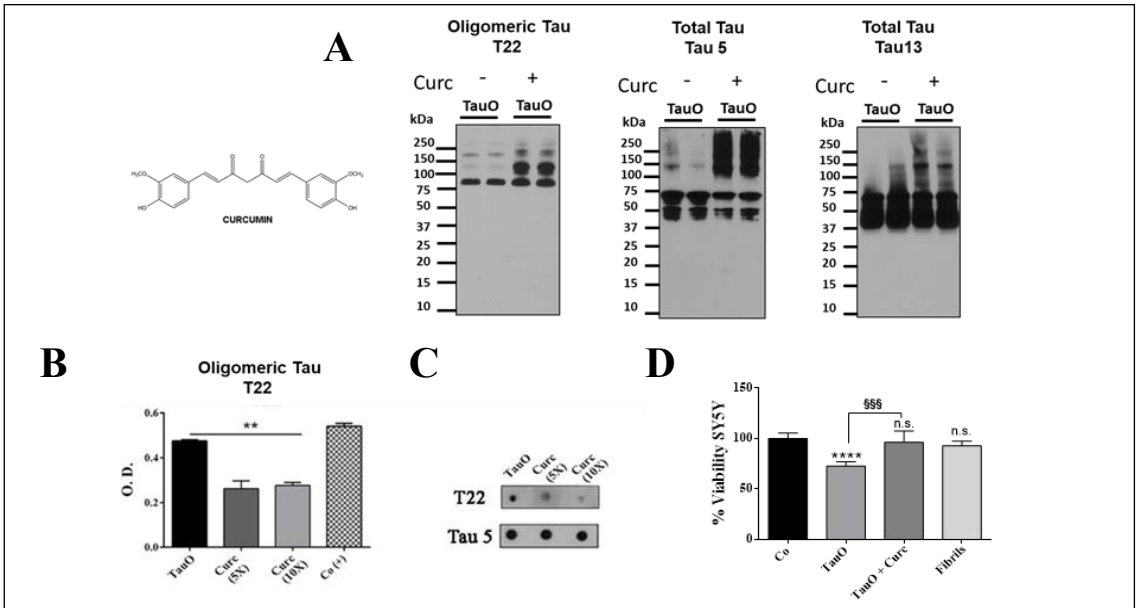


Figure 4.2 Biochemical and cytotoxicity analysis of oligomeric Tau treated with curcumin and untreated control.
(A) Western blot analysis of tau oligomers probed with the oligomeric tau antibody T22 and total tau antibodies, Tau 5 and Tau 13. Curcumin interacts and alters the aggregation states of preformed tau oligomers as compared to the untreated control, TauO. **(B)** ELISA analysis of oligomeric tau treated with increased concentration of curcumin showing a significant decrease in T22 immunoreactivity as compared to tau oligomers alone. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: **p<0.01 **(C)** Dot blot analysis show decreased levels of oligomeric tau in the presence of curcumin. **(D)** Viability percentage of cultured SH-SY5Y human neuroblastoma cells exposed to 2µM of tau oligomers, 2µM of tau oligomers pre-incubated with curcumin and controls. SH-SY5Y cells given TauO pre-treated with curcumin had significantly higher cells viability when compared to TauO alone and Ctrl. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: Co vs TauO, TauO+Curc, Fibrils:****p<0.001; TauO vs TauO+Curc: §§§ <0.001. Bars and errors represent the mean and standard deviation.

Western blot analysis showed that curcumin interacts with tau oligomers promoting the formation of larger tau aggregates (**Figure 4.2A**). In addition, direct ELISA and dot blot analyses showed a significant decrease in oligomers, as seen by the decrease in T22 immunoreactivity (**Figure 4.2B-C**). Next, the toxicity of these aggregated tau species, resulting from the co-incubation of TauO with curcumin, was assessed by MTT using the human neuroblastoma cell line, SH-SY5Y. Cells were exposed to tau oligomers alone (2μM) or in the presence of curcumin (final concentration 10 μM). SH-SY5Y viability decreased significantly after treatment with TauO, while the presence of curcumin rescued cells from TauO-induced toxicity as seen by the higher cell viability compared to the untreated control (Ctrl) (**Figure 4.2D**).

These exciting results led to a collaboration with medicinal chemistry experts to synthesize novel curcumin derivatives in an effort to improve curcumin's poor solubility in aqueous buffers and low bioavailability (**Figure 4.1**). The library of our newly synthesized curcumin-derived small molecules comprises four different groups of compounds with the potential to target and modulate tau oligomers aggregation state, thus neutralizing their toxicity and internalization potency in an effort to prevent or slow the spread of the pathology. Therefore, their efficacy was tested *in vitro* using recombinant tau oligomers and disease-relevant tau oligomeric strains were used to validate the effects of the most promising hit compounds, as shown in the following schematic (**Figure 4.3**).

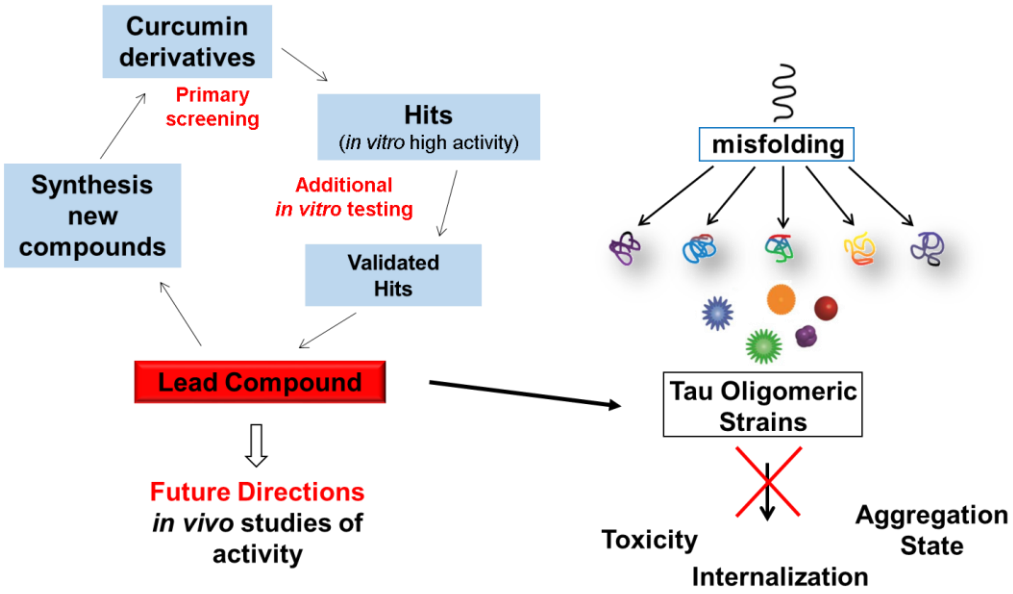
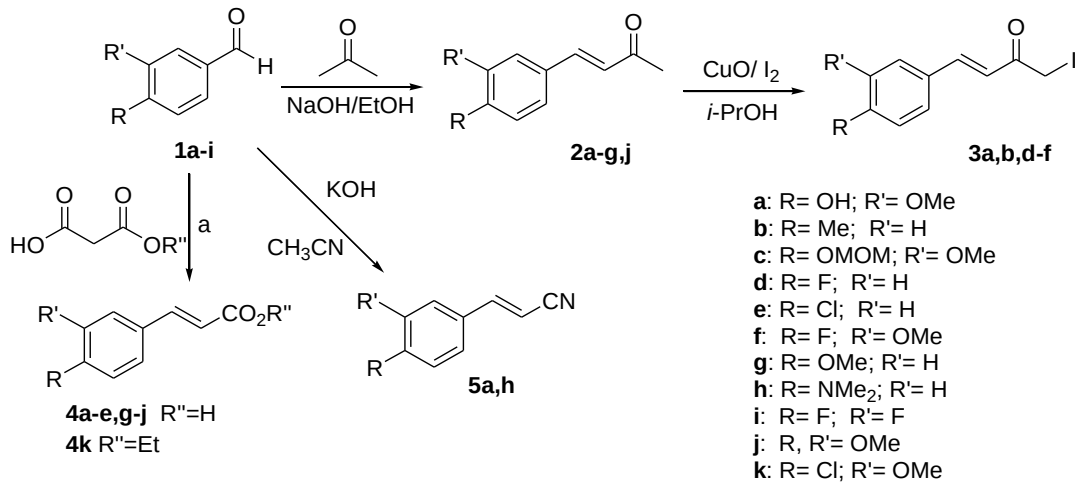


Figure 4.3 Flowchart describing the approach used to screen and develop biologically active curcumin derivatives.

Schematic representing the hypothetical model for the formation of tau oligomeric strains and the steps for developing active curcumin derivatives from initial *in vitro* screening using recombinant tau to the validation of the hits in disease-relevant tau oligomeric strains. Tau monomer misfolding leads to the formation of conformationally distinct misfolded monomers that aggregate into different oligomers. Toxic tau oligomeric strains can be targeted and modulated by active compounds inhibiting oligomers toxicity and internalization thus preventing further aggregation of tau and progression of tau pathology.

Synthesis

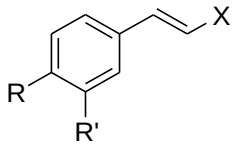
Hemi-curcuminoid analogs **2-5** were obtained by adapting previously reported condensation reactions (**Scheme 4.1**). *E*–α,β-Unsaturated ketones **2** were obtained through Claisen-Schmidt Aldol condensation (Agarwal, Srivastava et al. 2005), by treating commercial aldehydes **1** with acetone under basic conditions. In turn, reaction of compounds **2** with iodine, in the presence of CuO as catalyst, yields to iodo-derivatives **3** (Wang, Yin et al. 2008). *E*-Cinnamic acids **4** were obtained performing Doebner modification of Knoevenagel condensation (Mori, Wada et al. 2017), ethyl cinnamate **4k** was similarly obtained (Battisti, Palumbo Piccionello et al. 2017). Cinnamonitriles **5a,h** were obtained from benzaldehyde **1** condensation with acetonitrile, as previously reported (Khurana, Ali et al. 2014).



Scheme 4.1. Synthesis of Hemi-curcuminoid compounds. (a) R″=H: pyridine, aniline (cat.), toluene, reflux; R″=Et: pyridine, piperidine, reflux.

Among obtained compounds **2-5** were selected Hemi-curcuminoid compounds $HemiC_{1-10}$ (**Table 4.1**) which were tested as representative example of variously substituted derivatives. On the other hand, compounds **2-5** were used as building-block for the obtainment of other target compounds (see below).

