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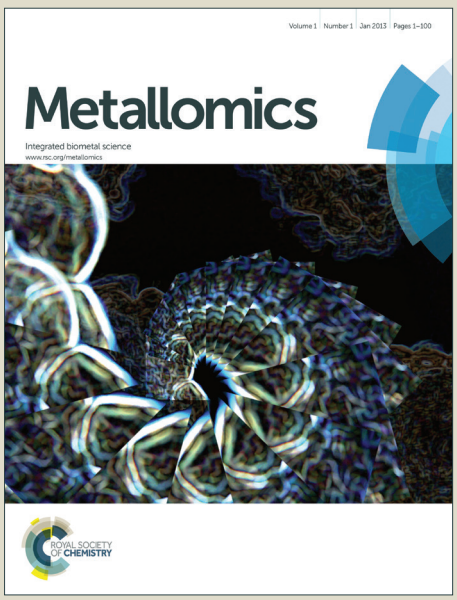

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A bifunctional non-natural tetrapeptide modulates amyloid-beta peptide aggregation in the presence of Cu(II)

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Amyloid-beta peptide (Aβ) aggregation is one of the hallmarks of Alzheimer's disease (AD), and metal ions such as Cu(II) have been proposed to play a role in amyloid formation and the onset of this progressive neurodegenerative disorder. This study reports the design and characterization of a novel bifunctional non-natural tetrapeptide, Met-Asp-D-Trp-Aib, that is capable of binding copper, competing with Aβ for Cu(II), and modulating Aβ aggregation. The study of this tetrapeptide provides further insights into the role of Cu(II) in the Aβ aggregation pathway, and into the design of compounds with therapeutic potential for Alzheimer's disease.

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects more than 30 million people worldwide, a number that will duplicate every twenty years[1]. Although the exact etiology of AD is not understood, one of the hallmarks of AD is the aggregation of amyloid-beta peptide (Aβ), which forms amyloid plaques in the brain cortical areas, particularly the hippocampus and neocortex[2]. Understanding the mechanism of formation of amyloid plaques from Aβ monomers is essential to propose therapeutic agents.

The interaction of metal ions with Aβ has been implicated in the neuropathological effects of Aβ[3]. Studies in brains of Alzheimer's patients show that senile plaques contain elevated concentrations of zinc, iron and copper with respect to healthy individuals[3-5], suggesting the loss of metal ion homeostasis in AD, particularly for copper. However, the molecular mechanisms implicated in Aβ aggregation in the presence of metal ions and their roles in the onset of AD have not been elucidated.

In recent years, several efforts have been devoted to the design of bifunctional molecules capable of chelating metal ions and modulating Aβ aggregation[6-8], with the aim of developing chemical tools that would help understand the role of metal ions in the etiology of AD, and ultimately, pave the road towards drug development. In some bifunctional molecules the chelating agent is inspired in clioquinol, and the interaction with Aβ is given by stilbene[9-11] while others combine structural features of clioquinol and thioflavin T (ThT) [12]. Additionally, bifunctional compounds combining the metal chelating properties of N-(2-pyridylmethyl)amine with 2-phenylbenzothiazole and o-vanillin have also been designed[13].

In this study, we report the design of a novel bifunctional non-natural tetrapeptide (TP), with sequence Met-Asp-D-Trp-Aib (Fig 1a). TP combines the beta-breaker properties of the oligomerization inhibitor D-Trp-Aib (previously reported by Frydman-Marom and co-workers[14]) with the Cu(II) specific chelating properties of α-synuclein, a protein implicated in

