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A long story for a short peptide: therapeutic efficacy of a cleavage-specific tau antibody

Giuseppina Amadoro*, Valentina Latina, Pietro Calissano

Tau is the prime participant in the Alzheimer's disease (AD) neurodegeneration: AD, the main cause of dementia in elderly people, is a multifactorial neurodegenerative disorder characterized by a long prodromal phase (starting more than two decades before clinical symptoms appear) with brain accumulation/misfolding of amyloid β (Aβ) in insoluble amyloid plaques and of tau protein in neurofibrillary tangles. Even though the slow-progressing clinical development of the disease opens important diagnostic and therapeutic perspectives for the preventive medicine, there's a general consensus that the amyloid deposition reaches early a plateau and does not change over time. On the contrary, the tau pathology is tightly linked with synaptic deterioration and neuronal death which eventually lead to the manifestation of classical symptomatology (Jack et al., 2018). Indeed, several lines of evidence support the notion that alterations of tau homeostasis actually drive the neurodegeneration in human tauopathies, including the most common AD where no genetic mutation in microtubule associated protein tau (MAPT) has been reported up to now. To this regard genetic, clinical and histopathological studies have undoutebly shown that abnormalities in tau protein(s) are sufficient to cause, both *in vitro* and *in vivo*, synaptic dysfunction, motor/sensorimotor and cognitive deficits indicative of loss of selective vulnerable neuronal populations (Spillantini and Goedert, 2013). Secondly, compelling evidence have also demonstrated that tau proteins have necessary, not-dispensable role in Aβ-dependent neurodegeneration, both in cellular and animal AD models and in elderly individuals (Iqbal and Gong, 2016). Moreover, both the "amyloid-cascade hypothesis" (i.e. Aβ is the initial insult driving tau pathology) and the "dual hit-model" (i.e. tau pathology is independent of Aβ which just provokes the tau spread to neocortex), which have been proposed to explain the etiopathogenesis of AD, posit a crucial place (i.e. induttive and/or permissive) of tau pathobiology in the chains of events ending in synaptic derangement and irreversible loss of neuronal viability (Small and Duff, 2008). Consistently, tau pathology correlates much more strongly than Aβ pathology with neurodegeneration and cognitive impairment, both spatially and temporally. In fact, the amyloid deposition does not cause the pronounced synaptic and neuronal loss which typically characterize the progressive clinical course of AD. Finally, tau is a common downstream effector both in Aβ-dependent and -independent pathogenic mechanisms such as increased amyloid precursor protein (APP) gene dosage/APP-derived-C-Terminal fragment (CTF,C99), cholesterol metabolism/endocytic, trafficking microglial immune activation, apoliprotein E allele epsilon 4.

Collectively, these findings outline that removal of Aβ is not sufficient *per se* to improve brain function and that tau targeting can be more clinically relevant than Aβ-directed disease-modifying therapies for the AD cure, once the clinical symptoms become evident (Busche and Hyman, 2020).

The N-terminal end of tau and its emerging contributing role in the AD onset and progression: Tau is a soluble, highly flexible, intrinsically disordered protein which is predominantly expressed in the neurons where it binds and stabilizes the microtubules track. Tau primary structure includes three different regions designed as: (i) the N-terminal acidic projection domain (amino acids 1–150); (ii) the stretch of tau encompassing residues 151–243 (the proline-rich domain); (iii) the microtubule binding domain consisting of four imperfectly repeated motifs (R1–R4), separated by flanking regions, which together are devoted to interact with and promote the tubulin heterodimer assembly; (iv) the amino acids 370–441 forming the C-terminal tail. Historically, the interest of scientific community has been mainly drawn by the hexapeptide motifs (Paired Helical Filaments, PFHs) located in the R2 and R3 of tau. These short sequences exhibit a strong propensity to form ordered β-sheet structures and are proposed to be the core nucleation sites necessary for the intracellular self-assembly of tau into insoluble filaments, which usually characterize the neurofibrillary pathology of AD and other, non-AD human related tauopathies (Mandelkow and Mandelkow, 2012). Recently however, a growing number of studies have highlighted the important role of N-terminal extremity provided with toxic properties such as misfolding/aggregation, activity-dependent synaptic release and cell-to-cell propagation of proteophatic seeds. To this regard, compelling evidence have demonstrated that the N-terminal projection domain of tau: (1) interacts with the plasma membrane and with molecules involved in signal transduction pathways; (2) undergoes early conformational changes and post-translational modifications in human tauopathies, including AD; (3) is prone to come into higher order of oligomerization and promote in cells the liquid-liquid phase separation, a mechanism along which droplets of disordered proteins undergo transition into aggregates; (4) is a mediator of Aβ-dependent and -independent synaptic dysfunction, acting both in pre- and post-synaptic compartments; (5) is preferentially secreted from synaptosomes of AD brains and in conditioned media from patient-derived induced pluripotent stem cell cortical neurons; (6) is largely detected in cerebrospinal fluid (CSF) and in plasma from AD and mild cognitive impairment patients. Besides, by measuring the phosphorylated tau at threonine-181 with an N-terminal partner antibody (N-phospho-tau181) in CSF and in plasma, recent strong data indicates this novel ptau biomarker markedly increases in preclinical AD when only subtle changes of Aβ pathology are evident, further stressing the clinical importance of specifically targeting the N-terminal tau specie(s) at very early stages in the disease development (Karikari et al., 2020; Suárez-Calvet et al., 2020). Taken together, these studies underlining the unexpected physio-pathological role of the N-terminal extremity of tau provide the proof-of-concept that the not-microtubule bindings region of protein can be an attractive target for the cure of AD and, possibly, of other non-related human tauopathies (Polanco et al., 2018; Brandt et al., 2020).