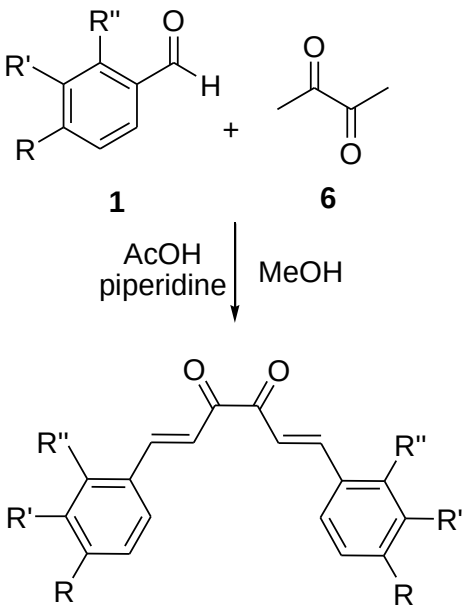
Table 4.1. Structures of tested Hemi-curcuminoid compounds (HemiC).



Entry ID	Compound	X	R	R′
$HemiC_1$	**2a**	COMe	OH	OMe
$HemiC_2$	**5a**	CN	OH	OMe
$HemiC_3$	**3a**	$COCH_2I$	OH	OMe
$HemiC_4$	**2g**	COMe	OMe	H
$HemiC_5$	**4a**	CO_2H	OH	OMe
$HemiC_6$	**2j**	COMe	OMe	OMe
$HemiC_7$	**4k**	CO_2Et	OMe	Cl
$HemiC_8$	**5h**	CN	NMe_2	H
$HemiC_9$	**2b**	COMe	Me	H
$HemiC_{10}$	**2c**	COMe	OMOM	OMe

Cinnamils (1,6-diarylhexa-1,5-diene-3,4-diones) **CL** are Curcumin-like analogs lacking of active methylene group and therefore, of associated tautomeric equilibria of the β-diketone moiety, partially responsible for curcumin's metabolic instability and poor pharmacokinetic properties (Sardjiman, Reksohadiprodjo et al. 1997). The synthesis of $\mathbf{CL_{1-12}}$, was performed through two aldol-condensation of aromatic aldehydes **1** on diacetyl

6 with the formation of both double bonds with *E* geometry (**Scheme 4.2**) (Sinu, Padmaja et al. 2013).

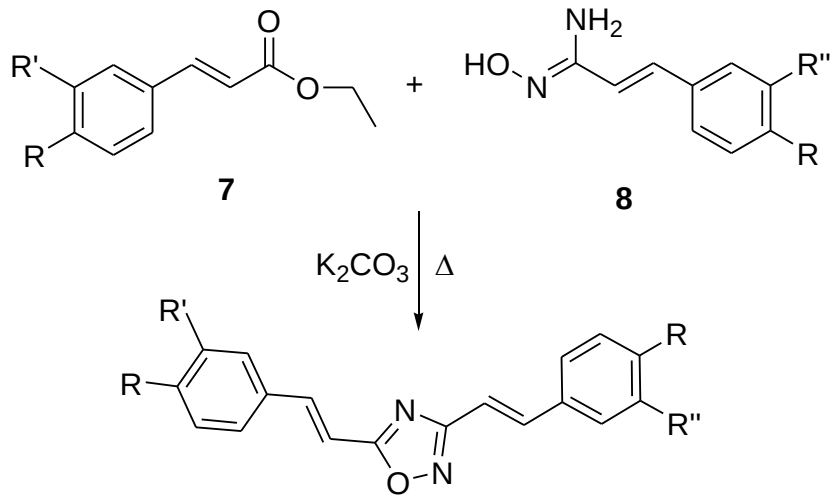


CL_1: R= OMe; R',R''= H
CL_2: R, R',R''= H
CL_3: R= F; R',R''= H
CL_4: R= NMe_2; R'= H
CL_5: R= Cl; R',R''= H
CL_6: R= F; R'= F
CL_7: R'= Cl; R,R''= H
CL_8: R''= Cl; R,R'= H
CL_9: R'= OMe; R,R''= H
CL_{10}: R= OMOM; R'= OMe; R''= H
CL_{11}: R,R'= OCH_2O; R''= H
CL_{12}: R= CO_2H; R',R''= H

Scheme 4.2. Synthesis of Cinnamils **$CL_{1\text{-}12}$**.

Another possible strategy is the substitution of the curcumin central core with heterocyclic rings, as previously reported for the design novel scaffolds able to target Aβ oligomers (Battisti, Palumbo Piccionello et al. 2017). In particular, we previously constructed a

database of structures endowed with a more stable and planar heterocycle. The virtual screening was accomplished through the calculation of molecular descriptors able to highlight the drug-like profile based on Lipinski's rules (rule of five) and by taking into account the molecular descriptors such as log BB, which allows the evaluation of BBB permeation ability (Battisti, Palumbo Piccionello et al. 2017). From this screening, were selected two scaffolds, 1,2,4- and 1,3,4-oxadiazole regio-isomers, two heterocyclic nuclei widely studied for AD treatment (Mangione, Palumbo Piccionello et al. 2015, Martorana, Giacalone et al. 2016). In particular, following Scheme 4.3, the 1,2,4-oxadiazole derivatives **CH$_{1-4}$**, were obtained by adopting the conventional amidoxime route (Pace, Buscemi et al. 2015), starting from the esters **7** and amidoximes **8**.

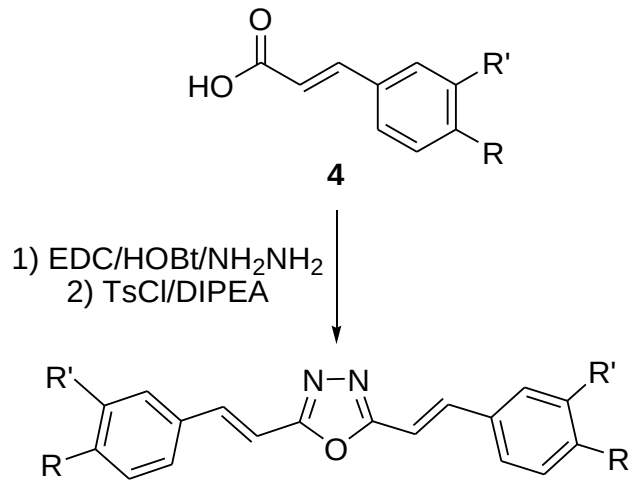


CH$_1$: R,R',R''= OMe
CH$_2$: R= OMe; R',R''=H
CH$_3$: R,R',R''= H
CH$_4$: R,R''= OMe; R'=Cl

Scheme 4.3. Synthesis of Heterocyclic curcumin-like 1,2,4-oxadiazoles **CH$_{1-4}$**.

The 1,3,4-oxadiazole regio-isomers **CH$_{7\text{-}11}$**, from Scheme 4.4, were obtained from the one-pot construction of a diacylhydrazine intermediate, followed by cyclization and starting from the cinnamic acid analogue **4** (Stabile, Lamonica et al. 2010) .

All compounds were region-selectively obtained in *E* geometry in good overall yields.

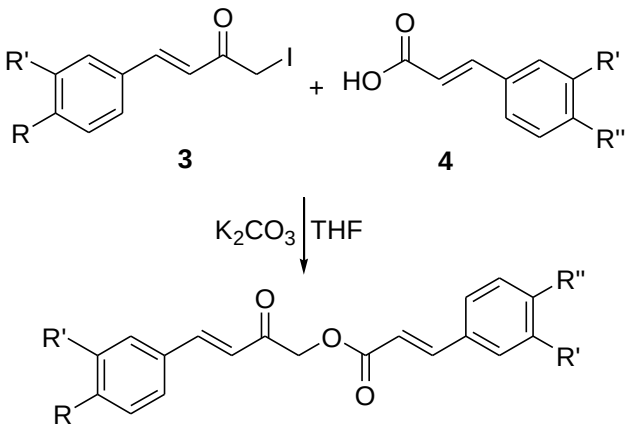


CH$_5$: R,R'= F
CH$_6$: R= Cl; R'= H
CH$_7$: R,R'= OCH_2O
CH$_8$: R= NMe_2; R'=H
CH$_9$: R,R'= OMe
CH$_{10}$: R= OMe; R'=H
CH$_{11}$: R= OMOM; R'= OMe

Scheme 4.4 Synthesis of Heterocyclic curcumin-like 1,3,4-oxadiazoles **CH$_{7\text{-}11}$**.

The last group of curcumin derivatives that were synthesized are the Calebin-A analogs. Calebin-A is a polyphenol compounds derived from turmeric of *Curcuma Longa* and was previously reported as neuroprotective compounds active toward Aβ peptide (Park and Kim 2002). The synthesis of Calebin-A and its analogs **Cal$_{1\text{-}9}$** was accomplished by coupling, through a nucleophilic substitution reaction, iodo-derivatives **3** and cinnamic

acids **4** [Majeed, M.; Nagabhushanam, K.; Majeed, A.; Thomas, S. M. Eur. Pat. Appl. 2016, EP 2963007], avoiding the use of protective groups (**Scheme 4.5**).



Cal_1: R, R', R''= OMe
Cal_2: R, R''= OMe; R'= H
Cal_3: R, R''= Me; R'= H
Cal_4: R = OMOM; R', R''= OMe
Cal_5: R, R'= CH_2OCH_2; R'', R'= CH_2OCH_2
Cal_6: R, R''= F; R'= H
Cal_7: R, R''= Cl; R'= H
Cal_8: R, R''= OH; R'= OMe
Cal_9: R = F; R', R''= OMe

Scheme 4.5. Synthesis of Calebin-A like compounds **Cal_{1-9}.**

All these newly synthesized compounds were screened and tested to evaluate and assess their efficacy in interacting and altering tau aggregation pathways using recombinant tau oligomers.

