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SMALL MOLECULES TARGETING AND MODULATING TOXIC TAU OLIGOMERIC STRAINS

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"Nella vita non bisogna mai rassegnarsi, arrendersi alla mediocrità, bensì uscire da quella "zona grigia" in cui tutto è abitudine e rassegnazione passive"

(Rita Levi-Montalcini)

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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

Aβ56: 56 kDa Aβ oligomers

BBB: Brain Blood Barrier

BDTOs: Brain-Derived Tau Oligomers

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

D9: clone D9F1-IgG1

DLB: Dementia with Lewy bodies

E7: clone B3E7-IgG1

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

H12: clone H12C10-IgG2a

H5: clone B3H5-IgG1

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

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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 curcumin derivatives. Interestingly, AC 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.

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 number and type of cells damaged. This leads to motor, behavioural and cognitive dysfunctions, besides 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 more than 130 million people by 2050 (http://www.alz.co.uk/research/world-report-2016). 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)[(4)]. 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)[(5)].

1. Neurodegeneration and proteinopathies

The study of the aetiology 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 [(6)]. 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 [(7, 8)], 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. Lysosomes act to degrade protein aggregates, while the proteasome would degrade ubiquitin-tagged proteins recognized by heat shock proteins. Nevertheless, these mechanisms can be compromised in many neurodegenerative diseases therefore failing to maintain proteostasis, resulting in misfolding and aggregation of abnormal proteins and formation of insoluble and fibrillar amyloid inclusions. 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 [(6, 8)].

1.1 Alzheimer's disease and Tauopathies

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[(9)]. AD is characterized clinically by progressive loss of memory, language problems, social withdrawal, deterioration of executive functions and eventually death [(10)]. Histopathologically, as Alzheimer's progresses, the brain shrinks dramatically and is characterized by a serious cortex damage, with progressive degeneration of limbic and cortical brain structures, mainly in the temporal lobe. This atrophy affects also the cortical association areas and the hippocampus, which is critical for the formation of new memories. 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. 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) [(11)], whereas NFTs are composed of hyperphosphorylated forms of the microtubule-binding protein, tau (**Fig. 1**).

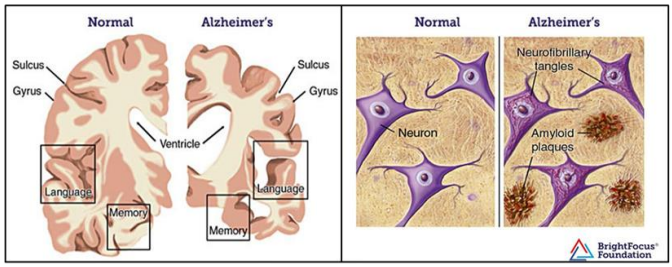

Fig. 1 Brain cross-sections of normal and AD brains and schematic showing amyloid plaques and neurofibrillary tangles.

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 remain to be established. 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 [12]. 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 [13]. Despite a strong body of evidence supporting an important role of tau in AD [2, 14, 15], the amyloid hypothesis [9, 16] proposes that Aβ is the sole cause of AD and that tau aggregation is one of many downstream events triggered by Aβ aggregation and deposition. However, the disappointing outcome of amyloid-reducing pharmacological agents, particularly clinical trials of anti-Aβ immunotherapy [17] , has revitalized research on the role of tau in AD. In addition, neurofibrillary tangles are not exclusive inclusions of AD, as these lesions are characteristic also of other pathologies [18]. Hence, tau aggregation plays an important role in many other neurodegenerative diseases, collectively referred to as tauopathies including PSP, Pick's disease, PD, FTD and several others [19, 20]. Although, the neuropathological hallmark of this large group of diseases is the presence of deposits of the microtubule-associated protein tau in the brain, they are diversified.

2. Tau in neurodegeneration

Neuropathological features of tauopathies include filamentous neuronal, or neuronal and glial tau inclusions found in association with focal neurodegeneration.

2.1 Tau deposits and its causal role in AD and tauopathies

Protein aggregation and deposition in AD and related neurodegenerative diseases have been studied extensively. Evidence from post-mortem brains showed that size, appearance and distribution pattern of amyloid deposits vary considerably between individual AD brains and correlate poorly with the disease severity. On the other hand, neurofibrillary pathology tends to develop at specific sites and follows a characteristic pattern depending on regions and cell types affected. NFTs in AD patients are highly correlated with disease progression and can be used to stage AD by post-mortem brain histopathology. Since amyloid pathology, in the absence of NFTs, is not necessarily associated with loss of cognitive function or appreciable neurodegeneration, tau pathology appears to be essential for AD [(21-23)]. Furthermore, mutations in the tau gene, MAPT, cause familial Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), implicating tau dysfunction in the neurodegenerative processes [(20, 24, 25)]. Interestingly, amyloid plaques are not found in individuals with Frontotemporal lobar degeneration- tau, FTLD-Tau. This finding suggested that abnormal forms of tau are sufficient for neurodegeneration causing memory loss and other neurological deficits. In mouse models, aged mice expressing non-mutant human tau in the absence of mouse-tau (Htau mice) develop NFTs and extensive cell death [(26)]. Mice that conditionally express a mutant human tau gene showed accumulation of NFTs in neurons as well

as learning deficits and neurodegeneration associated with tau expression; suppression of the mutant tau gene expression improved memory and halted neuronal loss [27]. Hippocampal neurons from tau knockout mice are resistant to β-amyloid-induced cell death, implicating tau's role in Aβ-related neurodegeneration in AD [28]. Reducing endogenous tau ameliorates Aβ-induced deficits in an AD mouse model; mice with normal tau levels showed age-related memory loss, behavioural abnormalities and deposition of amyloid plaques, while mice with reduced levels of tau showed a typical pattern of amyloid plaque accumulation with no memory loss or behavioural abnormalities [29, 30]. Reducing Aβ burden alone by immunotherapy is inadequate to reverse cognitive deficits in mice (3xTg-AD) that contain both hallmarks of AD, plaques and NFTs [31].

Taken together, these observations suggest that tau aggregation is a critical mediator of neurodegeneration and has a causal role in AD and other tauopathies. Due to the rise in life expectancy, finding an effective prevention and treatment strategy available for tauopathies, becomes increasingly important. Therefore, understanding the physiological and pathological functions and roles of tau is a challenge to identify new therapeutic targets and approaches [32].

2.2 *MAPT* GENE

Human tau is encoded by a single gene, *MAPT*, which is located on the long arm of chromosome 17 (17q21) (**Fig. 2**). 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 amount, in

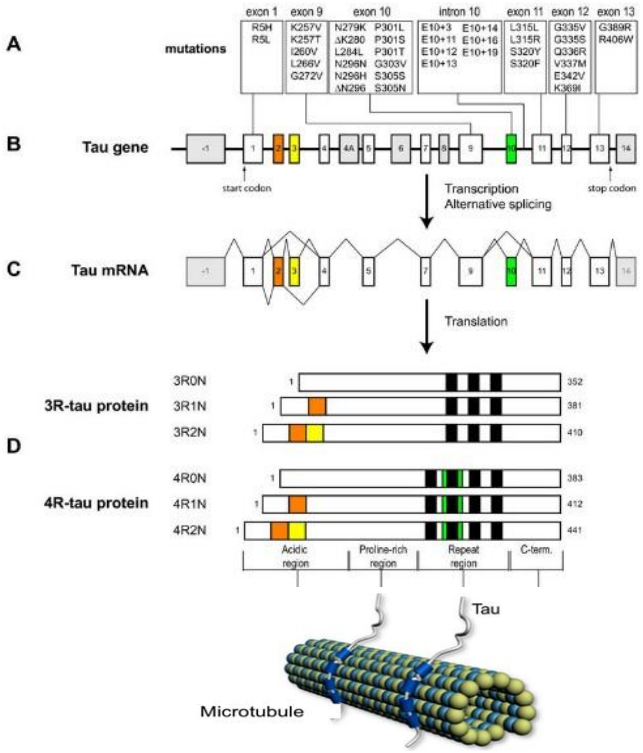

Fig. 2 Schematic representation of the human tau gene, mRNA and protein isoforms [(1)]. The human tau gene is located on chromosome 17q21 and contains 16 exons (**B**). White boxes represent constitutive exons and the grey or coloured boxes represent alternatively spliced exons. Identified mutations 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.

oligodendrocytes and astrocytes. Tau has a ubiquitous expression in immature neurons, whereas in mature neurons it is found primarily in the axonal compartment [(33)]. 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 [(34)]. The *MAPT* gene is comprised of 16 exons with two non-coding, 0 and 14, and 14 coding or partially coding exons [(35)]. 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 [36, 37]. Each repeat comprises 30-31 amino acid sequences and each one is separated from the other by a 13-14 amino acids insert [38]. 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 [5, 39]. Tau expression varies in different brain regions; cerebellum has less 0N3R tau isoform compared to other brain regions and globus pallidus show increased amount of 4R tau isoforms [40, 41]. Alternative splicing of *MAPT* exons 2 and 3 result 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 [42]. 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 expressed in foetal brain. The tau molecule is comprised of four major regions: the acid amino-terminal domain (amino acids 1-150) projects away from microtubules but it is involved in regulating the microtubule dynamics [43], the central region (amino acids 151-243) is comprised of the proline-rich domain (PRD) followed by the microtubule-binding domain with three or four repeats. Tau binds and stabilizes microtubules through this domain and it has been suggested that isoforms with 4R microtubule-binding repeats promote microtubules assembly more efficiently compared to the ones with 3R microtubule-binding repeats [44, 45]. 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 [46]. The fourth and last domain is the carboxyl-terminus (amino acids 370-441) which is common to all six human CNS tau isoforms [43, 47]. 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 [(48)].

2.2.1 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.

2.2.1a 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 foetal 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. Adult human brain contains 2-3 moles of phosphate per mole of tau. This seems to be the optimal condition for the interaction of tau with tubulin and the consequent microtubules assembly [(49)]. 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 [(50)]. 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 [(51)]. 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 [(52)]. Other tau kinases include the microtubule-associated regulatory kinase (MARK) [(53)], cyclin-dependent kinase 2 and 5 (cdk2, cdk5) [(54)].