Antibody-mediated targeting of the N-terminal domain of tau is a valuable disease-therapy for AD: At present, a large part of pharmacological and immunological approaches targeting A i.e. secretase inhibitors and anti-A antibodies), have failed to reach the final end-points of AD clinical trials, mainly due to the appearance of toxicity and/or the lack of efficacy (Giacobini and Gold, 2013). Therefore, alternative tau-based interventions have been attempted, including inhibition of kinases, targeting of aggregation, modulation of degradation, microtubule stabilization and passive or active immunotherapy relying on the antibody-mediated clearance of, both intracellular and extracellular, deleterious tau species (Polanco et al., 2018). In contrast to active vaccination depending on the direct stimulation of immune response to develop antibodies, passive immunization is safer and more adaptable, being provided with a reduced risk of eliciting an adverse immune reaction. Besides, it also offers a great flexibility to target the epitopes profile changes which evolve over the disease progression. It is also worth mentioning that the tau antibodies are amenable for a proper selection of specific targeted epitopes and/or

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their conformation(s) and are also endowed with ability of successfully penetrating the cerebral structures. Indeed, tau antibodies can partecipate to the elimination of toxic tau species by promoting their net efflux from brain parenchyma to peripheral CSF, following the "peripheral sink" route. Alternatively, it has been shown that tau antibodies can even cross the blood-brain barrier and be readily uptaken by neurons, promoting the intracellular sequestration of toxic forms of tau and/or preventing their extracellular secretion and spreading throughout the brain. Nevertheless, in order to develop an effective and safe tau-directed immunotherapy, the identification of the molecular identity (soluble *versus* prefibrillar/aggregated, full-length versus truncated, unmodified versus post-translational modified) or and/or nature (vesicular *versus* naked) of the neurotoxic forms to be targeted is only the first step. An ideal, antibody-mediated treatment should also provide the specific neutralization of these harmful species, in the absence of unwanted detrimental side-effects resulting from interference ("loss-of-function") with the normal physiological form of protein. Relevantly, tau protein has been recognized to deal into neurons with numerous important functions beyond the control of microtubule stability and dynamics (Sotiropoulos et al., 2017). In this context, we have first identified and deeply characterized a fragment of 20–22 kDa molecular weight generated by aberrant truncation at D25 and R230 aminoacids located in the N-terminal end of the longest human tau isoform (2N4R, 441 amino acids). By means of a comprehensive battery of biochemical, morphological and functional analyses, we found out that this short peptide (aka NH$_2$htau) turned out to be endowed with a potent "gain-of-function" action, for the most part centered on the synaptic compartments (Amadoro et al., 2020) (**Figure 1A**). In summary, we demonstrated that the NH$_2$htau is: (i) neurotoxic in mature hippocampal neurons, (ii) detected into