Hemi-curcuminoids (HemiC1-10)

The first group of curcumin analogs are the Hemi-curcuminoids (HemiC1-10). These compounds were synthesized using ferulic acid as a reference, since it structurally correlates to a half portion of curcumin. Therefore, the Hemi-curcuminoids, that have been obtained, are variously substituted and functionalized styrene derivatives with a very low

molecular weight (MW from 160 to 260 Da). Tau oligomers were incubated alone or in the presence of curcumin and Hemi-curcuminoids derivatives (5X) for 16 hours under oligomerization conditions and reactions were assessed using T22 antibody. Western blot analysis in Figure 4.4A showed the altered aggregation of preformed tau oligomers after incubation with Hemi-curcuminoids. Co-incubation with these derivatives showed the

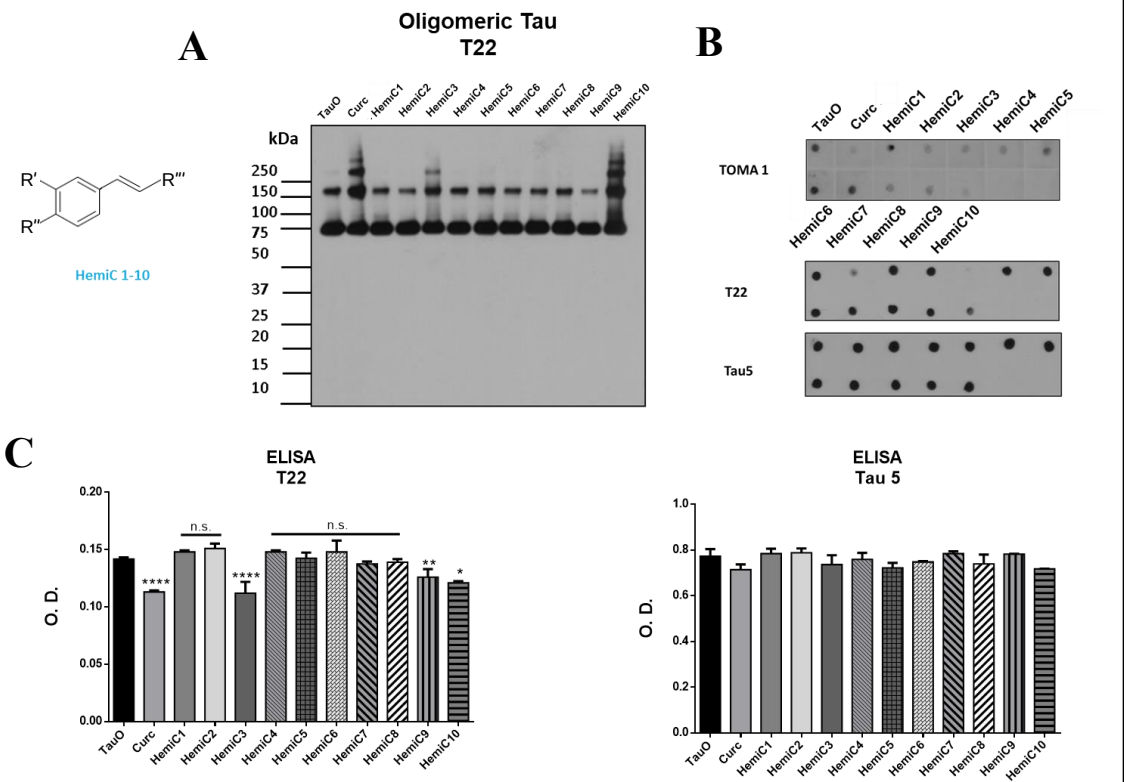


Figure 4.4 Biochemical analysis of oligomeric Tau treated with compound HemiC derivatives and untreated control.

(**A**) Western blot analysis of 3 ug/ul of tau oligomers alone and pretreated with curcumin and Hemi-urcuminoid analogs probed with T22, shows that some of the compounds are able to alter the aggregation states of preformed tau oligomers. (**B**) Dot Blots analysis of oligomeric tau alone and in the presence of HemiC, probed with TOMA1, T22 and Tau5, shows that some of the HemiC compounds are able to decrease tau oligomer levels as compared to the untreated control. (**C**) ELISA analysis of oligomeric tau with and without HemiC analogs shows that some HemiC are able to affect tau aggregation pathways reducing tau oligomer levels as compared to the untreated control while there is no change in total tau protein using Tau5. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: (*p<0.05; **p<0.01****p<0.0001) Bars and errors represent the mean and standard deviation.

capability of some Hemi-curcuminoids to reduce tau oligomer levels and others to induce the formation of higher molecular weight non-toxic aggregates.

Dot blots analysis of tau oligomers alone or in the presence of the HemiC compounds showed reduction in TauO after incubation with some Hemi-curcuminoids, as seen by the decreased TOMA1 and T22 immunoreactivities (**Figure 4.4B**). TOMA1 is a conformational monoclonal antibody that recognizes conformational epitopes that do not depend on linear amino acid sequences and displays distinct preferences for different subsets of tau oligomer (Castillo-Carranza, Sengupta et al. 2014), suggesting that the treatment with the HemiC compounds led to a conformational changes in the preformed oligomeric tau species. The potency of these analogs was also confirmed by direct ELISA showing a significant decrease in oligomers detection by T22 antibody with no differences using total tau antibody, Tau 5 (**Figure 4.4C)**.

Taken together, our results suggest that Hemi-curcuminoids interact and modulate the aggregation of preformed oligomeric tau species promoting the formation of larger non-toxic tau aggregates or decreasing tau oligomers levels.

Curcumin-like (CL1-12)

The second group of curcumin derivatives (CL1-12) displays the same structure of curcumin with different substitutions and functionalizations. Tau oligomers, incubated alone or in the presence of curcumin and Curcumin-like analogs (5X), were biochemically assessed by western blot using T22 as well as the total tau antibody, Tau 5. Figure 4.5A shows the capability of each curcumin-like derivate to interact with preformed tau oligomers modulating their aggregation states, resulting in the formation of larger and higher molecular weight non-toxic aggregates. Dot blots assay showed reduction in TauO

levels after incubation with Curcumin-like derivatives, as assessed by the decreased TOMA1 and T22 immunoreactivities and no changes were observed in total tau, once probed with Tau5. Direct ELISA confirmed the previous results; untreated tau oligomers showed strong immunoreactivity with T22 while, in the presence of the compounds, there was a reduced immunoreactivity suggesting their capability to modulate the aggregation pathway of preformed tau oligomers aggregation (**Figure 4.5**).

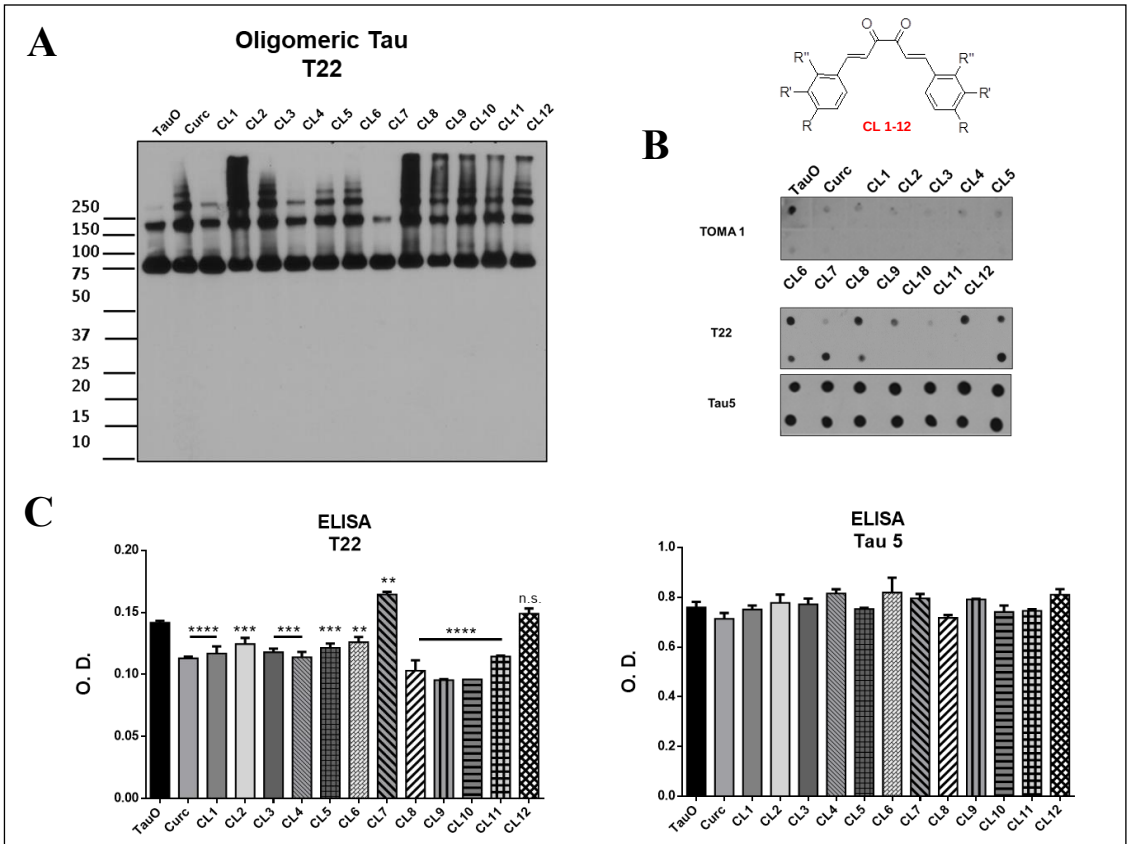


Figure 4.5 Biochemical analysis of oligomeric Tau treated with Curcumin-like (CL) derivatives and untreated control.

(A) Western blot analysis of 3 ug/ul of tau oligomers alone and pretreated with curcumin and CL analogs, probed with T22, shows that the compounds are able to alter the aggregation states of preformed tau oligomers. **(B)** Dot blot analysis probed with anti-oligomeric monoclonal and polyclonal tau antibodies, respectively TOMA1 and T22, and total tau antibody, Tau 5. **(C)** ELISA analysis of oligomeric tau shows a significant decrease in the tau oligomer levels in the presence of the CL compounds as compared to the untreated control, TauO. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: (**p<0.01; ***p<0.001****p<0.0001). Bars and errors represent the mean and standard deviation.

Heterocyclic curcumin analogs (CH1-11)

The next group of newly synthesized derivatives are the Heterocyclic curcumin analogs that display the same structure of the lead compound curcumin with the introduction of a heterocyclic moiety e.g. imidazole, pyridine and pyrazole among others. These compounds have been synthesized following Lipinski's rule of five to obtain active molecules that can easily pass through the BBB. Heterocyclic curcumin derivatives' effects on recombinant tau oligomers were evaluated biochemically (**Figure 4.6**). Western blot analysis showed that the treatment with Heterocyclic derivative induces the formation of larger tau species (**Figure 4.6A**). Dot blot and filter trap analyses showed decreased T22 immunoreactivity after co-incubation with the compounds as compared to the untreated tau oligomers. Moreover, some derivatives were also able to reduce TOMA1 immunoreactivity, suggesting that conformational changes have occurred in the preformed oligomeric tau species after treatment with the Heterocyclic analogs (**Figure 4.6B**).

Dot blot and filter trap assays probed with Tau5 as control, showed no changes in total tau protein. These results were also confirmed by direct ELISA (**Figure 4.6C**).

