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Fulvic Acid Inhibits Aggregation and Promotes Disassembly of Tau Fibrils Associated with Alzheimer's Disease

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Abstract. Alzheimer's disease is a neurodegenerative disorder involving extracellular plaques (amyloid-β) and intracellular tangles of tau protein. Recently, tangle formation has been identified as a major event involved in the neurodegenerative process, due to the conversion of either soluble peptides or oligomers into insoluble filaments. At present, the current therapeutic strategies are aimed at natural phytocomplexes and polyphenolics compounds able to either inhibit the formation of tau filaments or disaggregate them. However, only a few polyphenolic molecules have emerged to prevent tau aggregation, and natural drugs targeting tau have not been approved yet. Fulvic acid, a humic substance, has several nutraceutical properties with potential activity to protect cognitive impairment. In this work we provide evidence to show that the aggregation process of tau protein, forming paired helical filaments (PHFs) *in vitro*, is inhibited by fulvic acid affecting the length of fibrils and their morphology. In addition, we investigated whether fulvic acid is capable of disassembling preformed PHFs. We show that the fulvic acid is an active compound against preformed fibrils affecting the whole structure by diminishing length of PHFs and probably acting at the hydrophobic level, as we observed by atomic force techniques. Thus, fulvic acid is likely to provide new insights in the development of potential treatments for Alzheimer's disease using natural products.

Keywords: Alzheimer's disease, atomic force microscopy, disassembly, fulvic acid, tau aggregation

INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia that is characterized by the formation of two main protein aggregates in the brain: senile plaques consisting of amyloid-β (Aβ) and neurofibrillary tangles, which are composed mainly of the microtubule-associated protein tau [1]. Tau accumulates in a hyperphosphorylated state forming intracellular deposits in AD called paired helical filaments (PHFs) [2]. Physiologically tau stabilizes the microtubule structure, but in the neurons of patients with AD the microtubule system is believed to be disrupted, with concomitant axonal transport deficits and degeneration [3].

Recently, several data have shown that tau aggregation is the main event involved in the neurodegenerative process. This process in AD correlates with the clinical

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progression of the disease and cognitive impairment [4, 5]. The identification of mutations in the tau gene in hereditary frontotemporal dementia has established that dysfunction of tau protein is central to the neurodegenerative process [6]. Interestingly, improvement in the cognition of a transgenic model displaying both neurofibrillary tangles and senile plaques needs the reduction of soluble tau, but not of senile plaques of Aβ [7]. Moreover, evidence for cytotoxicity of intracellular aggregates comes from cellular models, where overexpression of tau is deleterious to the neuron [8].

In the search of new molecules for the treatment of AD, many drugs that have focused on Aβ aggregation have failed to stop the progression of the disease. Immunization against Aβ was effective in reducing amyloid plaque load, but it had little effect on improving cognitive function in patients [9, 10]. Also, a recent study shows the failure of a Phase III clinical trial with a γ-secretase inhibitor [11]. In this context, it seems timely to consider alternative drug discovery strategies for AD based on approaches directed at reducing misfolded tau and compensating for the loss of normal tau function [12]. Therefore, the development of small molecules that inhibit the aggregation of tau appears to be a valid therapeutic target for treatment of AD and other tauopathies [13]. This hypothesis has been favored by current findings on the compound *methylthioninium chloride* (MTC, also known as methylene blue), a previously described inhibitor of the aggregation of tau of the phenothiazine's family [14, 15]. A recent study with this compound in Phase II clinical trial shows an 81% reduction of cognitive decline with the use of the compound compared to placebo [16].

Compounds described for their anti-aggregating capacity in the formation of amyloid aggregates are the polyphenols [17, 18]. In this context, synthetic polyphenols have proved effective in the inhibition of heparin-induced tau aggregation [15]. Following this approach, current therapeutic strategies are aimed at natural phytochemicals and polyphenolic extracts that can either inhibit or disaggregate tau filament formation. It has been suggested that naturally occurring phytochemicals have the potential to prevent AD based on their anti-amyloidogenic, anti-oxidative, and anti-inflammatory properties [19]. Despite this, there are few phytocomplexes emerging to prevent tau aggregation. Only a cinnamon extract and a grape seed polyphenolic extract have been described for this purpose [20, 21].