cellular and animal AD models; (iii) expressed in human autoptic AD specimens mainly into the synaptic compartment(s) and in peripheral CSFs from patients affected by AD and other not-AD tauopathies; (iv) able to alter the normal synaptic function(s), both *in vitro* and *in vivo*. More recently, we have also developed a monoclonal antibody-named 12A12mAb (DRKD(25)-QGGYTMHQDQE epitope, phosphorylation-independent state) which *in vivo* selectively neutralizes this harmful specie(s) without altering the full-length normal tau. 12A12mAb, when intravenously (i.v.)-injected into 6-month-old transgenic AD animals (Tg2576 mice, APP KM670/671NL Swedish; 3XTg mice, APP Swedish/MAPT P301L/PSEN1 M146V), markedly alleviates the disease-associated signs, such as deficits in spatial memory and orientation, APP/
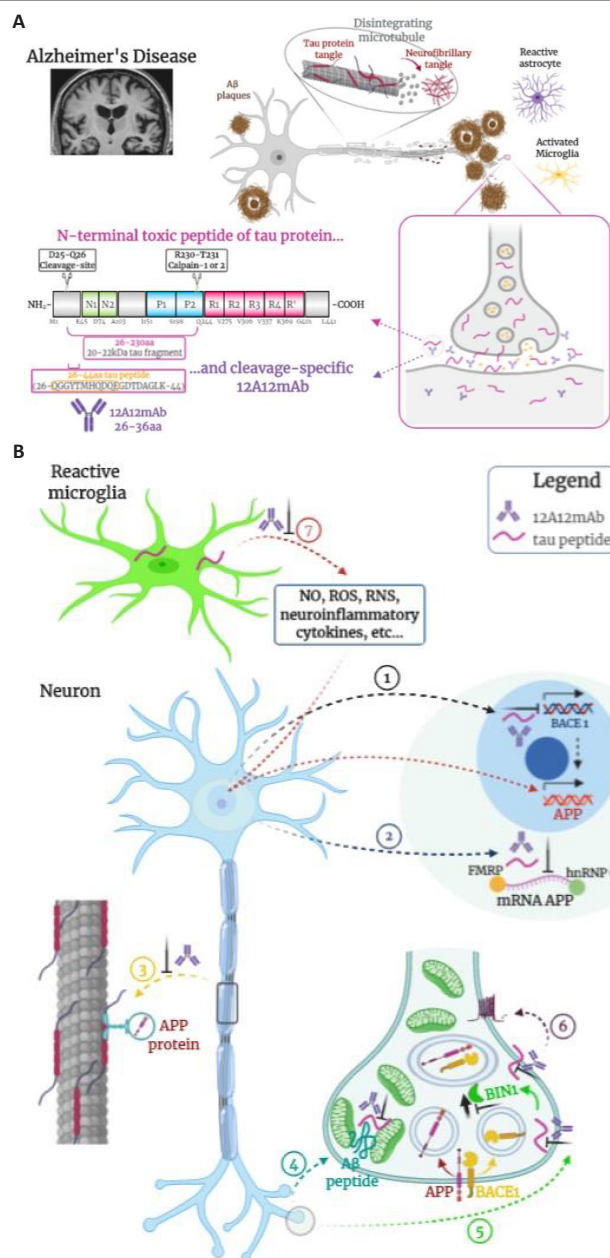

Figure 1 | N-terminal tau truncation in AD pathogenesis.
(A) The neurotoxic NH$_2$htau contributes to the synaptic and cognitive decline associated with AD symptomatology: Picture showing the two typical lesions which characterize the AD, the senile plaques, composed of fibrillar Aβ peptide, and the neurofibrillary tangles, composed of tau which, following pathological post-translational modifications, detaches from microtubules and undergoes aggregation. Reactive astrocytes and microglia surrounding neurons also contribute to the neuronal death by activation of pro-inflammatory pathways. The 20–22 kDa tau peptide, which is generated by N-terminal truncation of full length tau protein, is toxic for synaptic function(s) and its deleterious effects are specifically antagonized by the cleavage-specific 12A12mAb which targets the 26–36 aminoacidic stretch of the protein. (B) Picture depicting the possible signaling pathway(s) by which the 12A12mAb neutralizing the pathogenic NH$_2$htau interferes with APP/Aβ metabolism(s): 12A12mAb can operate into brain parenchyma at transcriptional ①, translational ② and post-translational (③, ④, ⑤) levels and along both cell-autonomous (①–⑥) and non cell-autonomous mechanims ⑦: ① by preventing the traslocation of NH$_2$htau which stimulates into nucleus the BACE1 gene transcription/expression and, then, the APP amyloidogenic processing with Aβ generation; ② by intercepting into cytosol the NH$_2$htau which, directly and/or indirectly, controls the translational efficiency of APPmRNA; (③, ④, ⑤) by sequestring the NH$_2$htau which affects the turnover/processing/endocytic-secretory trafficking of APP/Aβ (motor-driven neuronal axonal transport of APP; direct binding with the APP C-terminus and/or Aβ into synaptic compartments; interaction with BIN1 which reduces the trafficking of BACE-1 to APP-containing endocytic compartment); ⑥ (extracellular level): by removing the extracellularly-released NH$_2$htau which induces neuronal excitability which increases the generation and secretion of Aβ; ⑦ by blocking the glia-neuron cross-talk whereby NH$_2$htau activates the microglial release of pro-inflammatory mediators which upregulate into neurons the level of APP gene expression and its metabolism. Images have been assembled by means of Biorender (https://biorender.com). AD: Alzheimer's disease; APP: amyloid precursor protein; Aβ: amyloid β; NO: nitric oxide; RNS: reactive nitrogen species; ROS: reactive oxygen species.