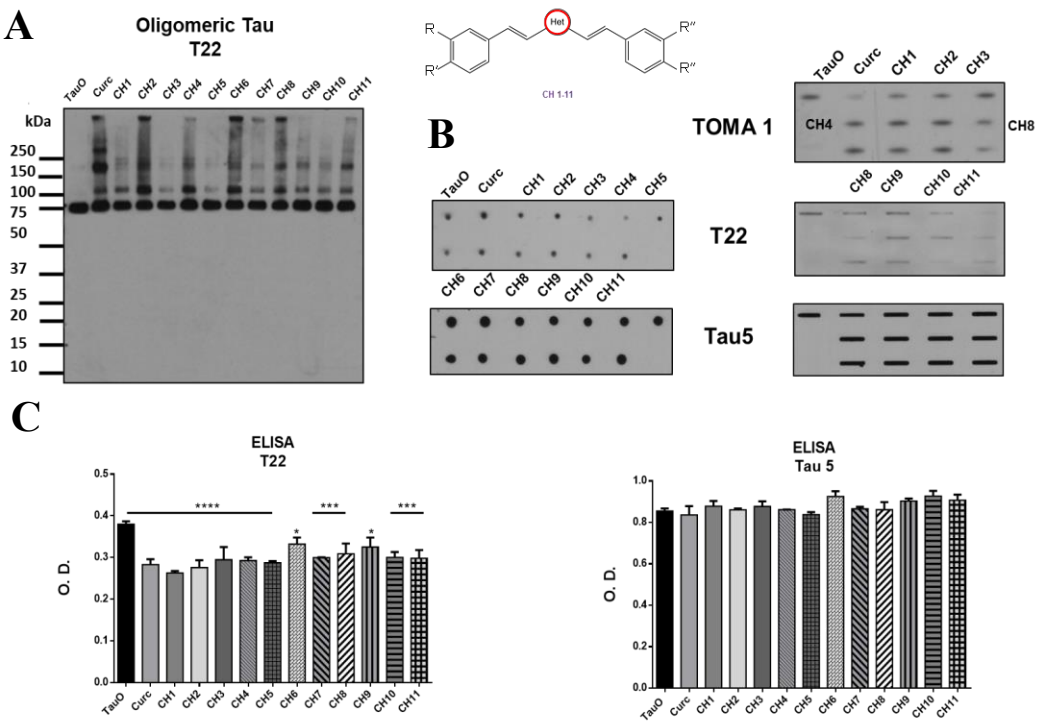


Figure 4.6 Biochemical analysis of oligomeric Tau with and without Heterocyclic curcumin (CH) derivatives treatment.

(A) Western blot analysis of 3 ug/ul of tau oligomers alone and pre-treated with curcumin and Heterocyclic curcumin analogs probed with T22, shows that the incubation with the compounds modulates the aggregation states of preformed tau oligomers as compared to the untreated TauO. **(B)** Filter Trap and Dot blot analyses of tau oligomers alone and pre-treated with curcumin and CH analogs probed with T22 and Tau 5. Some of the compounds are able to alter the aggregation states of preformed tau oligomers resulting in decreased tau oligomer levels as compared to tau oligomers alone. CH analogs are able to reduce the Tau Oligomer Monoclonal Antibody TOMA1 immunoreactivity. **(C)** ELISA analysis of oligomeric tau with and without CH derivatives show decreased T22 immunoreactivity after treatment with the compounds and no changes in total tau protein using Tau 5. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: (*$p<0.05$; ***$p<0.001$****$p<0.0001$) Bars and errors represent the mean and standard deviation.

Calebin-A analogs (Cal1-9)

The last group of curcumin derivatives screened are the Calebin-A derivatives. Calebin-A is a natural occurring small molecule obtained from the rhizome of *Curcuma Longa* like curcumin. Calebin-A was previously reported as neuroprotective compounds

active against Aβ insult (Park and Kim 2002). The structural difference with curcumin is the lacking of the 1,3 diketonic structure. However, Calebin-A as well as curcumin showed to have poor solubity in water and low bioavailabity, thus derivatives were synthesized to improve these shortcomings (Oliveira, Martinez et al. 2015).

Calebin-A derivatives were incubated with preformed tau oligomers and their effects were evaluated by western blot and dot blot analyses showing the potency of the compounds in altering the aggregation pathways of preformed tau oligomers (**Figure 4.7**).

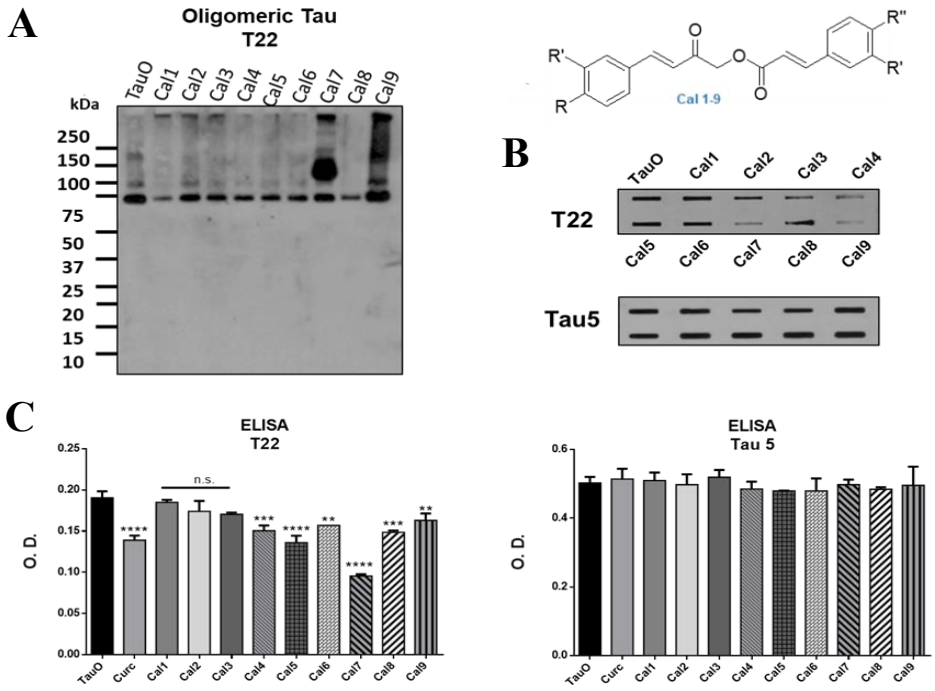


Figure 4.7 Biochemical analysis of oligomeric Tau with and without Calebin-A (Cal) derivatives treatment.

(A) Western blot analysis of 3 ug/ul of tau oligomers alone and pre-treated with curcumin and Calebin-A analogs probed with T22, shows that the incubation with the compounds modulates the aggregation states of tau oligomers as compared to the untreated TauO. (**B**) Filter Trap assay, probed with T22 and Tau 5, show that some of the compounds are able to decrease T22 immunoreactivity as compared to the untreated TauO. (**C**) ELISA analysis of oligomeric tau after treatment with Cal derivatives shows that some of the compounds decrease tau oligomer levels as seen by the reduced T22 immunoreactivity and no changes in total tau protein using Tau 5. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: (**p<0.01; ***p<0.001****p<0.0001) Bars and errors represent the mean and standard deviation.

Biochemical anylysis of tau oligomers after incubation with Calebin-A derivatives shows that the Calebin-A-derived small molecules are able to decrease the oligomer levels and promote the formation of higher molecular weight aggregates as seen by western blot as well as filter trap assay analyses. Furthermore, direct ELISA show significant decrease in tau oligomer levels after treatment with some of the Calebin-A derivatives as assessed by the reduced T22 immunoreactivity.

Based on the biochemical screens, we selected three compounds of each group showing higher activity with recombinant tau oligomers for additional *in vitro* testing, listed below (**Table 4.2**).

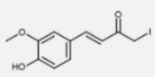
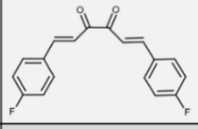
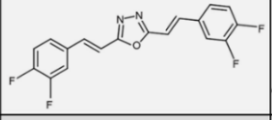
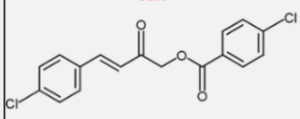
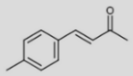
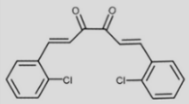
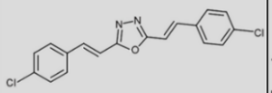
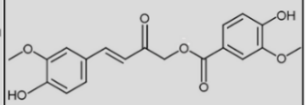
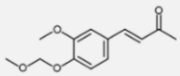
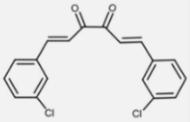
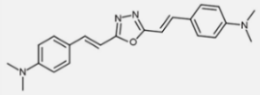
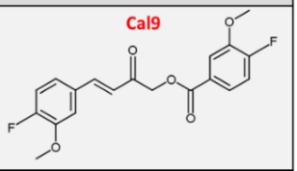
HemiCurcuminoids	Curcumin-like	Heterocyclic Curcumin	CalabinA-like
HemiC3 	CL3 	CH5 	Cal7 
HemiC9 	CL7 	CH6 	Cal8 
HemiC10 	CL8 	CH8 	Cal9 

Table 4.2 Selected compounds for each group of curcumin derivatives.

Therefore, the curcumin derivatives selected were further tested biochemically using preformed recombinant tau oligomers to evaluate their effects in parallel, side by side and under the same conditions. Indeed, oligomeric tau species were incubated with

and without curcumin derivatives (final conc. 5µM) and were evaluated biochemically using the oligomer-specific antibody T22 and generic tau antibody, Tau 5 (**Figure 4.8**).

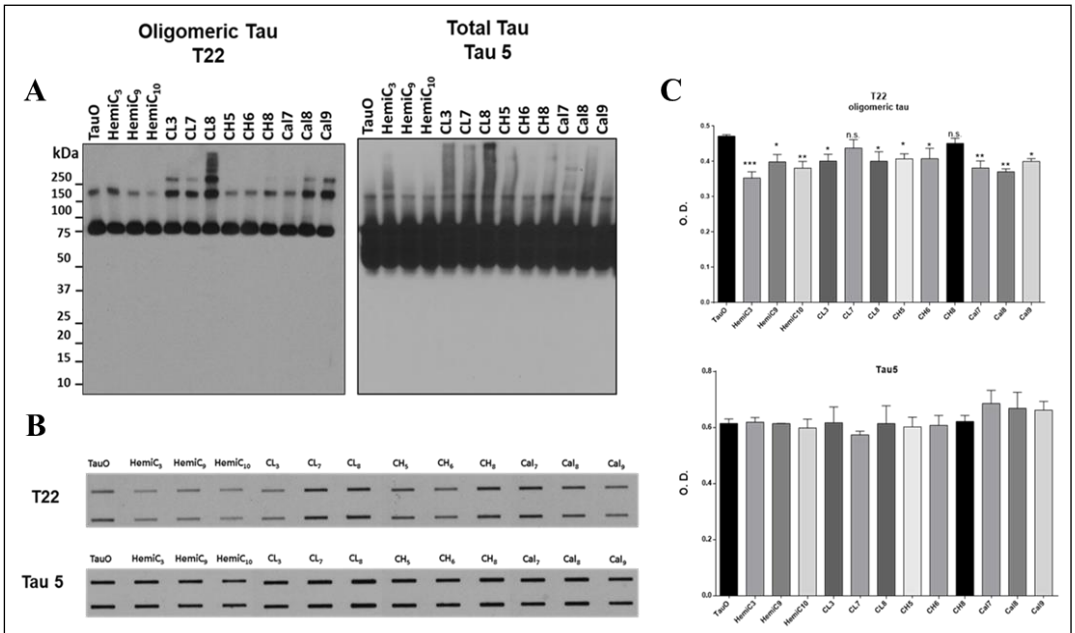


Figure 4.8 Biochemical analysis of oligomeric tau with and without curcumin derivatives treatment.