Among the phosphatases involved in tau dephosphorylation, protein phosphatase 2A (PP2A) appears to be the principal tau phosphatase *in vivo* [(55)]; PP1, PP2B and PP2C are also capable of dephosphorylating tau *in vitro* [(56, 57)]. Inhibition of tau kinases, as well as activation of tau phosphatase PP2A [(58)], have shown to be beneficial. However, unintended consequences for other proteins and harmful side effects are important concerns for these potential therapeutic strategies [(59-61)].

2.2.1b 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 [(62-64)]. Glycated tau has been shown to be

elevated in AD [65] and associated with high toxicity [66, 67]. 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[68]. In addition, *O*-GlcNAcylation can suppress 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[68].

2.2.1c 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 [69-73].

2.2.1d Nitration

AD patients have increased tau nitration at Tyrosines 18, 29, 197, and 394. Nitration of these residues have been shown to significantly decrease the binding to 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 [(74)].

2.2.1e Truncation

Aberrant fragmentation of tau is also associated with increased formation of tau aggregates [(75-77)], making it an important potential mechanism for toxicity in disease. Truncation, occurs at several site-specific tau cleavages, including Glu391 or Asp421 and may facilitate tau aberrant aggregation [(78)].

Further research is needed to better understand the upstream modulators of tau aggregation and the efficacy and potential risks of targeting them in disease.

2.3 Tau aggregation

In its native functional state, tau is an unfolded monomeric protein which plays 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 hexapeptide motifs in the repeat domain [(2, 79)]. Unfolded proteins tend to be in highly disorganized states and would become stable through aggregation (**Fig. 3**).

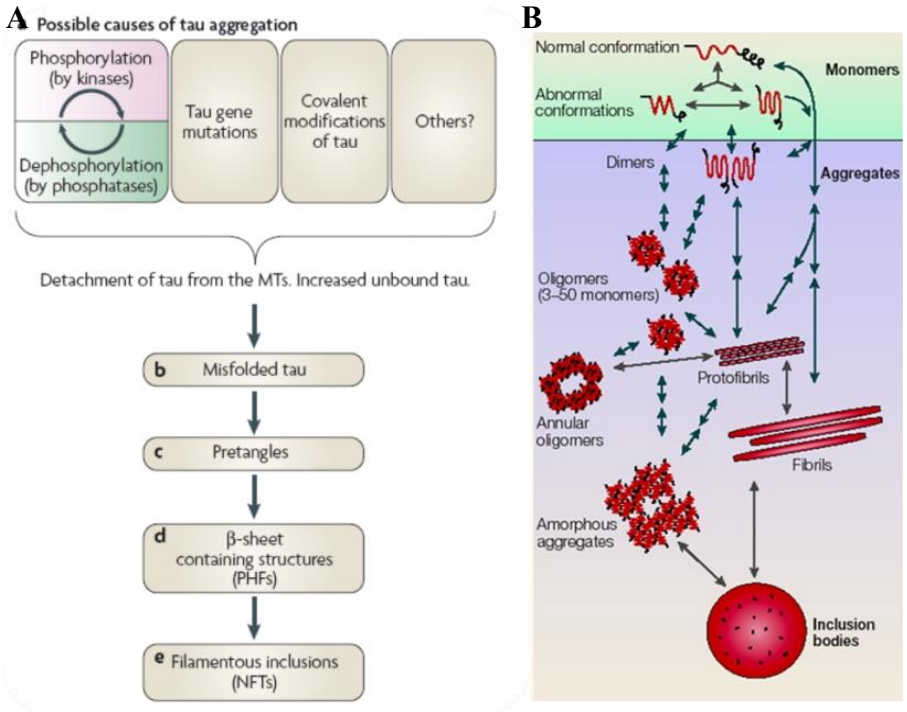

Fig. 3. Schematic representation of the different stages of the formation of pathological tau aggregates[(2)]. **A.** Abnormal disengagement of tau from the MTs and a concomitant increase in the cytosolic concentration of tau are likely to be the key events that lead to tau-mediated neurodegeneration. 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. **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[(3)].

2.3.1 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, but evidence demonstrating that hyperphosphorylation is sufficient for filament formation is 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 [(80)]. 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 [(80)]. 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 [(81-83)].

2.3.2 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 [26, 27, 84-90]. Furthermore, NFTs-containing neurons can survive for years in both human and mouse brain [91, 92]. Synaptic dysfunction and neuronal loss precede or are independent of NFTs formation [93-98], suggesting that other soluble tau oligomeric species exert effects during the early stage of AD and other tauopathies [99, 100]. Hence, the correlation between NFTs in the brains of AD patients with the disease progression remains contentious [91, 101-109].

In the last half decade, data emerging from biochemical, cell-based and transgenic mouse studies suggest that pre-filament forms of tau may be the most toxic and pathologically significant form of tau aggregates [90, 110]. Analogous to other amyloid oligomers, tau oligomers have been shown to be neurotoxic when applied extracellularly to cultured neuronal cells and provoke an increase in intracellular calcium levels [111-113]. Innovative work using animal models suggests that tau oligomers play a key role in eliciting neurodegeneration and behavioural impairments. These phenotypes are concurrent with accumulation of soluble aggregated tau species and dissociated from the accumulation of intracellular insoluble tau aggregates [90]. Cell death occurred independently of NFTs formation in aged Htau mice expressing non-mutant human tau [26];

Furthermore, hippocampal synapse loss, impaired synaptic function and microgliosis precede the formation of NFTs in the P301S mutant human tau transgenic mouse model (P301S Tg) [88]. Tau oligomers were biochemically characterized in the JNPL3 mice expressing human tau with the P301L mutation, and the conditional model (rTg4510) expressing the same P301L human tau mutant; Surprisingly, the accumulation of oligomeric tau species correlated better with neuronal loss and behavioural deficits in these models, whereas NFTs did not.

These findings suggest that the accumulation of tau oligomers, behavioural deficits, and neuronal loss precede the formation of NFTs [84, 114]. Moreover, tau oligomers were biochemically characterized in post-mortem human brains, and a correlation between disease progression and the accumulation of granular tau oligomers in the brains of AD patients was reported. In addition, increased levels of tau oligomers were detected in the frontal cortex at very early stages of the disease (Braak stage I), when clinical symptoms of AD and NFTs are believed to be absent.

Taken together, all the above recent findings suggest that an increase in tau oligomer levels occurs before NFTs formation and before the clinical manifestation of AD symptoms [96, 115]. Therefore, the small, hydrophobic, soluble and dynamic tau aggregates are believed to be highly toxic *in vitro* and the cause of synaptic and mitochondrial dysfunction *in vivo*. Moreover, they are present intra- and extracellularly and are elevated in disease brains, playing a crucial role in neuronal cytopathology [86, 97, 116, 117].

2.3.3 Tau Strains

The concept of prion-like induction and spreading of pathogenic proteins has been proposed for many neurodegenerative diseases [118-120]. Recently, researchers started to consider tau as well as other amyloid proteins, including fibrils of Aβ[121, 122] and α-synuclein (α-syn) [123] as "prion-like" in their characteristics due to their ability to template the misfolding and 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 [124, 125].

2.3.4 Prion-like spread of tau

One of the greatest challenge and point of interest in neurodegenerative tauopathies is determining the mechanism behind the propagation of misfolded tau and the stereotypic spread of pathology from initial brain regions throughout the brain in a trans-synaptic pattern as disease progresses. Understanding how tau strains seed pathological forms of the protein that propagates to different brain regions is critical to devising a solution to stop the disease. Numerous studies have shown that tau is capable of seeding the spread of pathology throughout the brain [(126-128)]. Studies *in vivo* show that tau conditionally expressed in the entorhinal cortex and injected tau aggregates can spread to synaptically connected brain regions (**Fig. 4**) [(127-129)].

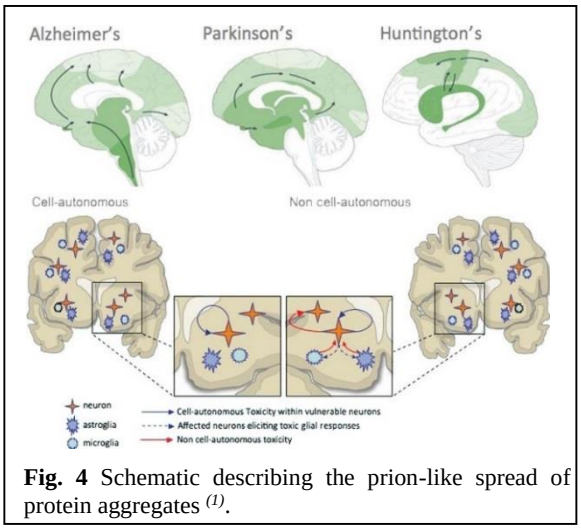

Fig. 4 Schematic describing the prion-like spread of protein aggregates [(1)].

2.3.5 Mechanism of tau internalization

Currently, there are very few insights into how tau aggregates are internalized. In the specific case of AD, NFTs progressively spread throughout the brain in an anatomically stereotypical manner. The first regions affected by NFTs are the hippocampus, the basal nucleus of Meyer and the brain stem [(21, 130)]. Based on these

and several others studies, it has been postulated that tau proteins spread in a prion-like mechanisms in the tauopathies [124, 126, 131]. Intracerebral injections of brain extract from mice with a filamentous tau pathology (P301S mutation) induces the formation and spreading of silver-positive aggregates made of hyperphosphorylated tau in transgenic mice for human wild type tau (ALZ17 mouse model), 15 months post injection [126]. Even more, they also observed endogenous tau aggregates and spread of pathology to neighbouring brain regions 12 months post injection with brain extract in wild type mice.

2.3.5.a 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 [132, 133]. Macropinocytosis has been implicated in the internalization of tau fibrils [134]. Heparan sulfate proteoglycan is a receptor that triggers macropinocytosis and has been associated with tau fibrils and trimers uptake [134-136]. Macropinocytosis has been the favored mechanism for aggregates internalization due to the size of the internalized vesicle, which is larger than other forms of internalization. Nevertheless, caveolae- and clathrin-mediated endocytosis may still play critical roles in the uptake of smaller oligomeric tau, which has not been previously characterized.