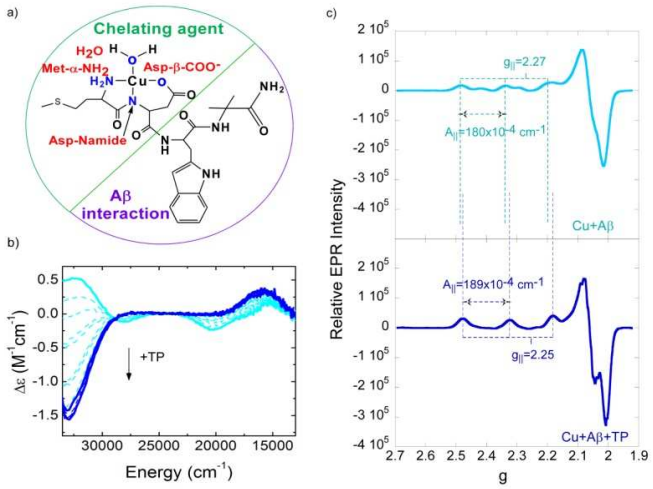


Fig. 1 TP competes with Aβ(1–16) for Cu(II) binding. a) TP structure and design; b) Titration of the Cu(II)-Aβ(1-16) complex at pH 7.4 (solid light blue line) by TP, as followed by CD; sequential additions of 0.125 equiv of TP (dashed light blue lines) were made up to 1 and 1.5 equiv of TP (dark blue lines). c) EPR spectra of Cu(II) bound to Aβ(1-16) (light blue) and the end-point of its titration by TP shown in b).

Parkinson´s disease[15]. The aromatic non-natural part of TP provides metabolic resistance and aromatic recognition to Aβ, in fact the dipeptide D-Trp-Aib (DP) has been shown to reduce Aβ aggregation significantly[14]. On the other hand, the residues Met-Asp are the minimal sequence required to reproduce the highest affinity Cu(II) binding site in α-synuclein. This binding site is highly specific for Cu(II) over other divalent metal ions, as demonstrated by Binolfi, et al.[15] Cu(II) binds at this site with high affinity (conditional K_d= 5x10^{-9} M), using the N-terminal group, a deprotonated amide, the carboxylic group of Asp2 and

a water molecule[15] as shown in Fig. 1a. Thus, TP is designed to specifically target Cu(II)-Aβ interactions and to inhibit Aβ oligomerization and aggregation.

Consistent with our design, a titration of TP by Cu(II) at pH 7.5, as followed by circular dichroism (CD) and electron paramagnetic resonance (EPR), shows that TP binds copper in a 1:1 ratio, leading to spectroscopic features that are characteristic of Cu(II) bound to Met-Asp in α-synuclein (Fig. S1). On the other hand, a titration of the Cu(II)-Aβ(1-16) complex (formed at a 1:1 ratio of Cu(II):peptide) at pH 7.4 with TP, as followed by CD, clearly shows that it only takes 1 to 1.5 equivalents of TP to completely chelate the Cu(II) ions out of Aβ, leading to the spectroscopic features of Cu(II) bound to the Met-Asp construct in TP (Fig. 1b), namely, a positive d-d transition at 15900 cm^{-1} and a negative ligand to metal charge transfer band at 33000 cm^{-1}. Consistently, the EPR spectrum of the final point of this titration shows the characteristic parallel g and A values for Cu(II) bound to TP (2.25 and 189x10^{-4} cm^{-1}), while signals associated to the Cu(II)-Aβ(1-16) complex are no longer observed (Fig. 1c). Altogether, these results clearly show that TP binds Cu(II) in an identical fashion as the highest affinity binding site in α-synuclein, consistent with our design. And most importantly, TP can effectively compete for Cu(II) ions with soluble Aβ species.