(A) Western blot analysis of 3 ug/ul of tau oligomers alone and pre-treated with curcumin analogs probed with T22, shows that the incubation with the compounds modulates the aggregation states of preformed tau oligomers as compared to the untreated TauO. **(B)** Filter Trap assay of tau oligomers alone and pre-treated with curcumin derivatives probed with T22 and Tau 5. Some of the compounds are able to alter the aggregation state of preformed tau oligomers resulting in decreased T22 immunoreactivity as compared to the untreated TauO. **(C)** ELISA analysis of oligomeric tau after treatment with curcumin derivatives show that the selected compounds decrease tau oligomer levels as seen by the reduced T22 immunoreactivity and no changes in total tau protein by using Tau 5. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: (*p<0.05; **p<0.01; ***p<0.001) Bars and errors represent the mean and standard deviation.

Western blot analysis showed that curcumin-derived small molecules interact with recombinant tau oligomers resulting in decreased oligomer levels or leading to tau structures with higher molecular weight. In addition, filter trap assay confirmed that some of the compounds modulate the aggregation pathway of preformed tau oligomers resulting

in decreased T22 immunoreactivity as compared to the untreated oligomers. Moreover, direct ELISA showed that curcumin derivatives interactions with tau oligomers resulted in decreased oligomer level as detected by T22 oligomeric-specific tau antibody. As a result from these additional screenings, we selected six promising compounds, showing to affect the aggregation state of toxic tau oligomers. (**Figure 4.9**). These hit compounds were further tested biophysically as well as cytotoxicity screens were performed to evaluate their ability to modulate tau oligomers associated neurotoxicity.

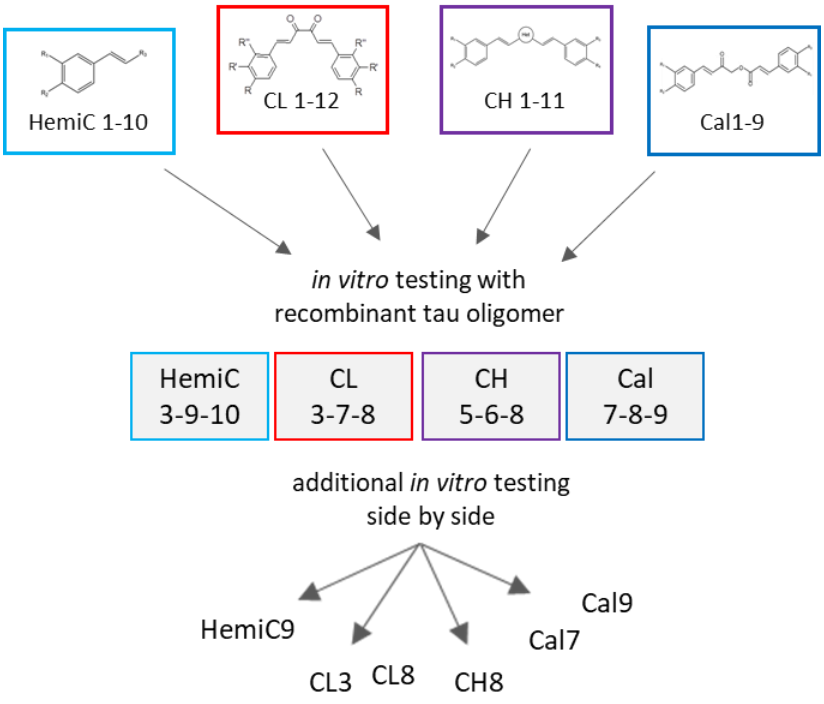


Figure 4.9 Schematic describing the steps to select *in vitro* active curcumin derivatives.

Therefore, tau oligomers with and without the selected active compounds were also characterized biophysically (**Figure 4.10**). Fast protein liquid chromatography (FPLC) was used to purified tau oligomers detecting a main peak at ~120 –150 kDa (tau dimer/trimer). Atomic force microscopy was performed to assess the morphology of purified tau oligomers before and after treatment with the curcumin derivatives. AFM images of tau oligomers alone displayed their classically homogeneous spherical morphology and, in the presence of the curcumin-derived small molecules, they are converted into larger tau aggregates as seen in Figure 4.10B.

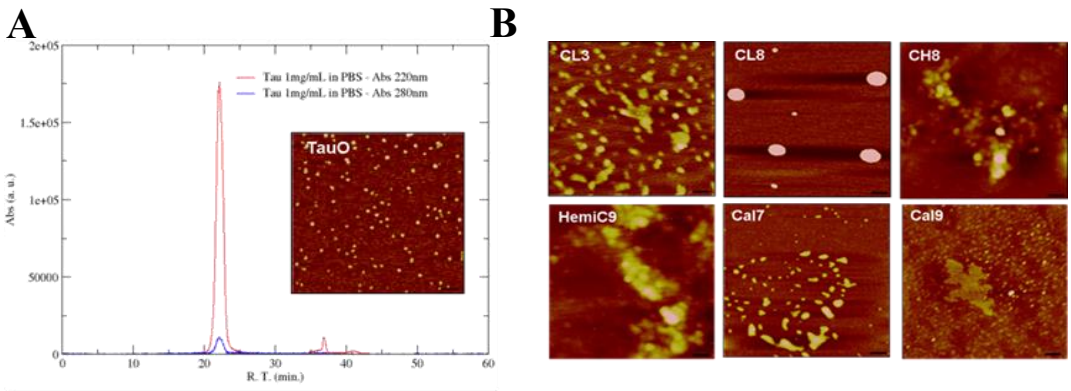


Figure 4.10 Biophysical characterization of tau oligomers.

(**A**) FPLC chromatogram of tau oligomers; the main peak is ~120 –150 kDa (tau dimer/trimer). (**B**) Atomic Force Microscopy images of TauO after treatment with 5µM of curcumin derivatives for CL3, CL8, CH8, HemiC9, Cal7 and Cal9. AFM analysis show the ability of the compounds to modulate TauO aggregation states converting TauO into much larger aggregates. Scale bars = 100 nm.

In addition, the cytotoxicity of each selected compound was evaluated using MTT assays in cultured human SH-SY5Y neuroblastoma cell line by exposing cells for 24 hours with increasing concentrations of the hit compounds within the range 0-800 µM. Our results showed that the curcumin derivatives have a very low toxic profile as shown by the dose-response curves in Figure 4.11.

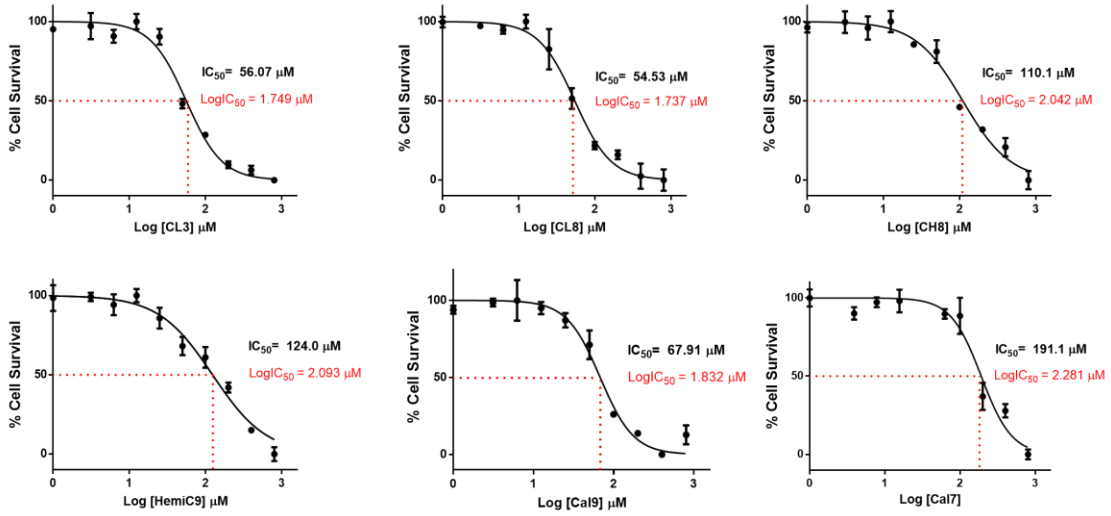


Figure 4.11 Curcumin derivative effects on cell viability.

The cytotoxicity of curcumin derivatives on human neuroblastoma SH-SY5Y cell line was determined by MTT assay. MTT assay was used to determine the IC50 values for CL3, CL8, CH8, HemiC9, Cal7 and Cal9 compounds following treatment with increasing concentration of the compounds (0-800 µM) for 24 hours. Values are presented as the mean ± SD (n = 3).

Next, the toxicity of the curcumin derivative-induced aggregates was evaluated by using primary cortical neurons isolated from embryos of Htau mice, expressing non-mutant human tau. Cells were exposed to tau oligomers alone or in the presence of curcumin derivatives and Aβ oligomers (AβO) were used as a control (**Figure 4.12**). Cell viability significantly decreased after treatment with untreated TauO, while treatment with curcumin

derivatives (final concentration 5 μM) reduced their toxicity significantly as seen by the higher level of cell viability using MTT assay.

Interestingly, curcumin derivatives were also incubated with Aβ oligomers and toxicity screens in primary neurons showed that the compounds were not able to rescue neurons from Aβ oligomers-induced toxicity (**Figure 4.12B**).

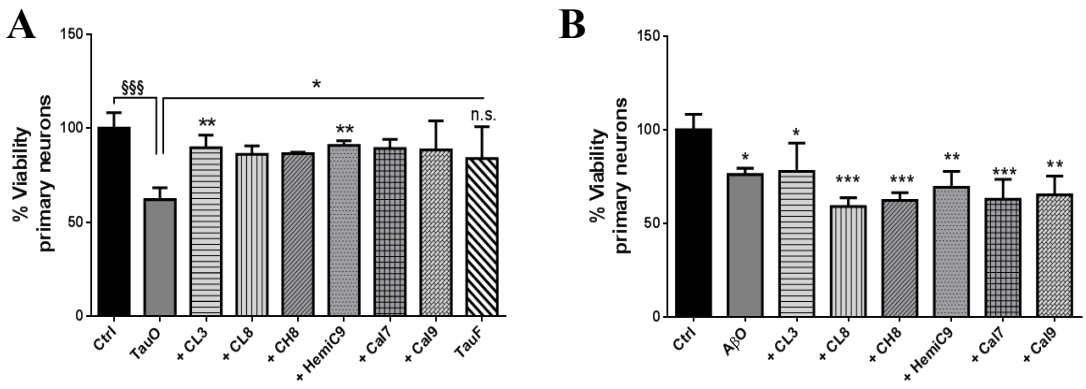


Figure 4.12 Curcumin derivative effects on primary cortical neurons cell viability.