2.3.5.b Caveolae-mediated endocytosis

Caveolae are membrane invaginations resulting from the assembly of caveolins, cavins, and other proteins[137]. Caveolae have been suggested to play a role in mechanical stress protection and sensing due to their ability to flatten under tension

[137, 138]. 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 [137, 139]. Prion proteins have been shown to be internalized by caveolae-mediated endocytosis[139]. However, other amyloid proteins have not been investigated. The vesicles that result from caveolae-mediated endocytosis are 50-100 nm in diameter [140]. Due to the smaller size of oligomers, caveolae may play a role in oligomer internalization.

2.3.5.c 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 [141, 142]. Clathrin assembles into 200 nm vesicles [143]. 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 [141]. 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 [142]. Polymorphisms in clathrin adaptor protein have been associated with the presence of PHF-tau and increased risk for developing AD [144].

3. Therapeutics targeting tau aggregates

The strong body of evidence supports the important role of tau in neurodegenerative diseases [2, 14] as a potential target for the development of disease modifying therapeutics. 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[145, 146]. 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 [147-150]. Thus, strategies targeting the extracellular aggregates responsible for the spread of disease are one of the most promising techniques against tauopathies (**Fig. 5**).

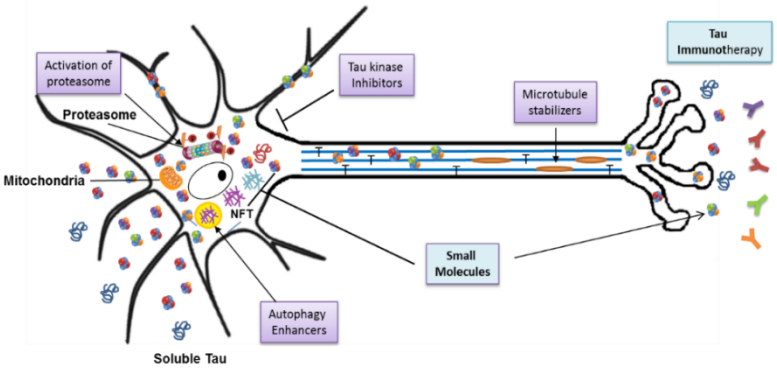

Fig. 5 Schematic representing therapeutic approach targeting tau.

Therapeutic approaches targeting tau include: interference with the splicing machinery to decrease the four-repeat tau isoforms; activation of proteolytic or proteasomal degradation pathways; prevention/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.

3.1 Inhibition of tau hyperphosphorylation

This therapeutic approach to treat AD was first introduced in 1998 [(151)]. A kinase inhibitor was shown to reduce tau hyperphosphorylation as well as the formation of soluble aggregated tau and to prevent motor deficits in mice expressing mutant human tau [(152)]. However, a major drawback of targeting kinases is the inhibition of the normal physiological functions of these common enzymes and the consequent side effects.

3.2 Activation of proteolytic or degradation pathway

Tau was found to be sensitive to calpain proteolysis [(153)]. Recently, puromycin-sensitive aminopeptidase (PSA), which was identified by a genetic screen as a modifier of tau pathology [(154)], was shown to be effective in degrading both recombinant and PHF tau purified from AD brain.

3.3 Stabilization of microtubules

Microtubule-binding drugs could be beneficial in treating tauopathies by functionally substituting the MT-binding protein tau [(155)]. Paclitaxel, a drug known to bind and stabilize microtubules, was tested in transgenic mice and showed to be effective in restoring axonal transport and ameliorating motor impairments [(155)].

3.4 Tau clearance by immunotherapy

Immunotherapy approaches designed to specifically eliminate the most toxic protein aggregate are a promising mode of treatment for neurodegenerative diseases [(156, 157)]. Immunotherapy may be divided into active immunization in which the antigen of interest is isolated and administered to activate the immune system to create its own antibodies against the toxin, and passive immunization in which antibodies are developed and administered to patients to fight the antigen of interest[(158, 159)]. While both strategies hold merit, initial active immunotherapy against Aβ caused encephalitis, forcing clinical trials to be halted, suggesting that similar strategies for tau protein should be approached with caution and careful evaluation of potential autoimmune effects [(160-163)]. Indeed, pre-clinical studies have found that active tau immunization induces dangerous levels of inflammation [(164, 165)]. Therefore, passive immunotherapy may be an effective, safer alternative approach; thus, a number of researchers are involved in this rapidly advancing field [(166-169)].

Passive immunotherapy against tau in the triple transgenic AD mouse model, expressing mutated APP and tau, led to cognitive benefits through lowering both total and hyperphosphorylated tau, even though it did not decrease levels of toxic Aβ [(170)]. As promising as these results are, conflicting studies showing negative effects in AD models with the lowering of total tau have been seen [(171)].

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 [(84, 117, 172-175)], the efficacy of immunization has been evaluated, using a tau oligomer-specific antibody, in two different tau transgenic mouse models and it was found to

significantly reduce behavioural deficits without effects on levels of either tau monomer or NFTs [176].

However, studies, that found massively detrimental effects with targeting total tau in an APP overexpressing mouse, highlighted the importance of testing tau immunotherapy in additional animal models, not only in tau transgenic mice [177]. Moreover, even successful immunotherapeutic approaches against tau in AD mouse models were previously only able to reduce levels of tau, but were ineffective against Aβ [170, 178]. Crucially, studies from our lab found that targeting tau oligomers in Tg2576 mice overexpressing mutated APP resulted in protection against memory deficits without evidence of side effects or inflammation [179]. Treatment with tau oligomer-specific antibody not only lowered levels of tau oligomers, but also led to a decrease in the toxic aggregate, Aβ*56 [179] which has been shown to be present early in AD and correlates with tau toxicity and may play a role in synaptic dysfunction [180-182]. The ability of a tau oligomer-specific antibody to mediate toxicity from Aβ as well suggests that passive immunotherapy against oligomeric tau may be able to reduce toxicity in mixed pathology diseases [183] more effectively than targeting proteins that aggregate upstream of tau alone, such as Aβ and α-syn[179, 184]. We have 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 [116, 185-187]. Therefore, depleting tau oligomers may disrupt amyloid structures formed from multiple proteins [179, 188].

3.5 Inhibition of tau aggregation by small molecules

An alternative and potential approach to the above therapeutic strategies is the use of small molecules that affect tau aggregation pathways and consequently, its toxicity [(189-191)]. Small molecule compounds can easily cross the blood-brain barrier (BBB) due to their low molecular weight [(192, 193)]. 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 membrane targeting both extracellular and intracellular tau oligomeric species[(192, 194-196)].

Beneficial therapeutic effects of small molecules can include modulation of amyloidogenic protein production [(197, 198)], 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 [(199-202)] or stable non-toxic tau fibrils [(183, 203)], anti-inflammatory effects [(204)] and antioxidant properties [(204-206)], among others [(207, 208)]. The last decade has witnessed a renaissance of interest in inhibitors of tau aggregation 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 [(209, 210)].

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 [189, 203, 209, 211-221]. In addition, a number of synthetic small molecules have also been designed to inhibit tau aggregation and toxic outcomes [222, 223]. Synthetic small molecules which have been found to inhibit tau aggregation include anthraquinones (e.g. Daunorubicin) and phenothiazines (e.g. Methylene Blue, MB) [209, 224-226]. *In vivo* studies show that methylene blue decreases tau pathology and toxic effects in mice and C. Elegans [227, 228]; However, some conflicting results have been seen [229], which may be due to its pleiotropic nature[230]. MB is also the first tau aggregation inhibitor (TAI) found, known also as methylthionium chloride. Phase III clinical trials of its reduced form, LMTX, show increased absorption compared to MB and is still ongoing [224, 231]. 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 [232].

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 approaches do not inhibit fibril formation at the cost of stabilizing the toxic oligomer, as in a number of cases [233]. Alternative approaches that have been used in the Aβ field in which fibrillization is accelerated may also be effective [234]. Additionally, combination approaches using both aggregation inhibitors and passive immunotherapy targeting toxic proteins for degradation may be more effective than either approach alone.

4. Specific aims

The research project presented here is aimed at testing the hypothesis that small molecules can be used to target and modulate tau oligomeric strains aggregation pathways, neutralizing their toxicity and affecting their internalization to prevent or slow the progression of the pathology (**Fig. 6**).

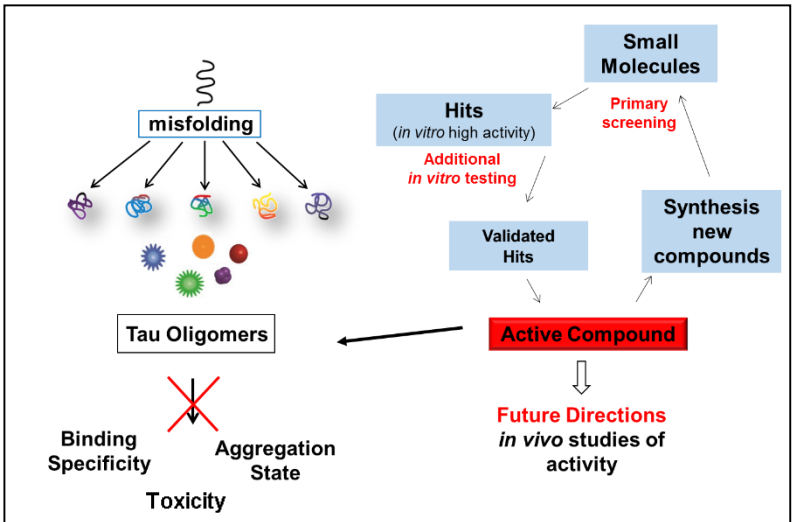

Fig. 6 Flowchart describing the hypothetical model for the formation of tau oligomers and the steps for developing curcumin derivative active compounds. Tau monomer misfolding leads to the formation of conformationally distinct misfolded monomers that aggregate into different oligomers. Toxic tau oligomers can be targeted and modulated by active compounds inhibiting oligomers toxicity and internalization thus preventing further aggregation of tau and progression of tau pathology.

To address the central hypothesis, two specific aims were pursued utilizing novel, highly specialized reagents and assays **1)** novel curcumin derived small molecules, **2)** methods developed to prepare homogeneous population of both wild type and mutated 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 human tau (Htau), and control mice to evaluate the toxicity and the uptake of tau oligomers, and **5)** a panel of Tau Oligomer conformation specific Monoclonal Antibodies (TOMA). These innovations

enabled the testing of the central hypothesis that "tau forms conformationally distinct toxic oligomeric strains that can be specifically targeted and modulated by small molecules".