The effect of TP in the aggregation of Aβ(1-40) was first evaluated in the absence of copper ions, using dynamic light scattering (DLS), thioflavin T (ThT) fluorescence assay, and transmission electron microscopy (TEM) (Fig. 2). Figure 2a shows that the incubation of Aβ 20 μM in aqueous buffer (10 mM NaCl, 20 mM NEM, pH 7.4) without agitation induces the slow growth of Aβ oligomers, as demonstrated by a steady increase in the hydrodynamic radius determined by DLS (black trace in Fig. 2a). When TP is present in the buffer, the formation of these early Aβ oligomers is abated (green trace in Fig. 2a). Indeed, TEM analysis at early times in the incubation of Aβ in aqueous buffer (Fig. 2c) shows that TP affects the size and morphology of the oligomers formed after 40 min. Consistent with our design, the effect of TP is similar to that of the oligomerization inhibitor DP (red trace in Fig. 2a). The kinetics of Aβ fibril formation is also affected by TP and DP, as determined by the ThT assay (Fig. 2b). In this assay, an increase in fluorescence intensity is indicative of the formation of rigid amyloid-like structures capable of ThT binding. Aβ aggregation follows a nucleated-growth mechanism[16], where the nucleation phase does not lead to ThT fluorescence changes, while the growth of mature fibrils is responsible for the drastic increase in fluorescence observed in the sigmoidal traces shown in Fig 2b. TP and DP increase the lag time for amyloid formation, although the effect is only statistically significant with TP (Fig. 2b, inset), suggesting that TP delays the nucleation process of Aβ. Finally, consistent with previous reports[14], the fibrils grown in the presence of DP and TP are thinner, smaller and less abundant as compared to those of Aβ(1-40) only (Figs S2 and 2c). Altogether, these results indicate that TP can delay the initial growth of Aβ(1-40) oligomers, and change the nature of the final aggregates.

The effect of Cu(II) in Aβ aggregation has been extensively studied, leading to controversial results[17, 18]. Here we evaluated the effect of Cu in the initial oligomerization of Aβ in aqueous solution, finding that the addition of 0.4 equiv of Cu(II) promotes the immediate growth of large Aβ(1-40) oligomers with hydrodynamic radius around 400 to 800 nm, as determined by DLS (Figs. S3a and 3a). TEM analysis at early times in the incubation of Aβ with Cu(II) confirms the presence of large oligomers (Fig. 3c) that are not observed in the absence of copper (Fig. 2c). Also, increasing concentrations of copper (0 to 1 equiv) delay the nucleation time and quench the fluorescence of fibril-bound ThT (Fig. S3b). Consistent with previous reports[19, 20], the final aggregates formed in the presence of Cu(II) are fibrilar, yet shorter than those of Aβ only, while less ordered aggregates are formed at higher Cu:Aβ ratios (Fig. S3c). Also, EPR data show that Cu(II) is bound to Aβ fibrils at the end point of the aggregation process (Fig. S4). Overall, these results suggest that Cu does not promote, nor inhibits Aβ aggregation, it simply takes Aβ through a different aggregation pathway that involves the rapid formation of large oligomers, increasing the lag time and leading to Cu(II)-bound fibrils with slightly different morphology (Scheme 1).

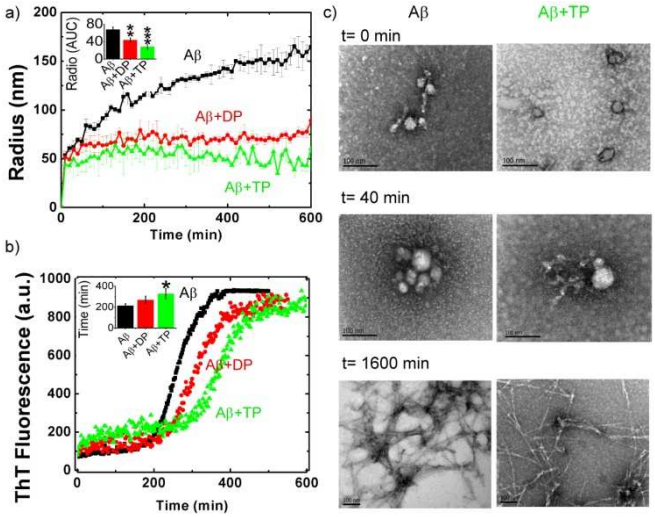


Fig. 2 TP modulates Aβ(1-40) aggregation. Particle hydrodynamic radius determined by DLS (a) and amyloid fibril growth detected by ThT fluorescence (b), as a function of time after mixing Aβ(1-40) (20μM) into aqueous buffer, in the absence (black line) and presence of DP (red line) or TP (green line) (40μM). TP and DP significantly inhibit initial growth of oligomers, as determined by one-way ANOVA post-hoc Tukey of the area under the curve (AUC) (inset a, **p<0.005 and ***p<0.0005). TP significantly increases the lag time for amyloid growth as plotted in inset b (*p<0.05). c) TEM images of Aβ(1-40) aggregates at 0, 40 min and in the end point of the kinetic traces shown in b).