Fulvic acid is one of the most interesting phytocomplex molecules. This is a mixture of polyphenolic acid compounds resulting from the long-term microbial degradation of lignin, among other sources [22]. It has several nutraceutical properties and is one of the most interesting naturally-occurring phytochemicals with its extremely high antioxidant properties and apparent neuroprotective effect [23, 24]. For instance, the interaction of prion protein with fulvic acid and its inhibitory effect on the content of β-sheet structure and the formation of protein aggregates has been described in detail [25].

Here, we investigated the effects of fulvic acid on heparin-induced tau aggregation *in vitro*. The main objective is the assessment of the inhibition of tau aggregation in presence of fulvic acid by means of three complementary techniques, namely thioflavin T fluorescence (ThT) analysis of aggregation, atomic force microscopy (AFM), and electron microscopy (EM). The observation of formed aggregates by AFM and EM allow us to conclude that fulvic acid inhibits heparin-induced tau aggregation *in vitro.* On the other hand, fulvic acid promotes the disassembling of tau preformed fibrils. Thus, fulvic acid could provide a new insight for developing treatments based on natural products for AD.

MATERIAL AND METHODS

Materials

Fulvic acid standard (Suwanne River I 1S101F) was obtained from the International Humic Substances Society (IHSS, USA). This standard was extracted from water of the Suwanne River in the Okefenokee Swamp in south Georgia. Several structures has been proposed by Leenheer and the third model is shown in Fig. 1B [22]. Heparin was purchased from Calbiochem and methylene blue was purchased from Sigma-Aldrich.

Protein expression and purification

The sequence of htau 40 (longest isoform) was donated gently by Dr. Eckard Mandelkow (Hamburg, Germany). This fragment was cloned into pET-28a vector (Novagen) to produce a His-tagged protein and htau 40 was expressed in *Escherichia coli* strain BL21(DE3). LB medium containing kanamycin was inoculated with a stationary overnight culture. The culture was grown at 37°C to OD_{600} of 0.5–0.6 and protein expression was induced by addition of 1 mM IPTG for 4 h. The cells were pelleted and sonicated. Recombinant tau was purified via a succession of

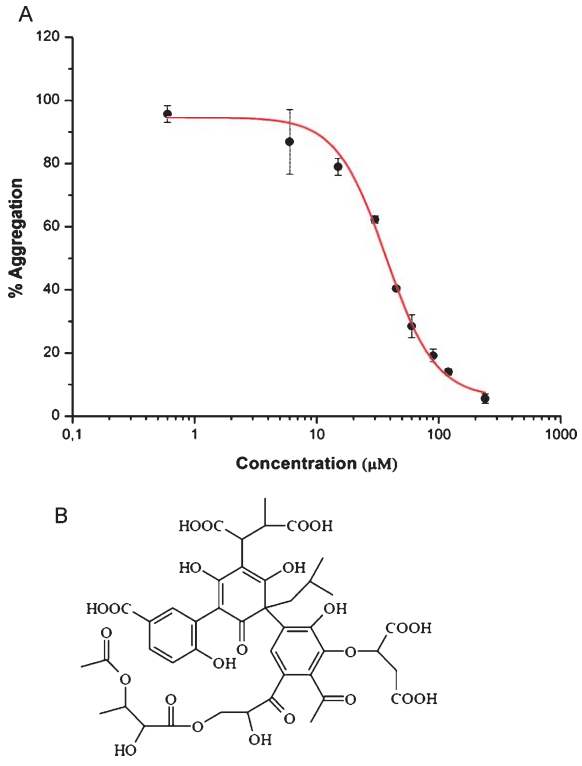

Fig. 1. Inhibitory effect of fulvic acid over PHF formation as monitored by Thioflavin assay (ThT). A) Aggregation of tau fragment 4RMBD in the presence of fulvic acid at different concentrations ranging between 0.1 to 200 μM. The degree of inhibitory effect was monitored by ThT and was plotted and represented as percentage of residual aggregation as a function of fulvic acid concentration. The IC_{50} value for inhibitory effect of Fulvic Acid was 37 μM. B) Suggested model for the fulvic acid structure from the Suwanne River standard [22].