Therefore, in this study I followed an alternative approach to evaluate the potency of small molecules in targeting and modulating pathological tau aggregates. Using different preformed recombinant tau oligomer preparations, 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 curcumin derivatives. I tested and evaluated the ability and potency of **Azure C** (AC) **(6.1)** and **Curcumin derivatives (7.)** to interact and alter tau aggregation pathways using different recombinant tau oligomer preparations. In addition, I tested the leading compounds using disease relevant **brain-derived tau oligomeric strains** from different tauopathies **(7.3)**.

Our lab has established the isolation of BDTOs [(172, 235)] 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) [(236, 237)]. Recent studies demonstrated that aggregated tau exhibits variable protease stability similar to prions [(125)]. Moreover, to better characterize tau oligomers and develop potential immunotherapies against them, we developed novel tau oligomer monoclonal antibodies, TOMA clones. Four clones that exhibit the best affinity and specificity against tau oligomers were sequenced and produced in large quantities using stable cell lines: clone H12C10-IgG2a (H12)[(99)], clone B3H5-IgG1 (H5), clone B3E7-IgG1 (E7), and clone D9F1-IgG1 (D9). We evaluated their binding preference for tau oligomers purified from

different tauopathies. Like the polyclonal antisera T22, novel TOMA clones and sequence specific tau antibodies, were used to characterize BDTOs by dot blot assay as well as direct ELISA (**Fig. 7 A-B**) [(99, 238)].

Therefore, using methods from the prion field, we found that tau oligomers purified from different disorders exhibit different aggregate compositions under atomic force microscopy (AFM) (**Fig. 7 C**) and specific PK digestion patterns (**Fig. 7 D**), indicating that tau oligomers purified from different disorders form structurally distinct strains.

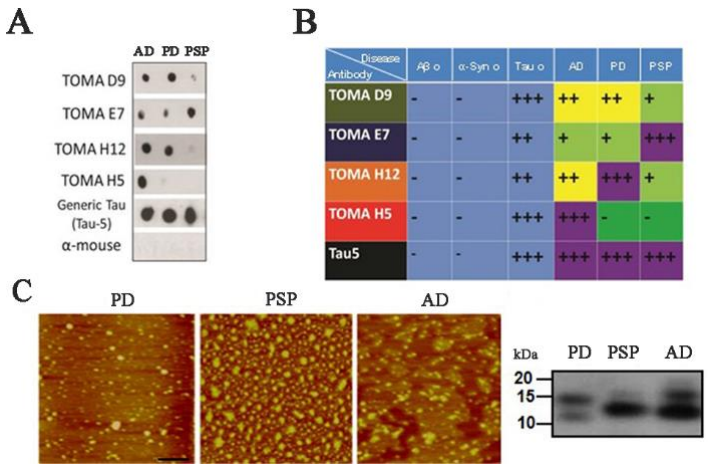

Fig. 7. Brain-derived tau oligomers from different tauopathies were assessed by TOMA clones in dot blot **(A)** and ELISA **(B)**, showing each TOMA clone detects different level of tau oligomers based on the disease. **(C)** BDTOs were characterized by AFM showing different morphologies. **(D)** BDTOs treated with K and evaluated by Western blot probed with anti-tau antibody Tau13, revealing different patterns of fragmentation. (**Gerson et al., under review**)

5. Materials and methods

Preparation of Tau Oligomers

Recombinant tau protein (tau-441 (2N4R) MW 45.9 kDa) was expressed and purified as described [239, 240]. 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

100 µl of tau oligomers (1µg/µl) were 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 and BDTOs were incubated with Curcumin (Sigma CAS 458-37-7) and Curcumin derivatives (1:5) and were dissolved in EtOH 75% at the final concentration of 5 mM and diluted in 1X PBS or ddH_2O either for incubation or toxicity assays. 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

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 [64]. 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[241]. 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[241]. 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 [(241)], 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 [(242-244)]. 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.

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.

Isolation of BDTOs

Oligomeric tau strains were isolated from brain extract by immunoprecipitation [172, 245]. 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 xg for 25min at 4°C. Tau concentration was measured using bicinchoninic acid protein assay (Micro BCA kit, Pierce)

Characterization of brain-derived oligomers

Immunoprecipitated tau oligomers were characterized using various biochemical methods as previously described [172, 245]. 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 isolated from embryos at embryonic day 16-18. Neuronal cells were then cultured in Neurobasal medium supplemented with 2% B27 and l-glutamine at 5×10^6 cells/ml. Neurons for toxicity and viability assays were plated at 30×10^4 cells/well in 96-well plates. Cells were grown for 10-12 days *in vitro* before experiments and 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 2.5μM) for two hours. The viability assay was performed as previously mentioned (MTT).

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).

6. Screening commercially available compounds

6.1 Azure C Binds and Modulates Toxic Tau Oligomer

Synthetic small molecules have been found to inhibit tau aggregation including phenothiazines (e.g. Methylene Blue, MB) [209, 224-226]. 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 [224, 231]. 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[246, 247]. MB has also been shown to affect tau aggregation through inhibiting the molecular chaperone hsp70 [248]. *In vivo* studies show that MB decreases tau pathology and toxic effects in mice and C. Elegans[227, 228]. However, there has been some conflicting results, may be due to tau's pleiotropic nature [229, 230]. MB has been shown to inhibit the aggregation not only of tau but also of other amyloidogenic proteins including TDP-43, alpha-synuclein and Aβ [232]. Another dye, belonging to the family of the phenothiazine as well as methylene blue, is Azure C (AC).

AC has previously been found to modulate hsp70 ATPase activity, consequently leading to the clearance of tau [249]. AC has also been shown to interact with and inhibit Aβ42 oligomerization without inhibiting Aβ42 fibrilization [250].

I investigated and evaluated the ability of AC to target and modulate oligomeric tau aggregation pathways

6.2 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 (**Fig. 8 A**) 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 (**Fig. 8 B-D**). The half-maximal activity concentration AC_{50} was determined from dose-response curves (**Fig. 8 B-F**). Incubation of TauO with 5 μM AC resulted in a significant decrease in TauO levels (**Fig. 8 B-D**), confirmed also by filter trap analysis (**Fig. 8 E-F**).

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 (**Fig. 9 A-C**). The data showed significant reduction of T22 immunoreactivity in the presence of AC compared to the untreated control. Direct ELISA confirmed the

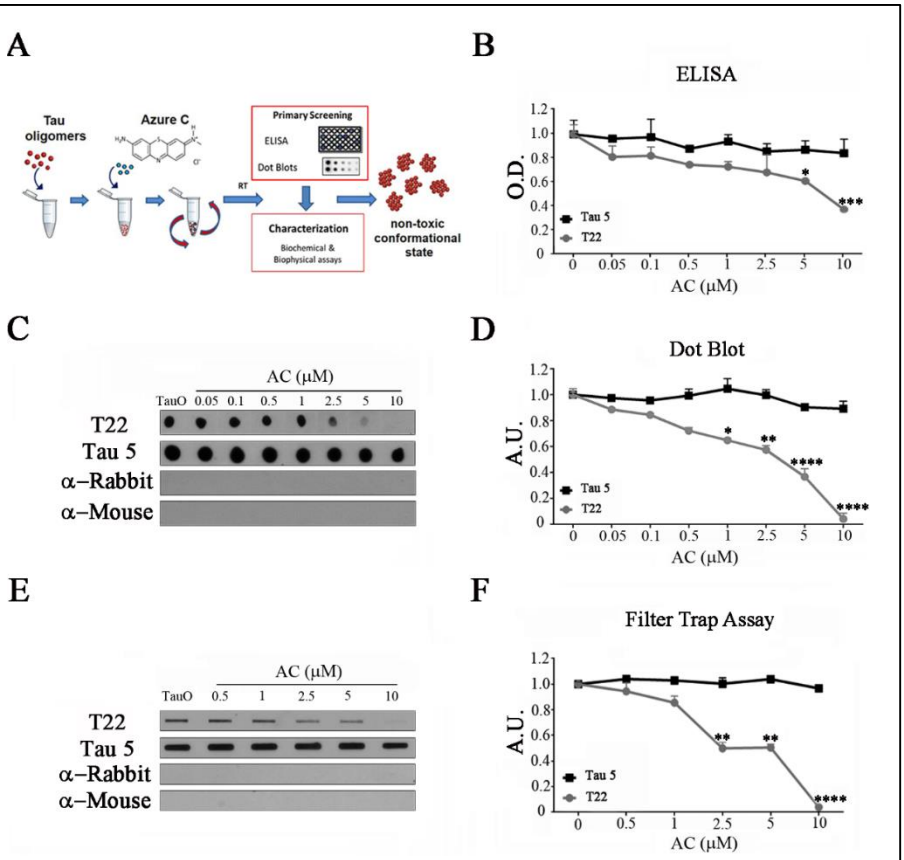

Fig. 8 Biochemical analyses of oligomeric tau after incubation substoichiometric concentrations of AC. (**A**) Schematic describing the general approach used to treat preformed tau oligomers with AC. The reactions were conducted at room temperature on an orbital shaker under oligomerization conditions. (**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)**.**

significant effects of 5 μM AC on TauO levels at micromolar concentrations (**Fig. 9 D**). MB and its derivatives, Azure A and B, have been shown to act through a reduction/oxidation mechanism [(246)]. 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 (**Fig. 9 F**), 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 (**Fig. 9 G**).