The effect of TP in Aβ(1-40) aggregation was also evaluated in the presence of 0.4 equiv of Cu(II), finding that TP partially abates the early formation of large Aβ oligomers that Cu(II) promotes, as observed by DLS (Fig. 3a) and TEM (Fig. 3c). In the ThT kinetic assay, TP recovers the sigmoidal behavior of Aβ(1-40) fibril formation that is characteristic of Aβ aggregation in the absence of Cu (Fig. 3b), however, the lag time is still increased with respect to Aβ only, indicating that TP chelates Cu(II), and it also delays the initial stages of fibril formation. It should be noted that the effect of TP is unique to its bifunctional design, as the control peptide DP, with no capability to chelate Cu(II) ions, has no effect in the aggregation of Aβ in the presence of Cu (Fig 3b).† Finally, TEM analysis at the end point of the aggregation assay shows that TP promotes the formation of longer and less fragmented fibrils. Overall, these data indicate that TP prevents Aβ from

undergoing the Cu(II)-induced aggregation pathway (Scheme 1), and it promotes the formation of longer and less fragmented fibrils, consistent with its bifunctional design.

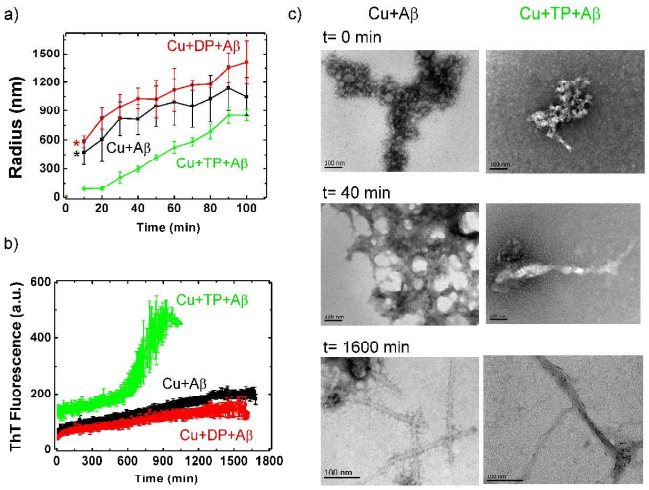
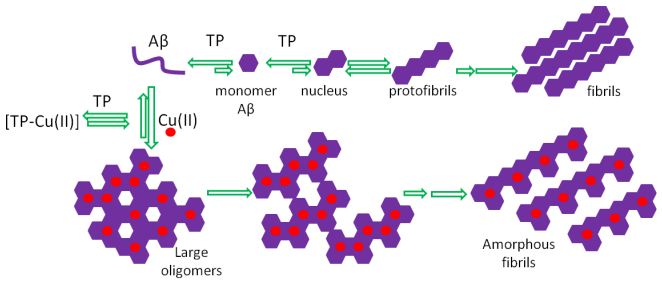


Fig. 3 TP modulates Aβ(1-40) aggregation in the presence of copper. Particle hydrodynamic radius determined by DLS (a) and fibril growth detected by ThT fluorescence (b) as a function of time after mixing Aβ(1-40) (20μM) into aqueous buffer with 0.4 equiv of Cu(II), in the absence (black line) and presence of DP (red line) or TP (green line) (40 μM). *Asterisks in (a) indicate that an accurate measurement of the hydrodynamic radius could not be achieved because the particle radius is much greater than 500 nm. c) TEM images of samples after 0, 40 min and at the end point of the kinetic traces shown in b).