(A) Viability percentage of neuronal culture exposed to 0.5 μM of tau oligomers, 0.5μM of tau oligomers pre-incubated with 5 μM curcumin derivatives and controls for 2 hours. Cells exposed to TauO pre-treated with curcumin derivatives had significantly higher cells viability as compared to TauO alone. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: Ctrl vs TauO $^{§§§}p<0.001$; TauO vs CL3, CL8, CH8, HemiC9, Cal7 and Cal9: *p<0.05; **p<0.01. Bars and errors represent the mean and standard deviation (n = 3). **(B)** Viability percentage of neuronal culture exposed to 0.5 μM of Aβ oligomers, 0.5μM of Aβ oligomers pre-incubated with 5 μM curcumin derivatives and controls. Cells exposed to AβO pre-treated with curcumin derivatives show no changes in cells viability as compared to AβO alone showing the selected curcumin derivatives were not able to rescue from AβO-induced toxicity. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: Ctrl vs AβO, CL3, CL8, CH8, HemiC9, Cal7 and Cal9: *p<0.05; **p<0.01; ***p<0.001. Bars and errors represent the mean and standard deviation (n= 3).

Furthermore, to further confirm our findings and gain a better understanding of the protective role of curcumin derivatives, SH-SY5Y human neuroblastoma cells were treated

with sub-lethal concentration of TauO or TauO after treatment with the curcumin compounds and imaged by fluorescence microscopy (**Figure 4.13**).

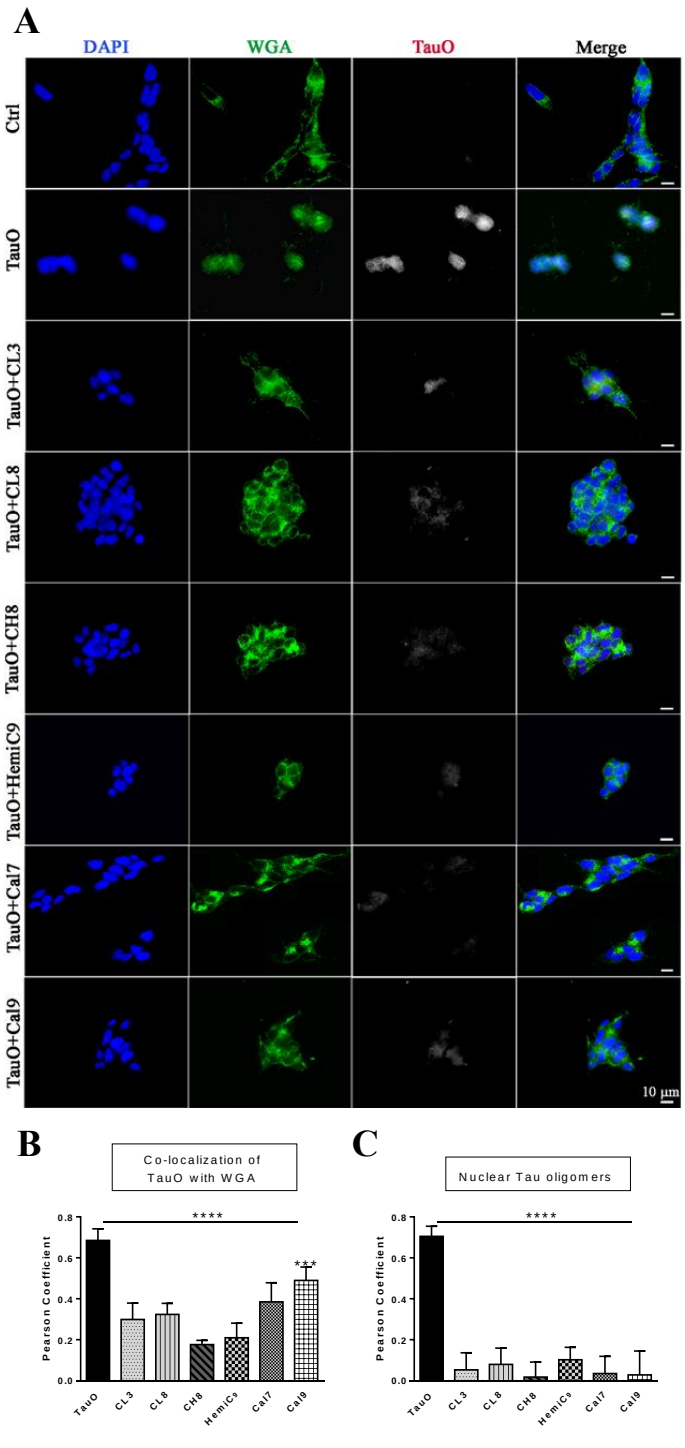


Figure 4.13 Representative epifluorescence images of human SH-SY5Y neuroblastoma cells after treatment with TauO or TauO in the presence Curcumin derivatives.
(**A**) SH-SY5Y cells were treated for 1 hour with sub-lethal concentration (0.5µM) of TauO in the absence and presence of 5 µM Curcumin derivatives. Cells were stained with DAPI (nuclei – blue), WGA - AF488 (plasma membranes – green) and TauO labeled with AF 568 (represented in grey). (**B**) PCC graph represent colocalization coefficient of TauO with WGA showing a significant reduction of TauO in the plasma membranes when cells are exposed to TauO in the presence of compounds. **(C**) Curcumin derivative-induced aggregates are less uptaken by the cells as shown by the decreased nuclear tau compared to untreated TauO. Analyses were performed on selected regions of interest characterized by same size and comparable number of cells. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: ***p<0.001, ****p<0.0001. Bars and error bars represent means and standard deviations.
Scale bar = 10µm.

Tau oligomers were observed in the plasma membranes as well as in the nuclei, as shown by PCC graph, indicating extensive cellular internalization of TauO.

Furthermore, cells exposed to untreated TauO, exhibit extensive loss of plasma membrane integrity, reflecting the toxic effect of tau oligomers. Interestingly, SH-SY5Y cells that were treated with TauO, co-incubated with curcumin derivatives, show a significant reduction in the percentage of area positive of TauO staining. Immunofluorescence analysis shows that the tau species, resulting from the incubation of curcumin derivatives, mostly co-localize with the plasma membrane (**Figure 4.13B**).

Altogether, these data suggest that curcumin derivatives-induced aggregates are less prone to be internalized by the cells, elucidating their reduced cytotoxicity.

In addition, curcumin derivatives, showing high activity with recombinant tau oligomers, were tested using disease-relevant brain-derived tau oligomers (BDTOs) from different tauopathies.

Our lab has established the isolation of BDTOs (Lasagna-Reeves 2012, Gerson, Sengupta et al. 2014) to directly test whether tau oligomers form conformationally distinct strains that depend upon individual and/or disease difference. One of the most common determinants of strain differences in the prion field is the stability of the protein core following exposure to Proteinase K (PK) (Legname, Nguyen et al. 2005, Ghaemmaghami, Watts et al. 2011). Recent studies demonstrated that also aggregated tau exhibits variable protease stability similar to prions (Sanders, Kaufman et al. 2014).

To characterize disease-relevant tau oligomeric strains, BDTOs were isolated by immunoprecipitation with the oligomeric tau antibody, T22, using brain homogenates from different neurodegenerative tauopathies. BDTOs were then purified by FPLC and

characterized, alone and in the presence of small molecules, biophysically and biochemically to evaluate the ability of each compound to affect BDTOs strains aggregation state and toxicity (**Figure 4.14**).

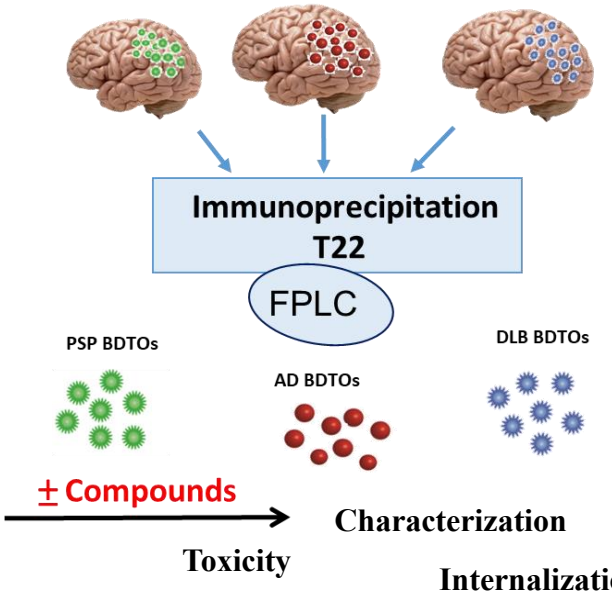


Figure 4.14 Schematic describing the steps by through BDTOs strains are isolated.

Brain homogenates from DLB, AD and PSP were isolated and characterized by AFM. Images from each BDTO displayed a different morphology (**Figure 4.15A**). One of the most common determinants of strain differences in the prion field is the stability of the protein core following exposure to PK. Therefore, BDTOs were exposed at 1µg/ml of PK and evaluated by western blot using the sequence specific anti-tau antibody, Tau 5. Western blot analysis revealed that each BDTO strain has different patterns of fragmentation (**Figure 4.15B**).

In addition, Tau strains toxicity was evaluated using primary cortical neurons, isolated from Htau mice, which better mimic the physiology of cells *in vivo*. Indeed, gene as well protein expression profiles in primary neurons better resemble those of the

differentiated cell *in vivo* and are also more appropriate for drug targeting validation. Hence, primary neurons, exposed to 0.5µM BDTOs for 2 hours, showed a significant decrease in cell viability as compared to untreated cells, Ctrl (**Figure 4.15C**).

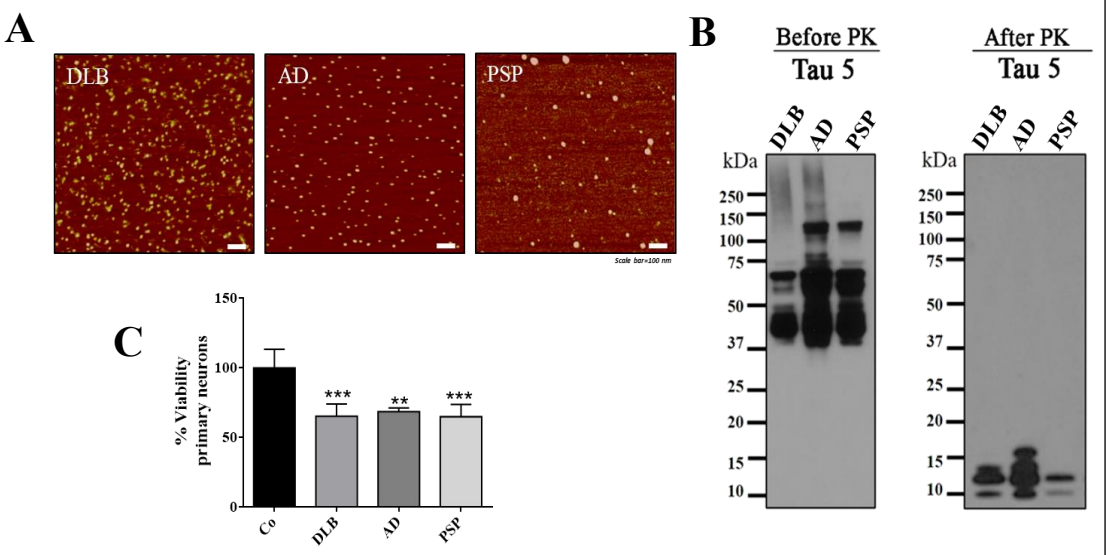


Figure 4.15 Characterization of BDTOs

(**A**) Brain-derived tau oligomers from different tauopathies were characterized by AFM showing different morphologies. Scale bars=100nm. (**B**) BDTOs were evaluated by Western blot, probed with anti-tau antibody Tau5, before and after treatment with 1µg/mL of PK. Western blot analysis revealed different patterns of fragmentation of each BDTO after exposure to PK digestion. (**C**) Viability percentage of cultured Htau primary neurons exposed to 0.5µM of BDTOs. Primary neurons given BDTOs reduced significantly cells viability when compared to the untreated control. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: (**p<0.01; ***p<0.001.) Bars and errors represent the mean and standard deviation.