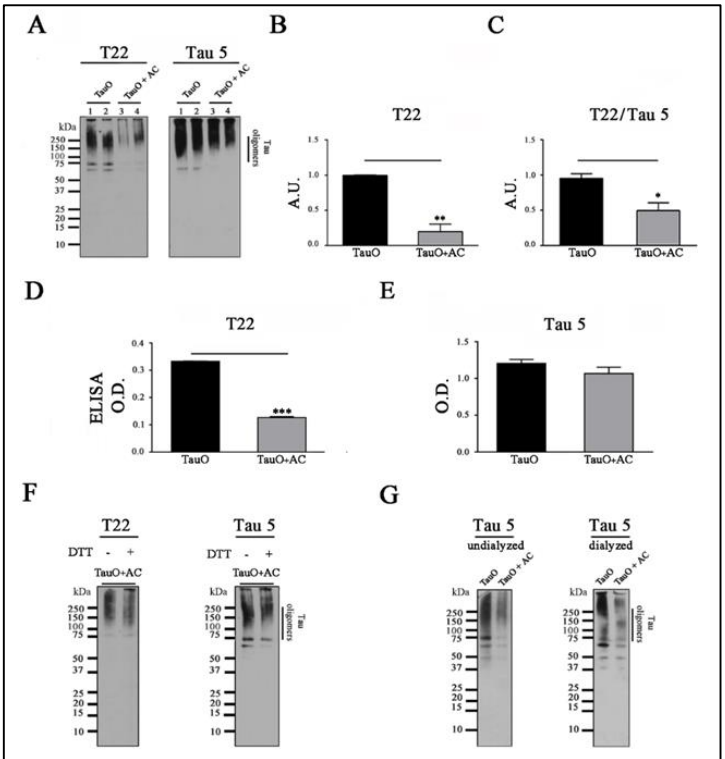

Fig. 9 Biochemical analyses of oligomeric tau incubated with 5 µM AC. (**A-C**) Western blot analysis: lanes 1 and 2 tau oligomers (TauO); lanes 3 and 4 tau oligomers incubated with 5 µM AC (TauO+AC) probed with oligomeric and total tau antibodies, T22 and Tau 5, respectively. (B) Results show decreased 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 (**Fig. 10 A-B**) while in the presence of AC **(Fig. 10 C-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

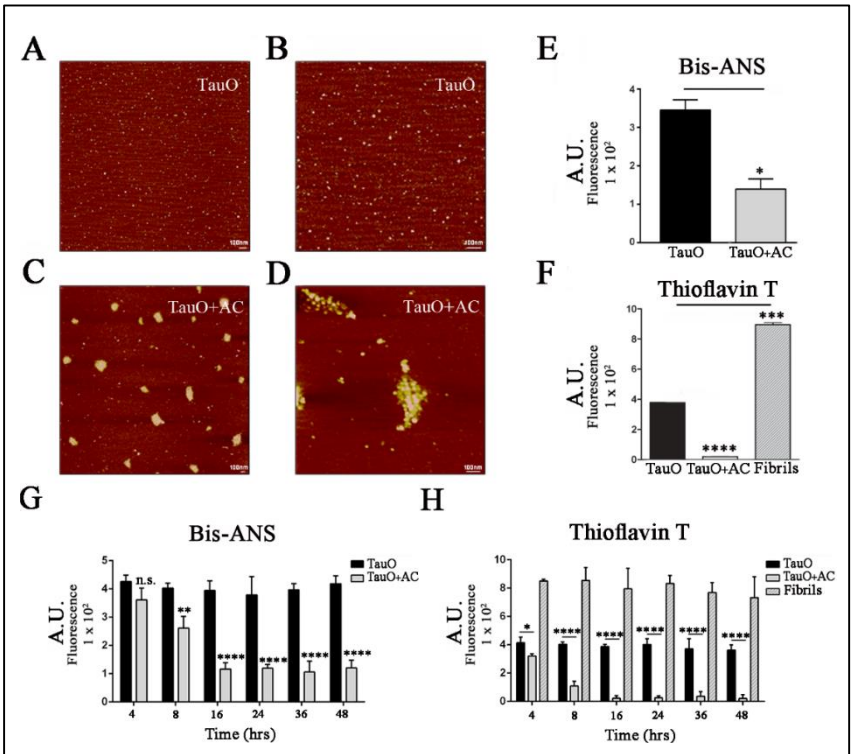

Fig. 10 Biophysical analyses of oligomeric tau in absence or presence of AC. (**A-D**) Atomic Force Microscopy images of TauO (A-B) and TauO incubated with 5 µM AC (C-D). AFM images show the ability of AC to form clusters of non-toxic tau aggregates. Scale bars = 100 nm. (**E-F**) Tau oligomers alone 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).

showed decreased binding of hydrophobic oligomers with bis-ANS as well as the absence of fibril formation with AC incubation, respectively (**Fig. 10 E-F**).

Initially, both assays were carried out for 48 hours with measurements being recorded at 4, 8, 16, 24, 36 and 48 hours (**Fig. 10 G-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.

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. My 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 *(97, 116, 238, 245, 251)*.

Cells were exposed to tau oligomers alone and in the presence of AC (**Fig 11**). 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 (**Fig. 11 A**).

Cells were also evaluated for morphological differences (**Fig. 11 B-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.

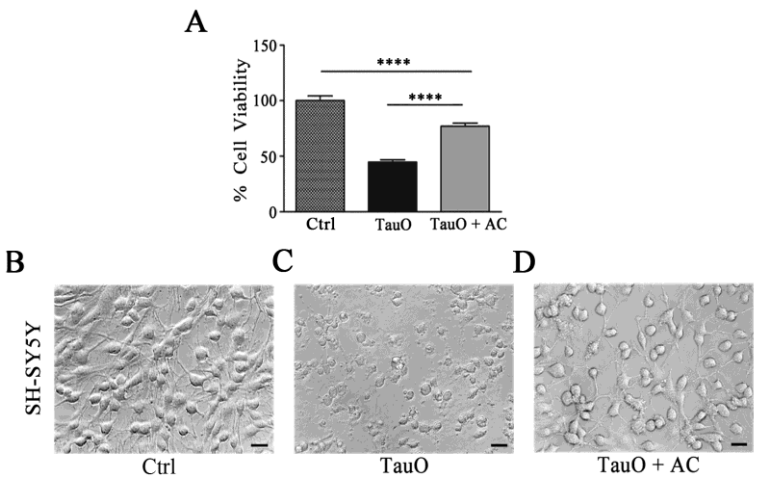

Fig. 11 SH-SY5Y neuroblastoma cells viability.
(**A**) SH-SY5Y human neuroblastoma cells viability after exposure to 2 µM tau oligomers, or 2 µM tau oligomers with 5 µM AC. Untreated cells were used as control (Ctrl). Treatment of SH-SY5Y cells with TauO+AC had significantly higher cell viability compared 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

Epifluorescence images of human SH-SY5Y neuroblastoma cells, after treatment with sub-lethal concentration (0.5µM) of tau oligomers in the absence and presence of AC (**Fig. 12 A**), 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 (**Fig. 12 B**). 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

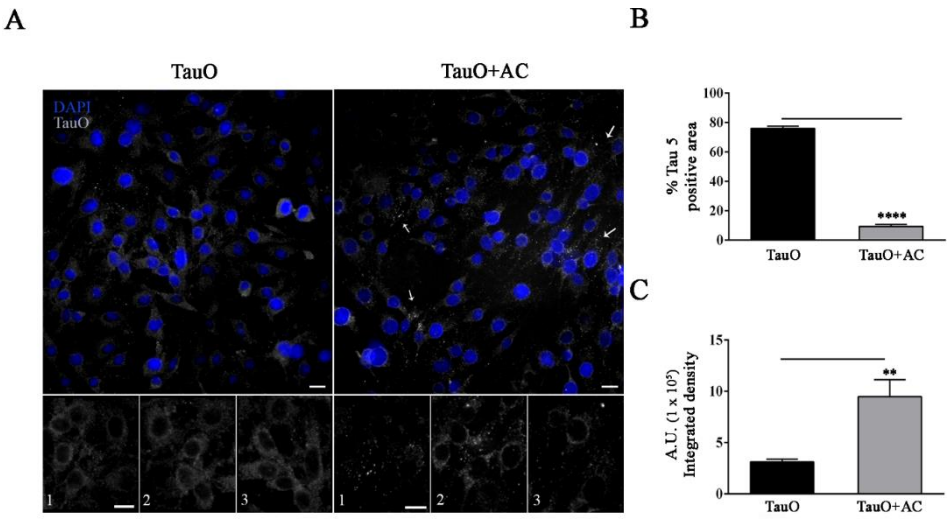

Fig. 12 Representative epifluorescence images of human SH-SY5Y neuroblastoma cells after treatment with TauO or TauO+AC.

(**A**) SH-SY5Y after 1 hour of treatment with sub-lethal concentration (0.5µM) of tau oligomers in the absence and presence of AC. TauO or TauO+AC are labelled with Tau 5 and DAPI, shown in grey and blue respectively. (**B-C**) Analyses of the percentage of area positive to Tau 5 (**B**) and integrated density (**C**) for each condition (TauO and TauO+AC) 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.

incubation with AC promote the formation of larger tau aggregates (**Fig. 12 C**). These results suggest that AC-induced aggregates are less prone to be uptaken by cells compared to tau oligomers.

Next, I investigated the selectivity and specificity of AC compared to a naturally occurring polyphenol found in grapes and red wine, resveratrol (RS) [(212)] **(Fig. 13)**. It has been shown that RS selectively remodels soluble Aβ oligomers as well as fibrillar intermediates and amyloid fibrils converting them into non-toxic aggregates