Scheme 1. Proposed model for Aβ(1-40) aggregation in the presence of Cu(II) and TP.

In summary, a bifunctional non-natural tetrapeptide (TP) has been designed to specifically chelate Cu(II) and modulate Aβ(1-40) aggregation. The beta-breaker properties of the aromatic non-natural part of TP abate the initial growth of Aβ oligomers and delay fibril formation. At the same time, TP can effectively compete for Cu(II) ions with soluble Aβ species, due to the Cu(II) specific chelating properties of the Met-Asp construct, inspired in α-synuclein. TP expands the pool of this type of bifunctional molecules to non-natural peptides, and it has proven to be a useful tool to investigate the role of Cu(II) ions in Aβ aggregation. While Cu(II) ions take Aβ through a different aggregation pathway that involves the rapid formation of large oligomers, TP prevents Aβ from undergoing the Cu(II)-induced aggregation pathway (Scheme 1).

There is growing evidence that Aβ oligomers are the neurotoxic species that lead to cell death in AD,[17] while several studies suggest that Cu(II)-induced oligomers may be more toxic due to their ability to generate reactive oxygen species.[17] Thus, the bifunctional properties of TP might prevent the formation of Cu-induced toxic Aβ oligomeric species. The effect of TP in Aβ citotoxicity remains to be studied, yet its Cu(II) specificity is a promising feature that may help gain further insight into the specific role of Cu ions in the molecular mechanism of Aβ-mediated neurotoxicity. This study provides insights into the role of Cu(II) in the Aβ aggregation pathway, and into the design of compounds with therapeutic potential for Alzheimer's disease.

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Notes and references

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† A second control was performed: The peptide MDV, containing the amino acids residues required for Cu(II) coordination and lacking residues that would confer it with β–breaker properties, recovers the aggregation pattern of Aβ in the absence of Cu, but it has no effect in the lag time associated to Aβ fibril formation (Fig S5).

Electronic Supplementary Information (ESI) available: [Experimental section and figures S1 to S5]. See DOI: 10.1039/b000000x/

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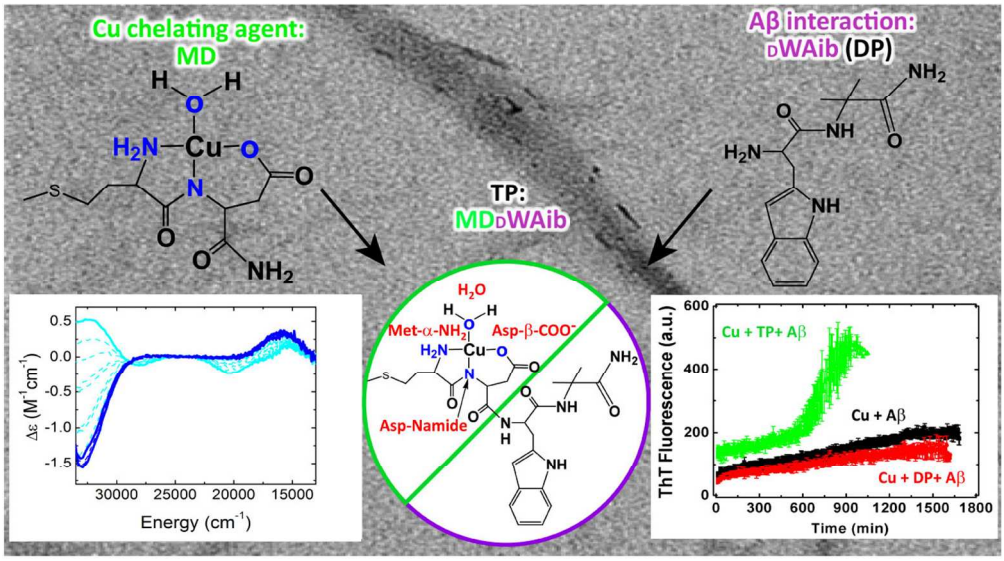

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