Therefore, using methods from the prion field, we found that tau oligomers purified from different tauopathies exhibit different aggregate compositions under atomic force microscopy (AFM) and specific PK digestion profile, indicating that brain-derived tau oligomers from different disorders form structurally distinct strains.

After characterizing biochemically and biophysically BDTOs, tau oligomeric strains isolated from PSP brain homogenates, were treated with three of the derived small molecules, CL1-3, showing high activity with recombinant tau oligomers.

Therefore, BDTOs were incubated alone or in the presence of curcumin analogs (final conc. 5µM) for 16 hours, under oligomerization conditions. PSP-derived oligomers were evaluated by western blot using T22 and Tau 5 antibodies (**Figure 4.16A**), revealing

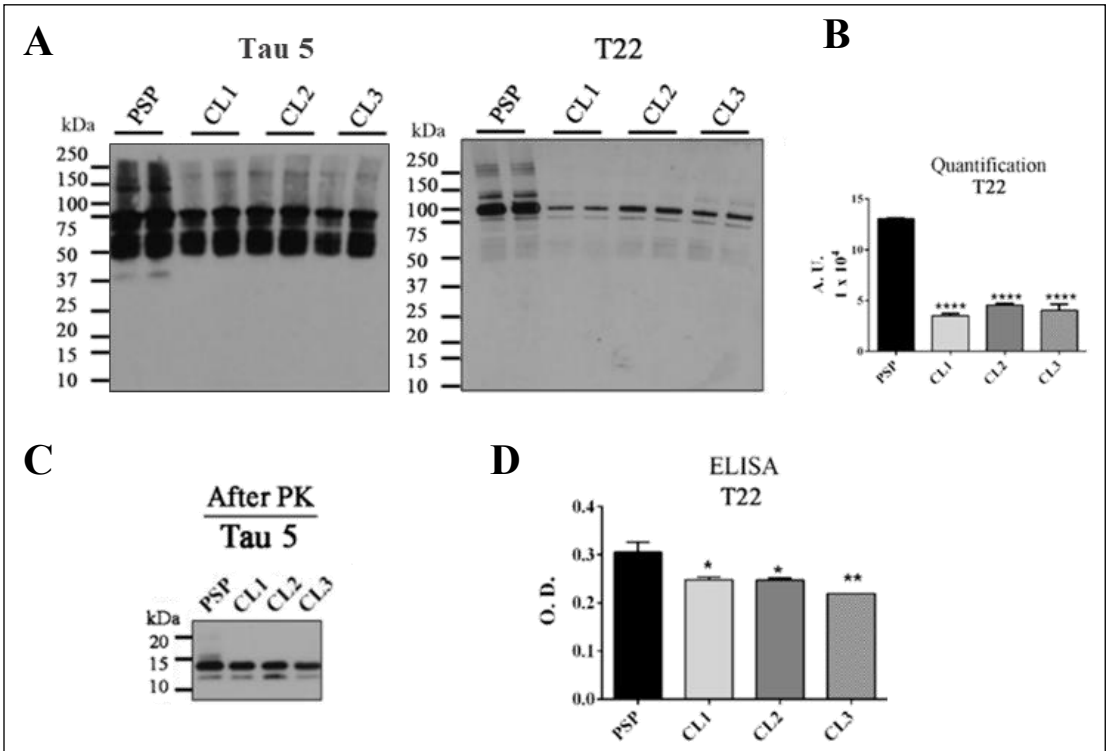


Figure 4.16 Biochemical analyses of PSP tau oligomers treated with Curcumin-like derivatives and untreated control.
(**A**) Western blots of BDTOs probed with total (Tau 5) and oligomeric (T22) tau antibodies showing decreased tau aggregates after treatment with CL analogs.
(**B**) Western blot analysis, using T22, revealed a significant decrease in tau oligomer aggregates in the presence of the derived small molecules as compared to BDTOs alone. (**C**) BDTOs, alone and in the presence of CL small molecules, were exposed to PK digestion. Representative Western blot using anti-tau antibody Tau 5, revealed the ability of the analogs to affect the protein core stability as compared to BDTOs alone. (**D**) Direct ELISA analysis of BDTOs alone and in the presence of CL derivativess confirmed the CL's ability to modulate toxic BDTOs decreasing the oligomer levels. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.) Bars and errors represent the mean and standard deviation.

that the aggregation state of BDTOs was modulated by incubation with the CL derivatives. Western blot analysis showed a significant decrease in T22 immunoreactivity when PSP derived oligomers were incubated with CL1-3 as compared to the untreated BDTOs (**Figure 4.16B**).

Furthermore, PSP Tau strains, alone or in the presence of the Curcumin-like derivatives, were also exposed to PK digestion and evaluated by Western blot using the generic tau antibody, Tau 5. Western blot analysis showed that Curcumin-like derived small molecules affect the protein core stability (**Figure 4.16C**). In addition, direct ELISA analysis confirmed the previous results, revealing a decreased T22 immunoreactivity when BDTOs were incubated with CL1-3 as compared to the untreated control (**Figure 4.16D**).

Next, the toxicity of these tau aggregated species, resulting from the co-incubation of BDTOs with CL3, was investigated to assess the ability of the newly synthesized small molecules to prevent and reduce brain-derived tau oligomer-induced toxicity in primary cortical neurons, isolated from Htau mice (**Figure 4.17A**).

Therefore, primary neurons were exposed to 0.5μM of untreated BDTOs from PSP and AD and incubated with CL3 (final concentration 5μM) and controls. Viability significantly decreased when cells were treated with BDTOs alone, while the treatment with CL3 reduced PSP-derived tau oligomers toxicity as seen by the higher cell viability.

Interestingly, CL3 showed to be able to rescue primary neurons from PSP BDTOs-induced toxicity and were not be able to modulate and neutralize AD BDTOs-induced toxicity, suggesting that this promising compound may specifically bind to PSP tau strain.

Furthermore, PSP and AD BDTOs alone and in the presence of CL3 were evaluated by AFM to assess their morphology and aggregation state (**Figure 4.17B**).

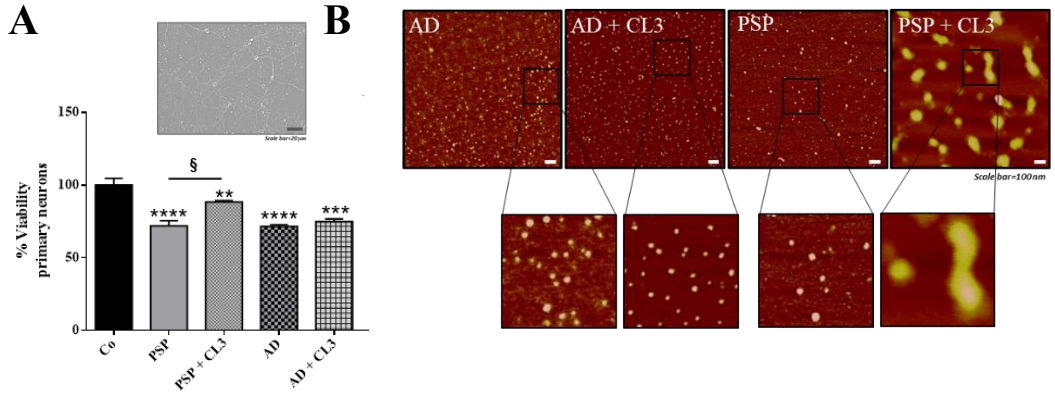


Figure 4.17 Cytotoxic and biophysical analyses of PSP and AD derived tau oligomers treated with Curcumin-like derivative and untreated control.

(A) Representative image of cultured primary cortical neurons from Htau mice and viability percentage of cultured neurons exposed to 0.5 µM of BDTOs or 0.5 µM of BDTOs pre-incubated with CL3 (final concentration 5 µM), and control. Each experiment was performed in triplicate (n = 3). Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: Co vs PSP, PSP+CL3, AD, AD+CL3: **p<0.01, ***p<0.001, ****p<0.0001; PSP vs PSP+CL3: §p<0.05. Bars and errors represent the mean and standard deviation. (B) Atomic Force Microscopy images of BDTOs without and with CL3. AFM images show the ability of CL3 to modulate PSP BDTOs aggregation pathways converting them into much larger tau aggregates while no changes are observed in the morphology of AD BDTOs. Scale bars = 100 nm.

Excitingly, AFM images confirmed the capability of CL3 to modulate the aggregation state of PSP BDTOs leading to the formation of larger tau aggregates while no morphological changes were observed in AFM images of AD BDTOs with and without treatment with the Curcumin-like derivate CL3.

Altogether, these results show the efficacy of Curcumin-like compounds to interact with BDTOs isolated from PSP homogenates, and modulate their aggregation states by promoting the formation of non-toxic larger tau aggregates. In addition, CL3 modulates

PSP BDTOs associated neurotoxicity and has no effect in preventing AD BDTOs-induced toxicity, suggesting that this promising compound may specifically bind to PSP tau strains.

CONCLUSIONS

Several small molecules, including small polyphenol molecules, have been shown to alter the misfolding of many amyloid proteins. Curcumin, which is a polyphenol extracted from the plant Curcuma Longa, has been demonstrated to have a broad spectrum of properties including anti-oxidant and anti-inflammatory effects with a very low-profile toxicity. Furthermore, it has been shown that curcumin plays an important role in the prevention and treatment of many diseases including neurodegenerative disorders (Maiti and Dunbar 2018, Rahmani, Alsahli et al. 2018). However, curcumin low cerebral bioavailability represents one of the major drawback which hampers its use as a potential therapeutic agent for AD and related diseases. Alternative formulation and drug delivery systems, including liposomes and nanoparticles, have been formulated to enhance its bioavailability. In addition, curcumin well-known limitations has prompted researcher to look for novel curcumin derivatives. Therefore, the rationale behind the synthesis of our curcumin derivatives is to remove the β-di keto moiety that is believed to be responsible for curcumin low solubility (Vyas, Dandawate et al. 2013). Hence, four different groups of curcumin analogs without β-di keto moiety were synthesized and screened against preformed tau oligomers in order to test their ability in altering and modulating the aggregation state of toxic tau oligomers by further promoting their aggregation and formation of larger tau structures with decreased toxicity. Indeed, for a long time the research has been focusing on biologically active inhibitor small molecules that could either inhibit tau aggregates assembly or disassemble pre-existing tau aggregates, rather than small molecules that could promote the formation of non-toxic high molecular weight aggregates (Lo Cascio and Kayed 2018).