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

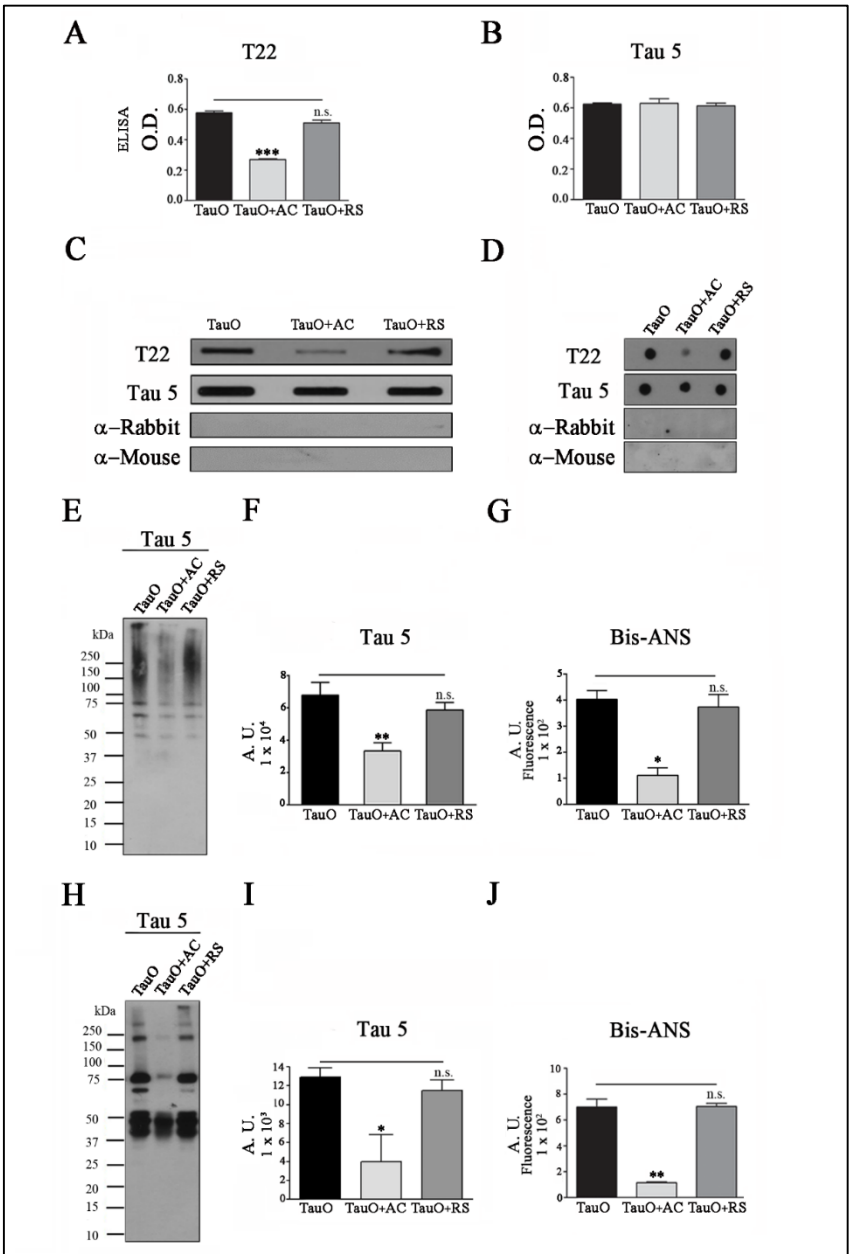

Fig. 13 Biochemical and biophysical analyses of oligomeric tu alone, pre-incubated with 5 µM AC, or 5 µM of RS. (**A-B**) ELISA analysis of oligomeric tau using T22 and Tau 5 antibodies. In the presence of AC but not RS, tau oligomers show a significant decrease in T22 immunoreactivity and no changes 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).

species. Therefore, I evaluated T22 immunoreactivity with TauO alone and after 16 hours incubation with AC or RS by direct ELISA (**Fig 13 A-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 (**Fig. 13 C-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 (**Fig. 13 5 E-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 (**Fig. 13 G**). 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 (**Fig. 13 H-J**) were similar to those obtained using purified oligomers and confirmed that AC does not disassemble oligomers into monomeric tau.

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.

7. Newly synthesized small molecules

7.1 Curcumin and curcumin derivatives

Curcumin, a polyphenol extracted from the plant *Curcuma longa,* has several broad biological activities such as antioxidant and anti-inflammatory effects with low-toxicity profile. Indeed, it plays an important role in the prevention and treatment of many diseases including neurodegenerative disorders [(252, 253)]. 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 [(254)]. 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. Curcumin significantly reduces β-amyloid and tau pathology in transgenic AD mouse models [(254, 255)]. 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 [(256)]. In addition, curcumin can decrease levels of tau hyperphosphorylation in cells and mice and can also bind to fibrillar tau. Recently, curcumin was also found to be able to selectively suppress soluble tau dimers in aged Htau mice [(255)].

However, curcumin displays poor solubility in aqueous buffers and low brain bioavailability following oral administration. Indeed, it is metabolized very rapidly via glucuronidation, primarily in the liver and intestine, before reaching the systemic circulation and the BBB [(253, 257)]. Therefore, its use as a potential therapeutic for AD and other neurodegenerative diseases has been a challenge.

Curcumin analogs were created to overcome its well-established shortcomings *(194-196)*.

7.2 Results and discussion

Highly purified oligomeric tau species were incubated with curcumin (5X and 10X) at room temperature on an orbital shaker, without stirring, for 16 hours under oligomerization conditions. Tau oligomers in the absence of curcumin were used as a control. Reactions were assessed using the oligomer-specific antibody T22 and assayed by dot blot and direct ELISA (**Fig. 14 A-B**). Results showed a significant decrease in oligomers, detected by T22 antibody, revealing the capability of curcumin to interact and alter the aggregation state of preformed tau oligomers.

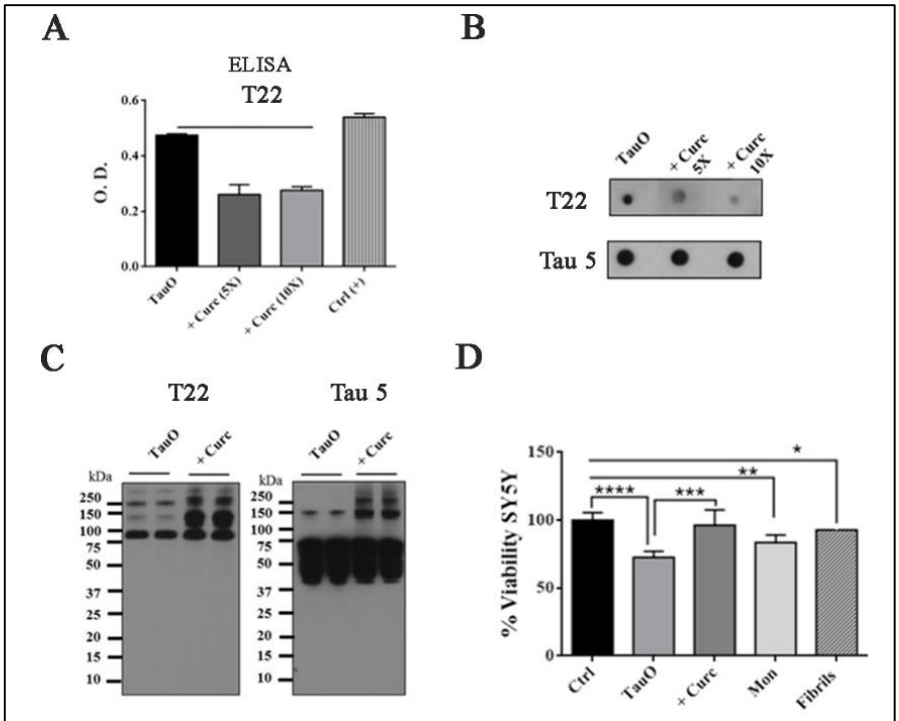

Fig. 14. Biochemical and cytotoxicity analysis of oligomeric Tau 4R treated with curcumin and untreated control.
(**A**) ELISA analysis of oligomeric tau, treated with increased concentration of curcumin, shows a significant decrease in the immunoreactivity of the oligomeric-specific antibody T22 as compared to tau oligomers alone. (**B**) Dot blot analysis show decreased levels of oligomeric tau in the presence of curcumin compared to the untreated tau oligomers. (**C**) Western blot analysis of TauO probed with oligomeric and total tau antibodies, respectively T22 and Tau 5. Curcumin interacts and alters the aggregation states of preformed tau oligomers as compared to the untreated control. (**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 control (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). Bars and errors represent the mean and standard deviation.

Western blot analysis of TauO alone or TauO in the presence of the naturally occurring small molecule, using T22 and Tau 5, showed reduction in tau oligomers as compared to the untreated control (**Fig. 14C**). I evaluated the toxicity of these tau aggregated species, resulting from the co-incubation of TauO with curcumin, to assess its ability in preventing and reducing tau oligomer-induced toxicity in 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 significant decreased after treatment with TauO, while the presence of curcumin rescued the cells from TauO-induced toxicity as seen by the higher cell viability compared to the untreated control (Ctrl) (**Fig. 14 D**).

These exciting results led to a collaboration with medicinal chemistry experts to synthesize novel curcumin derivatives in an effort to overcome curcumin's poor solubility in aqueous buffers and low bioavailability (**Fig. 15**).

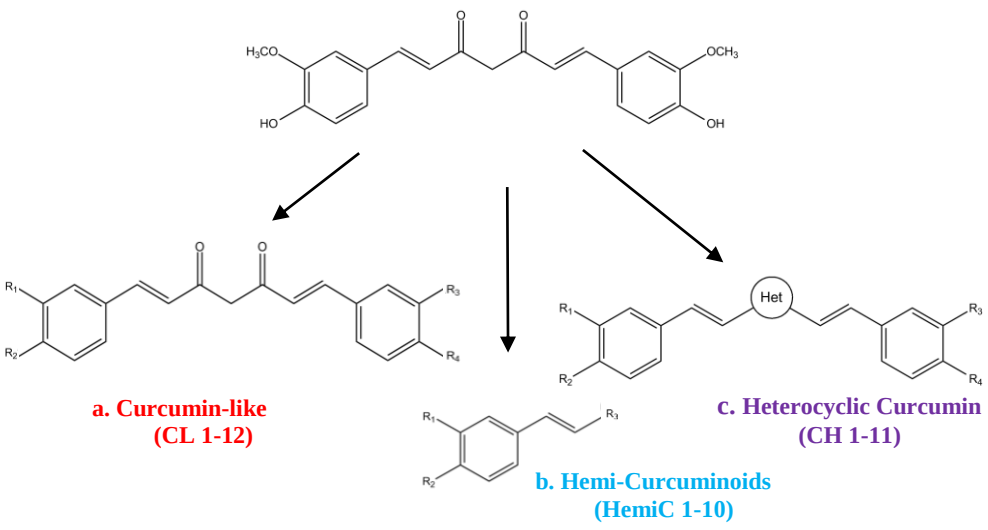

Fig. 15 Structure of curcumin and curcumin related compounds.

These novel compounds were synthesized to easily cross the BBB to target and modulate tau oligomers aggregation state, neutralizing their toxicity and

internalization in an effort to prevent or slow the spread of the pathology. The library of our curcumin derivatives consists of three different classes, as shown in Fig 15: curcumin-like (CL 1-12), hemi-curcuminoids (HemiC 1-10) and heterocyclic curcumin-like (CH 1-11). The latest have been synthesized by following Lipinski's rule of five to obtain active molecules that easily pass through the BBB. All these derivative compounds were tested to evaluate their ability to interact and alter tau aggregation using different recombinant tau oligomer preparations according to materials and methods (5).