Aβ and phosphotau accumulation, loss in dendritic spine density in pyramidal CA1 neurons, electrophysiological deficits in hippocampal long-term potentiation at the CA3-CA1 synapses, reactive astro-microgliosis (Corsetti et al., 2020). Importantly, we found out that systemic delivery of 12A12mAb inhibits *in vivo* not only tau but also APP/Aβ pathology suggesting that a positive, feed-forward regulation occurs at AD synapses. APP/Aβ dysmetabolism is more likely to trigger into hyppocampal neurons the disease pathway via generation of truncated N-tau which, in turn, further increases the APP/Aβ levels in a self-propagating noxious cycle, which can be specifically antagonized by the cleavage-specific 12A12mAb (**Figure 1B**). It is worth noting that the action mechanism(s) of 12A12mAb appears not to require the engagement of microglia offering thus *in vivo* a potent neuroprotective effect without induction of the excessive harmful neuroinflammatory response which, on the contrary, often halts the current prosecution of the clinical trials enrolling the anti Aβ-antibodies. Thus, our preclinical findings prospect that tau-based immunotherapy via the "humanized" version of parental murine 12A12mAb (h12A12mAb) could be: (i) an effective (truncated tau-specific); (ii) safe (normal tau-preserving and, thus, without adverse potential side-effects); (iii) non-invasive potential disease-modifying therapy to contrast the Aβ-dependent and -independent cognitive deficits in human beings affected from AD and, possibly, other not-AD human tauopathies. It is worth noting that the anti-pan tau antibodies binding all forms of tau usually suffer from low availability of their effective dose *in vivo* (i.e."target distraction" due to dilution of the antibody's action between healthy/irrelevant and pathological tau forms). Conversely, anti-tau antibodies which selectively target/intercept the pathogenic tau species, such as the h12A12mAb, would avoid unproductive binding toward the normal protein with an increase of overall therapeutic efficacy within a fixed treatment period time and a parallel decrease of the potential undesired side-effects. In addition to the advantages offered in terms of reduction in antibody dosage and in time of treatment -which are extremely important especially in the medical care of chronic diseases such as AD which usually require prolonged medicaments and multiple/high dose administrations of drugs- h12A12mAb administration is awaited to exhibit an improved efficacy in comparison to other therapeutic tau antibodies when tested in future clinical trials in human beings. To this regard, combined interventions aimed at reducing concomitantly both Aβ and tau pathology are expected to increase the loss of neuronal connectivity and amnestic disabilities associated with clinical symptomatology of AD more powerfully than monotherapy

altering either neuropathology alone. To this regard, it is widely recognized that AD has complex etiopathogenesis displaying numerous multifactorial and heterogeneous features which involve different, both intrinsic and extrinsic causative mechanisms (Busche and Hyman, 2020).

In view of these findings, we propose that early medical regimen with not-invasive and safe combined therapies aimed at restablishing the homeostasis of both APP/Aβ and tau, such as that potentially offered by h12A12mAb, might significantly increase the clinical management of AD whose rapid increase in our aging societies is bound to reach over 150 million in 2050. Finally, as the retina and other ocular structures, which share similar characteristics to the central nervous system, are considered a "window to the brain", h12A12mAb should be also evaluated as potential candidate tool to advance an early AD diagnosis of cerebral damage starting from inspection of patients' eye.

Giuseppina Amadoro[*],
Valentina Latina, Pietro Calissano
Institute of Translational Pharmacology (IFT)–National Research Council (CNR), Via Fosso del Cavaliere 100, 00133-Rome, Italy (Amadoro G)
European Brain Research Institute (EBRI), Viale Regina Elena 295, 00161-Rome, Italy (Amadoro G, Latina V, Calissano P)
*Correspondence to: Amadoro Giuseppina, PhD, g.amadoro@inmm.cnr.it.
https://orcid.org/0000-0003-2080-2951 (Amadoro Giuseppina)

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