Ni-Sepharose chromatography (equilibrated in 20 mM NaH_2PO_4, 500 mM NaCl, and 20 mM imidazole, pH 7.4, elution with buffer 200 mM imidazole) and side exclusion chromatography coupled to HPLC in a Proteema 100 column (PSS, Germany) with buffer 50 mM NaH_2PO_4, 300 mM NaCl, pH 6.5. The purity of the protein was verified on a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel. The protein was concentrated and stored at –20°C until use. The concentration of purified tau was determined using the extinction coefficient at 280 nm (7700 $M^{-1}cm^{-1}$).

Tau fragment 4RMBD ($htau_{244-372}$) was amplified by using the plasmid for htau 40 as a template. The PCR amplified sequence was subcloned into pET-28a vector (Novagen) to produce a His-tagged protein. The recombinant fragment 4RMBD was expressed in *Escherichia coli* strain BL21 (DE3) and purified as described above, via a succession of Ni-Sepharose chromatography and size exclusion chromatography coupled to HPLC. The concentration of purified 4RMBD was determined using the extinction coefficient at 280 nm (1520 $M^{-1}cm^{-1}$).

ThT fluorescence assay

The ThT fluorescence assay adopted here was modified from the reported by Pickhardt et al. [26] and Crowe et al. [27]. Briefly, to examine the inhibition of tau aggregation, the total volume of the reaction mixture was 100 μl, which included 20 μM 4RMBD, 5 μM heparin in 100 mM sodium acetate, pH 7.0 with the fulvic acid standard at different concentrations. After 20 h of incubation at 37°C, addition of 100 μl of a 25 μM solution of ThT was made and incubation continued for 1 h at room temperature prior to fluorescence reading. Then, the fluorescence was measured in a Biotek Sinergy 2 spectrofluorimeter (Bioteck Instruments, USA) with an excitation wavelength at 440 nm and emission wavelength of 508 nm in a 96-well plate. Each experiment was made at least in triplicate and the background fluorescence was subtracted when needed. To examine the disassembly of preformed PHFs-like of tau, the same reaction mixture was allowed to aggregate for 20 h and then, the pure fulvic acid was added at different concentrations.

Tau aggregation in vitro for EM and AFM

Aggregation for EM experiments was induced by incubating htau 40 typically in the range of 45 μM in volumes of 100 μL at 37°C in 10 mM Hepes, 100 mM NaCl, pH 7.4 buffer with the anionic cofactor heparin (molar ratio of tau to heparin = 4:1) for incubation time of 7 days with continuous shaking. Fulvic acid standard was resuspended in working buffer and applied to the reaction at 200 μM final concentration prior to heparin addition. 4RMBD and htau 40 aggregation experiments for AFM were induced separately. 4RMBD aggregation was incubated at 20 μM of protein and 5 μM of heparin with 20 mM Hepes pH 7.4, 25 mM NaCl for incubation time of 24 h with continuous shaking. Fulvic acid standard was resuspended in working buffer and applied to the reaction at 120 μM final concentration prior to heparin addition. Negative control was developed with only 4RMBD (without both heparin and Fulvic acid) and another with methylene blue at 100 μM. The inhibition experiments with htau 40 were developed as

described above. For disassemble experiments, full length tau was induced for 6 days and then fulvic acid was added at 200 μM for the same time. The preformed fibril control was induced for 12 days.