Therefore, in this study, we investigated the effects of newly synthesized curcumin derivatives on toxic tau oligomers and disease-relevant tau oligomeric strains. Curcumin derivatives were screened against preformed toxic recombinant tau oligomers and evaluated biochemically and biophysically. Our results suggest that curcumin analogs modulate the aggregation pathways of tau oligomers leading to the formation of larger non-toxic tau aggregates. Toxicity screens were assessed using cultured primary cortical neurons isolated from embryos of Htau mice, expressing non mutant human tau. Treatment with the selected active curcumin derivatives shows to protect primary cortical neurons from tau oligomer-induced toxicity, while the same compounds were not able to rescue neurons from Aβ oligomers-induced toxicity. In addition, internalization screens using SH-SY5Y human neuroblastoma cell line showed that the compounds are able to affect the preformed tau oligomers internalization, mechanism that mediates their uptake by cells.

Importantly, these results also suggest that curcumin derivatives showing high activity *in vitro* may be used as potential tau PET tracers. Indeed, since they can stabilize the highly dynamic and transient oligomers into larger and stable tau conformations, they can be used for diagnostic purpose as imaging agents to enhance the very weak imaging signals of small oligomers.

In addition, we have reported the existence of brain-derived tau oligomeric strains that can be differentiated by resistance to proteinase K. Indeed, the characterization of disease-relevant tau strains will be critical for the accurate study of tau pathology in disease as well as diagnostic and therapeutic applications. To evaluate differences in disease-relevant tau oligomeric strains, we isolated, purified and characterized brain-derived tau oligomers from different tauopathies, including Alzheimer's disease, Progressive

supranuclear palsy and Lewy body with dementia. Digestion with PK revealed differences in tau stability between diseases, as well as AFM images shown different morphologies within BDTOs from different tauopathy. The association of diverse tau oligomeric strains with different disorders suggests that they may be partly responsible for the diverse outcomes of tauopathies, explaining how the aggregation of the same protein can cause different diseases and diverse progression and phenotypes. Modulating their conformations and depleting the disease-relevant structures through the use of small molecules, including our novel curcumin analogs, could be a powerful therapeutic strategy that targets toxicity regardless of the many other factors involved in the formation of tau oligomeric strains.

Our result showed that Curcumin-like derivatives (CL1-3) modulate and alter the aggregation state of toxic tau oligomeric strains from PSP brain. In addition, CL3 was found to be able to rescue primary cortical neurons from PSP BDTOs-induced toxicity and not from AD BDTOs, suggesting the potential of this active compound to specifically bind to PSP tau strain.

Altogether, these results suggest that conformational diversity of tau oligomeric strains may impact disease outcomes and be a viable route for research into the design of biomarkers for diagnostics and personalized therapeutics for different neurodegenerative tauopathies. In addition CL3 and other promising compounds could aid both in the development of novel therapeutic approaches for AD and related diseases as well as in diagnostic field as PET imaging agents for the early detection of tau oligomers and differential diagnosis for each different tauopathies, thus providing the opportunity for prompt interventions.

CONCLUSIONS/SUMMARY

Age-related neurodegenerative disorders are one of the leading causes of death and disability in the elderly population. These diseases are characterized by synaptic dysfunction and progressive neuronal damage as well as cell death. The clinical manifestations depend on the afflicted brain region as well as the number and type of cells damaged. This leads to motor, behavioral and cognitive dysfunctions, along with dementia and psychological disorders with severely debilitating outcomes including the disruption of daily activities. Alzheimer's disease is the most common form of dementia and one of over 18 different disorders collectively known as tauopathies, characterized by pathological aggregation and accumulation of the microtubule-associated protein, tau.

The large body of evidence supporting the key role of tau in neurodegenerative diseases suggests the importance of tau as a potential target for the development of successful disease-modifying therapeutics. Unfortunately, the ability of aggregating proteins to spread and multiply makes treatment difficult and highlights the need to diagnose these disorders earlier and more effectively in order to begin treatment prior to the initiation of the massive spread of pathology. The recent evidences for the presence of extracellular tau aggregates and their importance in the spread suggests that extracellular treatments may be equally important in disease prevention.

Tau-related disease-modifying strategies are considered highly promising for the near future, perhaps in combination with the more well-investigated anti-amyloid approaches. While upstream targets of tau modifications may be useful in combination with other therapeutics, they likely will be unable to entirely control tau aggregation, as there are a number of factors involved in the process.

The most advanced strategies for targeting toxic tau aggregates are immunotherapeutic approaches, using antibodies for the clearance of extracellular tau aggregates seeds, as well as the use of small molecules, which can pass through the BBB more effectively than antibodies, thus targeting and neutralizing toxic tau aggregates.

To date, research on tauopathies focused primarily on tau aggregation inhibitors or small molecules with the property of disassembling tau aggregates. The focus should be on finding small molecules able to convert toxic tau aggregates into less toxic structures, promoting the formation of a non-toxic conformation or ones that can be more easily degraded by active cellular mechanisms thus preventing the progression of the pathology.

Therefore, in these studies was followed an alternative approach to evaluate the potency of small molecules in targeting and modulating pathological tau aggregates. Using recombinant tau oligomer preparation, I screened a large group of commercially available compounds, known to inhibit the aggregation and alter the misfolding of other amyloidogenic proteins, as well as novel synthesized small molecules. Therefore, I tested and evaluated the ability and potency of Azure C, Heparin like oligosaccharides and Curcumin derivatives to interact and alter tau aggregation pathways using recombinant tau oligomers. In addition, I also evaluated the efficacy of the promising compounds using disease-relevant brain-derived tau oligomeric strains from different tauopathies.

The results include:

1. AC is the first compound showing to prevent tau oligomers toxicity not by disassembling the oligomers into monomeric tau but rather converting them into clusters of aggregates. Indeed, AC interacts and modulates the aggregation pathway of preformed tau oligomers leading to the formation of clusters of aggregates,

conformational state shown to be non-toxic. The results suggest that AC-induced aggregates are less prone to be taken up by cells compared to the untreated tau oligomers. (Chapter 2)

Lo Cascio F. *& Kayed R. Azure C Targets and Modulates Toxic Tau Oligomers. ACS Chem Neurosci. 9, 1317-1326, (2018). PMID: 29378132*

2. Well-defined glycans were synthesized and evaluate against preformed toxic tau oligomers. We report the new finding that heparin like oligosaccharide, as short as tetrasaccharide, can bind strongly to toxic TauO. Their binding affinity can be further enhanced by increasing the length of the oligosaccharide to a decasaccharide. Our data show that heparin oligosaccharides convert tau oligomers into high molecular weight species and mitigate their associated cytotoxicity.

   In addition, the glycans significantly reduce tau oligomers cellular internalization, which is critical for the progression of the pathology. (Chapter 3)

   Wang, P,* ***F. Lo Cascio*****, J. Gao, R. Kayed and X. Huang (2018) "Binding and neurotoxicity mitigation of toxic tau oligomers by synthetic heparin like oligosaccharides. "* Chem Commun (Camb) ***54****(72): 10120-10123*

   ** These authors contributed equally to this project.*

3. Four different groups of curcumin derivatives were synthesized and tested against pure populations of preformed oligomeric tau species and discovered novel curcumin derivatives that bind and are capable of altering tau aggregation pathways, reshaping the conformation of toxic tau species and resulting in the formation of tau structures with decreased toxicity as assessed by cell toxicity assay and immunofluorescence internalization screens. (Chapter 4)

   Lo Cascio F *et al, Toxic Tau Oligomers Modulated by Novel Curcumin Derivatives. (In preparation)*

4. The efficacy of the most promising compounds was evaluated using disease-relevant tau oligomeric strains, isolated from different neurodegenerative tauopathies. I found that Curcumin-like derivatives (CL1-3) modulate and alter the aggregation state of toxic tau oligomeric strains from PSP brain, resulting in decreased tau oligomers levels and formation of non-toxic tau aggregates. Importantly, CL3 rescue primary cortical neurons from PSP BDTOs-induced toxicity and not from AD BDTOs, suggesting that CL3 may specifically bind to PSP tau strain (Chapter 4).

   ***Lo Cascio F** et al, Modulating Disease-relevant Tau Oligomeric Strains by Small Molecules. (In preparation)*

In this project were used novel, highly specialized reagents and assays including **1)** novel synthesized small molecules, **2)** methods developed to prepare homogeneous populations of both recombinant tau oligomers and brain-derived tau oligomers (BDTOs), **3)** optimized biochemical assays adapted from the prion field to tau aggregation, **4)** primary cortical neurons from Tg animal human tau (Htau) to evaluate the toxicity and the uptake of tau oligomers, and **5)** Tau Oligomer conformation specific Monoclonal Antibodies (TOMAs).

Collectively, the results presented here, suggest that different tau oligomeric strains may affect disease outcomes thus explaining variations in clinical manifestation as well as in neuropathological lesions in different tauopathies. In addition, our results show that conformationally distinct toxic oligomeric strains can be specifically targeted by small molecules. Therefore, small molecules can modulate tau oligomers aggregation pathways thus to neutralize their associated toxicity by affecting their internalization and preventing the progression of the pathology.

FUTURE DIRECTIONS

The results I obtained from this research project lay the foundation for future experiments to test the efficacy and beneficial effects of promising active compounds *in vivo* in animal models of tauopathies, thus to offer conclusive insights in their potential to target toxic tau oligomeric species. Therefore, the active small molecules, showing high activity *in vitro* with recombinant tau oligomers as well as diseases-relevant BDTOs, will also be tested in Htau mice model and controls.

In addition, tau oligomeric strains with and without compounds will be analyzed by CryoEM, an effective method for evaluating the structure of amyloidogenic proteins. This study will determine specific amino acid-binding sites of the active compounds to tau oligomeric strains. Thus, critically revealing new structural information that may open up the possibility of a protective tau species.

Furthermore, we are also planning to synthesize new derived small molecules, based on the structure of the biologically active compounds, thus to increase their binding affinity and screening additional compounds able to modulate tau oligomeric strain aggregation pathways and/or toxicity. These small molecules can be used to develop tau PET imaging agents to detect toxic tau oligomeric strains at the very early stages of the diseases when the clinical symptoms of AD and related diseases are not yet observed. Indeed, there is an urgent need to find accurate and safe methods for the detection of toxic disease-relevant tau oligomers, for a differential diagnosis of each tauopathy and to monitor the disease progression.

This research and future studies, suggested here, will advance the tau field as well will contribute to further clinical development of novel disease-specific and personalized therapeutics.

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