7.2.1 Curcumin–like derivatives

The first group of curcumin analogs (CL1-12) displays the same structure of curcumin with different substitutions and functionalizations.

Highly purified tau oligomers were incubated alone or in the presence of curcumin and curcumin-like analogs (5X) on an orbital shaker for 16 hours, under oligomerization conditions. Reactions were biochemically assayed by western blot using T22 as well as the total tau antibody, Tau 5. Fig. 16 A shows the capability of each curcumin-like derivate to interact with preformed tau oligomers and modulate their aggregation states resulting in either the reduction of tau oligomer levels or the formation of larger and higher molecular weight non-toxic aggregates. Direct ELISA assay of the untreated tau oligomers showed strong immunoreactivity with T22 while, in the presence of the curcumin related compounds, there was a reduced immunoreactivity suggesting their capability to interact and alter preformed tau oligomers aggregation (**Fig. 16 B**).

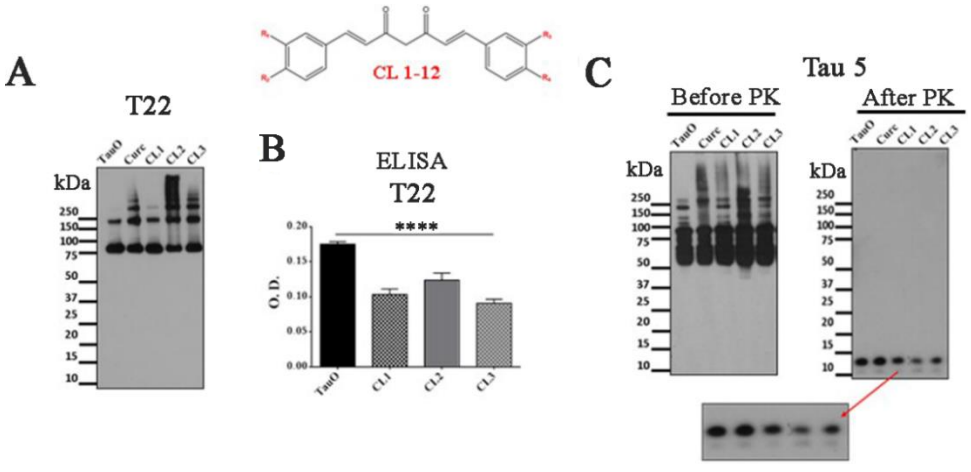

Fig. 16 Biochemical analysis of oligomeric Tau 4R treated with CL derivatives and untreated control.
Western blot analysis of 3 µg/µl of tau oligomers alone or incubated with curcumin and Cl analogs probed with T22, shows that the compounds are able to alter the aggregation states of preformed tau oligomers. **(B)** 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. **(C)** Western blot analysis probed with Tau 5, before and after Proteinase K digestion, shows the ability of the compounds to affect the protein core stability. (****p<0.0001) Bars and errors represent the mean and standard deviation.

Tau oligomers alone or in the presence of curcumin compounds were also exposed to PK digestion and evaluated by Western blot using Tau 5 (**Fig. 16 C**). Western blot analysis showed the capability of some of the compounds to alter the protein core stability.

7.2.2 Hemi-curcuminoids derivatives

The second 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 Fig. 17 A showed the altered aggregation of preformed tau oligomers after incubation with hemi-curcuminoids. Co-incubation with these small molecules derivatives showed the 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 T22 immunoreactivity (**Fig. 17 B**). 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 (**Fig. 17 C)**.

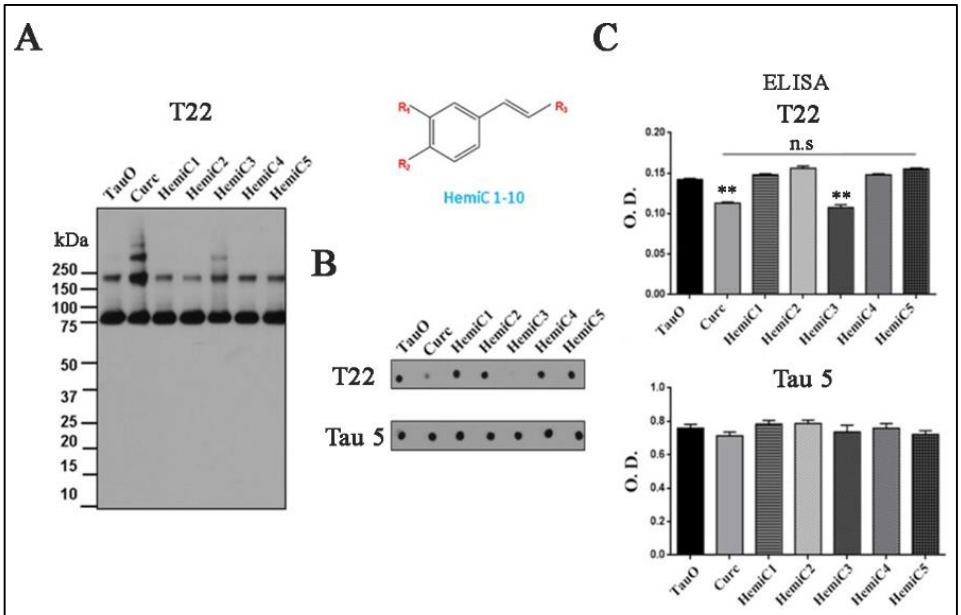

Fig. 17 Biochemical analysis of oligomeric Tau 4R treated with compound HemiC derivatives and untreated control.
(**A**) Western blot analysis of 3 μg/μl of tau oligomers alone or incubated with curcumin and HemiC derivatives probed with T22, shows that some of the compounds are able to alter the aggregation states of preformed tau oligomers. **(B)** Dot Blot analysis of oligomeric tau alone or in the presence of HemiC, probed with T22 and Tau 5, shows that some compounds are able to decrease tau oligomer levels as compared to the untreated control. **(C)** ELISA analysis of oligomeric tau with and without HemiC compounds shows that some HemiC compounds are able to affect tau aggregation reducing tau oligomer levels as compared to the untreated control while there is no change in total tau protein assayed by Tau 5. (**p<0.01) Bars and error bars represent the mean and standard deviation.

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

7.2.3 Heterocyclic curcumin derivatives

The third group are heterocyclic curcumin derivatives displaying 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. Highly purified tau oligomers were incubated alone or in the presence of curcumin and heterocyclic curcumin analogs (5X) on an orbital shaker for 16 hours, under oligomerization conditions. Reaction were assessed by dot blot and filter trap assays using T22 as well as TOMA clones and Tau 5 antibodies. Heterocyclic curcumin derivatives interact with preformed tau oligomers and are able to alter their aggregation states as shown by both dot blot and filter trap analyses (**Fig. 18A**) showing 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. TOMA1 is a conformational monoclonal antibody that recognizes conformational

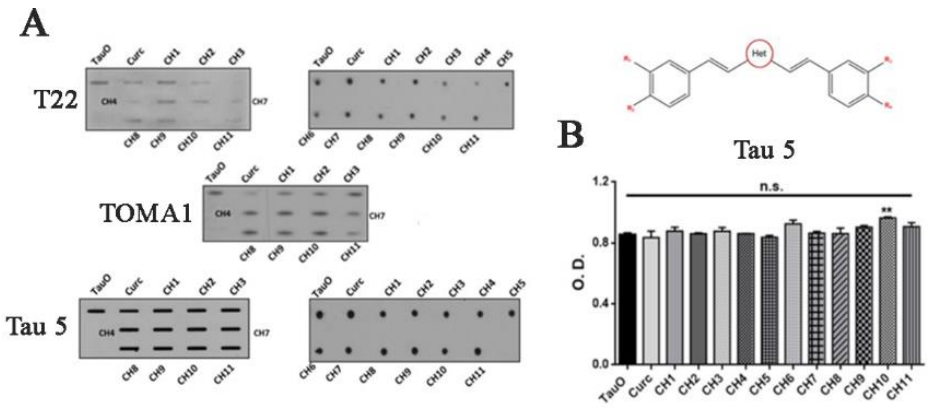

Fig. 18 Biochemical analysis of oligomeric Tau 4R with and without compound Heterocyclic Curcumin (CH) derivatives treatment.
A) Dot blot and Filter Trap analyses of tau oligomers alone or incubated with curcumin and CH analogs probed with T22 and Tau 5. Some of the compounds are able to alter the aggregation states of preformed tau oligomers resulting in decreased tau oligomer levels as compared to tau oligomers alone. Some CH analogs are able to reduce TOMA1 immunoreactivity. **(B)** ELISA analysis of oligomeric tau with and without CH derivatives show no changes in total tau protein after incubation with the compounds. (**p<0.01) Bars and errors represent the mean and standard deviation.

epitopes that do not depend on linear amino acid sequences and displays distinct preferences for different subsets of tau oligomer, suggesting that the treatment with the heterocyclic analogs led to a conformational changes in the preformed oligomeric tau species. Dot blot and filter trap assays probed with Tau 5 showed no changes in total tau protein, confirmed also by direct ELISA (**Fig.18B**).

Taken together these results show the ability of heterocyclic curcumin analogs to modulate toxic tau oligomers resulting in the formation of non-toxic aggregates.

7.3 Curcumin-like analogs and brains derived tau oligomeric strains from different tauopathies

In addition, according to the materials and methods previously described in (5), I tested the leading compounds, showing high activity with recombinant tau oligomers, using disease relevant BDTOs**.** To characterize 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 assess the ability of each compound to affect BDTOs strains aggregation state and toxicity (**Fig. 19**).

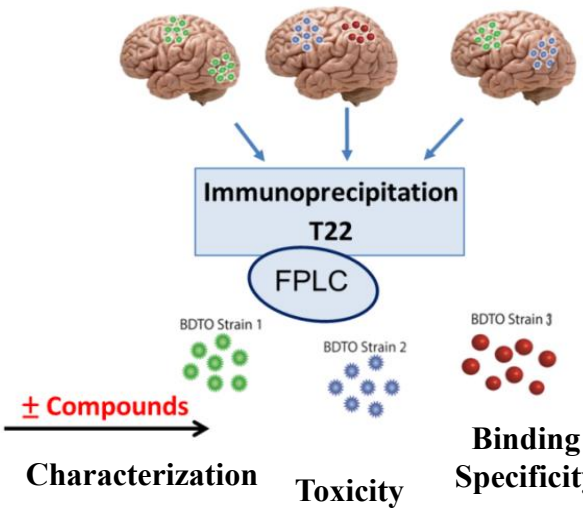

Fig. 19 Schematic describing the steps by through BDTOs strains are isolated.

Brain homogenates from DLB, AD and PSP were isolated and characterized by AFM. Images from each BDTO displayed a different morphology (**Fig. 20A**). 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/µl 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 a different patterns of fragmentation (**Fig. 20B**). Tau strains toxicity was evaluated using primary cortical neurons, isolated from Htau mice, to mimic the physiology of cells *in vivo.* Indeed, gene as well protein expression profiles in primary neurons better resembles those of the differentiated cell *in vivo* and are also more appropriate for drug targeting validation. Primary neurons were exposed to 0.5µM BDTOs for 2 hours and as seen in Fig. 20 C, resulting in a significant decrease cell viability after treatment with BDTOs compared to untreated cells (Ctrl).

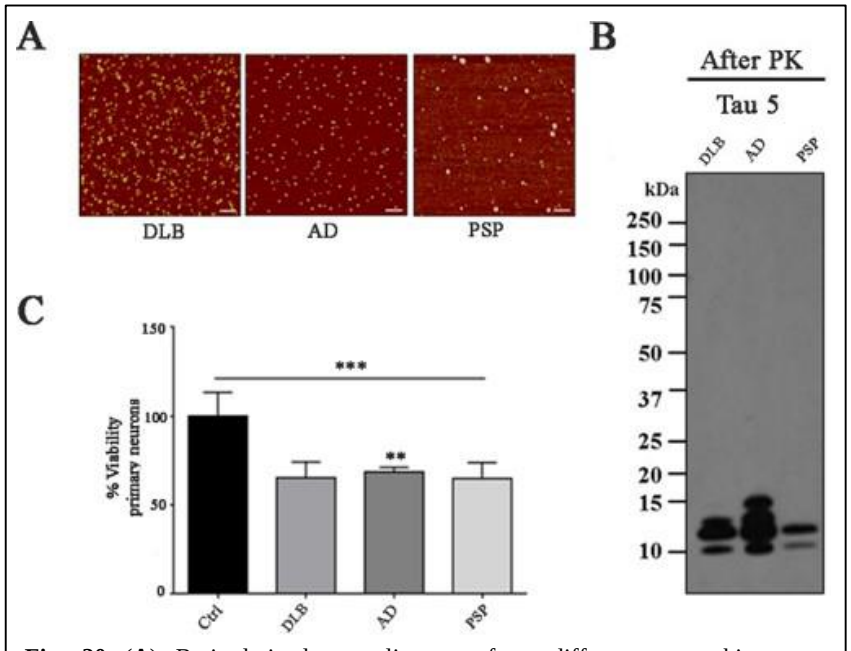

Fig. 20 (**A**) Brain-derived tau oligomers from different tauopathies were characterized by AFM showing different morphologies. Scale bars=100nm (**B**) BDTOs treated with 1µg/µl of PK and evaluated by Western blot probed with anti-tau antibody Tau5, revealing different patterns of fragmentation. (**C**) 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 (**p<0.01; ***p<0.001.) Bars and errors represent the mean and standard deviation.

7.3.1 Results and discussion

BDTOs, isolated from PSP brain homogenates, were treated with three of the derived small molecules, CL1-3, showing high activity with recombinant tau oligomers.

BDTOs were incubated alone or in the presence of curcumin analogs (5X) for 16 hours, under oligomerization conditions. Reaction were assessed by western blot using T22 and Tau 5 antibodies (**Fig. 21A-B**), revealing that the aggregation state of BDTOs was modulated by incubation with the curcumin-like derivatives. Western blot analysis showed a significant decrease in T22 immunoreactivity when PSP derived oligomers were incubated with CL1-3 compared to the untreated BDTOs (**Fig. 21 C**).

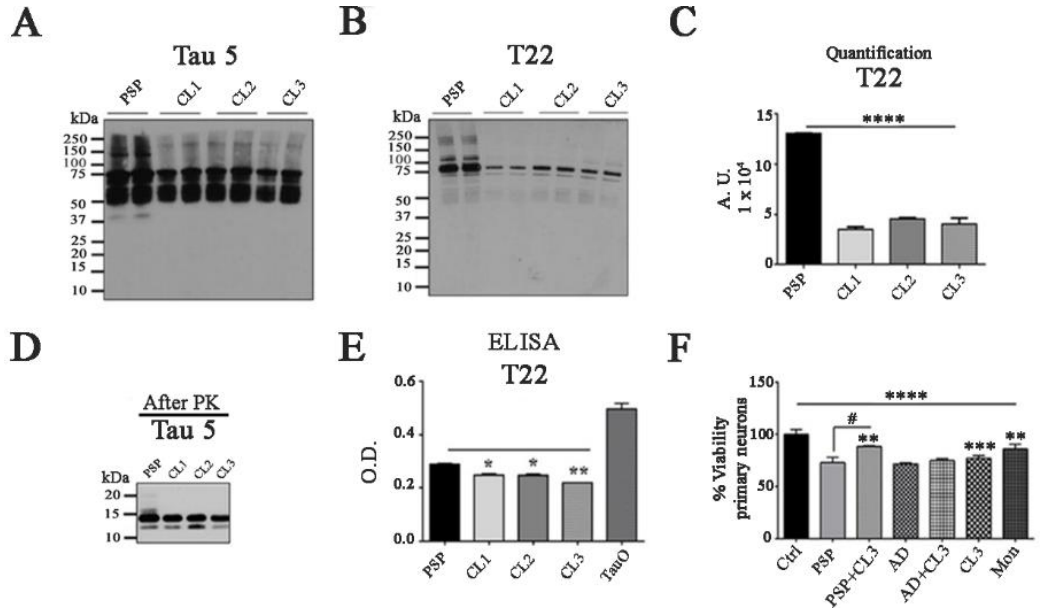

Fig. 21 Biochemical and cytotoxicity analyses of BDTOs from PSP brain homogenates treated with curcumin-like derivatives and untreated control.
(**A-B**) Western blots of BDTOs probed with total (Tau 5) and oligomeric (T22) tau antibodies showing decreased tau aggregates after treatment with CL analogs. (**C**) 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. (**D**) BDTOs, alone and in the presence of CL, exposed to PK and evaluated by Western blot using anti-tau antibody Tau 5, revealing the ability of the analogs to affect the protein core stability as compared to BDTOs alone. (**E**) Direct ELISA analysis of BDTOs alone and in the presence of CL analogs confirmed the CL's ability to modulate toxic BDTOs. (**F**) Viability percentage of cultured primary Htau neurons exposed to 0.5 µM of TauO, 0.5 µM of TauO pre-incubated with CL (final concentration 2.5 µM), and controls. ($^{\#}$p<0.05; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.) Bars and errors represent the mean and standard deviation.

Exposure of BDTOs, alone or in the presence of the curcumin-like analogs, to PK and evaluation by Western blot using the generic tau antibody, Tau 5, showed that curcumin derived small molecules were able to affect the protein core stability (**Fig. 21 D**). Direct ELISA analysis confirmed the previously results, revealing a decreased T22 immunoreactivity when BDTOs were incubated with CL1-3 as compared to the untreated control. The toxicity of 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 tau oligomer-induced toxicity in Htau primary cortical neurons. Cells were exposed to 0.5μM of untreated BDTOs from PSP and AD and incubated with CL3 (final concentration 2.5μM) and controls. Viability significant 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 (**Fig. 21 F**).

Moreover, CL3 showed to be able to rescue PSP BDTOs- induced toxicity and to do not be able to modulate and neutralize AD BDTOs- induced toxicity in primary cortical neurons.

Taken together, these results shown the potency of curcumin-like compounds to interact with BDTOs isolated from PSP homogenates, modulating their aggregation states and promoting the formation of non-toxic tau aggregates.

Conclusions

Millions of people worldwide are affected by age-related tauopathies which are characterized by the pathological accumulation of tau aggregates. Due to the rise in life expectancy, finding an effective prevention and treatment strategy becomes increasingly important.

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 seed, 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.

The focus should be on finding molecules that are able to convert toxic aggregates to less toxic structures or ones that can be more easily degraded by active cellular mechanisms. Herein, I found that:

1. AC is able to interact and modulate the aggregation pathway of preformed tau oligomers resulting in the formation of clusters of aggregates, conformation that is non-toxic. In the near future, I will further evaluate the ability of AC to rescue from tau oligomers-induced toxicity in primary cortical neurons from Htau and controls mice that better will recapitulate the properties of neuronal

cells *in vivo* and give more reliable insights in the potential of AC in neutralizing tau oligomers toxicity.

2. I screened three different group of curcumin derivatives 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.
3. I investigated the efficacy of the most promising compounds against disease relevant BDTOs from different neurodegenerative tauopathies. I found that Curcumin-like derivatives (CL1-3) were able to modulate and alter the aggregation state of toxic tau oligomeric strains from PSP brain, resulting in decreased tau oligomers levels when BDTOs were in the presence of the compounds as compared to the untreated control. In addition, CL3 was found to be able to rescue from PSP BDTOs-induced toxicity and not from AD BDTOs in primary cortical neurons, suggesting that this promising compound may specifically bind to PSP tau strain.
4. I am investigating other promising molecules for their efficacy and specificity for tau oligomers in AD and DLB. CL3 and other promising compounds could aid both in the development of novel therapeutic approaches for AD and other tauopathies as well as as in diagnostic field as PET imaging agents for the early detection of tau oligomers and differential diagnosis for each different tauopathies.

Future directions

The successful completion of this research project laid the foundation for future experiments to test the efficacy and beneficial effects of promising active compounds *in vivo* in animal model of tauopathies, thus to offer conclusive insights in their potential to target tau oligomers

Therefore, three of the derived small molecules, showing high activity *in vitro* with recombinant tau oligomers as well as diseases relevant BDTOs, will 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 allow to 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 planning to synthesize new derived small molecules, based on the structure of active compounds, thus to increase the binding affinity and screening additional compounds able to modulate tau oligomeric strain formation and/or toxicity to develop them as 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.

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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