Transmission electron microscopy

To prepare the samples for electron microscope observation, 5 μL of protein solutions and 5 μL of buffer (10 mM Tris-HCl, pH 7.4 KCl 50 mM) were placed on formvar/carbon coated copper grids and were left at room temperature for 5 min. Then, the samples were negatively stained with 2% (w/v) uranyl acetate for 15 s. All samples were examined with the help of a JOEL EM1200 electron microscopy at 80 kV.

Atomic force microscopy

Tau protein was diluted in 10 mM Tris-HCl, pH 7.4, 50 mM KCl and then immobilized onto highly ordered pyrolitic graphite (HOPG) to a final concentration of 5 μM. A drop of tau protein solution was deposited onto HOPG surface (30 μl) and adsorbed for 30 min. The excess of protein was removed by washing with abundant 10 mM Tris-HCl, pH 7.4, 50 mM KCl solution. In order to avoid spontaneous disassembly all atomic force images were obtained in liquid environment using 10 mM Tris-HCl, pH 7.4, 300 mM KCl.

AFM imaging was performed in tapping (intermittent contact) mode in fluid using a Nanoscope III (Veeco, CA) and Si_3N_4 cantilevers (NPS series, Veeco) exhibiting spring constants of 40–60 N/m at resonance frequencies in buffer of 6 to 10 kHz [28]. To achieve minimal imaging forces between AFM stylus and sample, the drive amplitude was set between 0.5 and 1.0 V, and the amplitude set point was adjusted manually to compensate for the thermal drift of the AFM. The force applied by the scanning process of AFM ranges from 80 to 100 pN.

Data analysis

The ThT fluorescence data were adjusted to a sigmoidal model and graphed with Origin 6.0 software. Fibril lengths were obtained with the program of Nanoscope III system from the AFM images and these data were analyzed with homemade software (developed at the University of Santiago) after being normalized.

RESULTS

Fulvic acid inhibits the 4RMBD tau fragment aggregation process

For screening the inhibition of tau protein aggregation, the fourth microtubule binding domain (4RMBD) was chosen instead of full length tau protein. Indeed, it is well known that 4RMBD repeat is more prone to aggregation and assembles into PHFs, the hallmark of AD. In order to test the capacity of fulvic acid (Fig. 1B) to inhibit aggregation we used three methods including fluorescence spectroscopy, AFM, and EM. Figure 1A shows the inhibition isotherm at different concentrations of fulvic acid, as monitored by ThT, a compound that is able to bind to fibrils containing β-sheet structure promoting changes in the fluorescence spectrum [29]. Fulvic acid in a concentration of 120 μM was able to inhibit in 85% the process of aggregation indicating that β structures diminish when fulvic acid is incubated with 4RMBD fragment. In order to check the correct formation of fibrils, we first induced the aggregation process, observing under AFM the several structures on the HOPG surface. These structures resemble oligomers and longest fibrils (Fig. 2A, Control). After treatment of fibrils with fulvic acid, only oligomeric structures were observed over the surface of HOPG (Fig. 2B). A bigger magnification, Fig. 2b inset, shows unstructured oligomers. The experiment was repeated ten times and twenty images took randomly were analyzed for each run. Fibrils formation was never observed on HOPG surface in the presence of fulvic acid, suggesting that the aggregation process was strongly inhibited. This fact indicates that fulvic acid could be interacting with different regions of 4RMBD avoiding almost completely the process of aggregation. Also a negative control is shown in Fig. 2C suggesting that 4RMBD in absence of heparin does not form either oligomers or fibrils. On the other hand, it is well documented that methylene blue has a strong inhibitory properties on tau aggregation process [15]. In this context we were interested on investigating whether methylene blue is still able to inhibit the 4RMBD at low concentration (IC_{50}) and to exhibit the same pattern on HOPG surface as fulvic acid does. Interestingly, the analysis of AFM images of methylene blue (Fig. 2D) shows oligomers and few fibrils on HOPG surface, suggesting that despite the high degree of inhibition of aggregation exhibited by methylene blue in ThT fluorescence assay [15], this inhibition does not avoid the filaments formation.

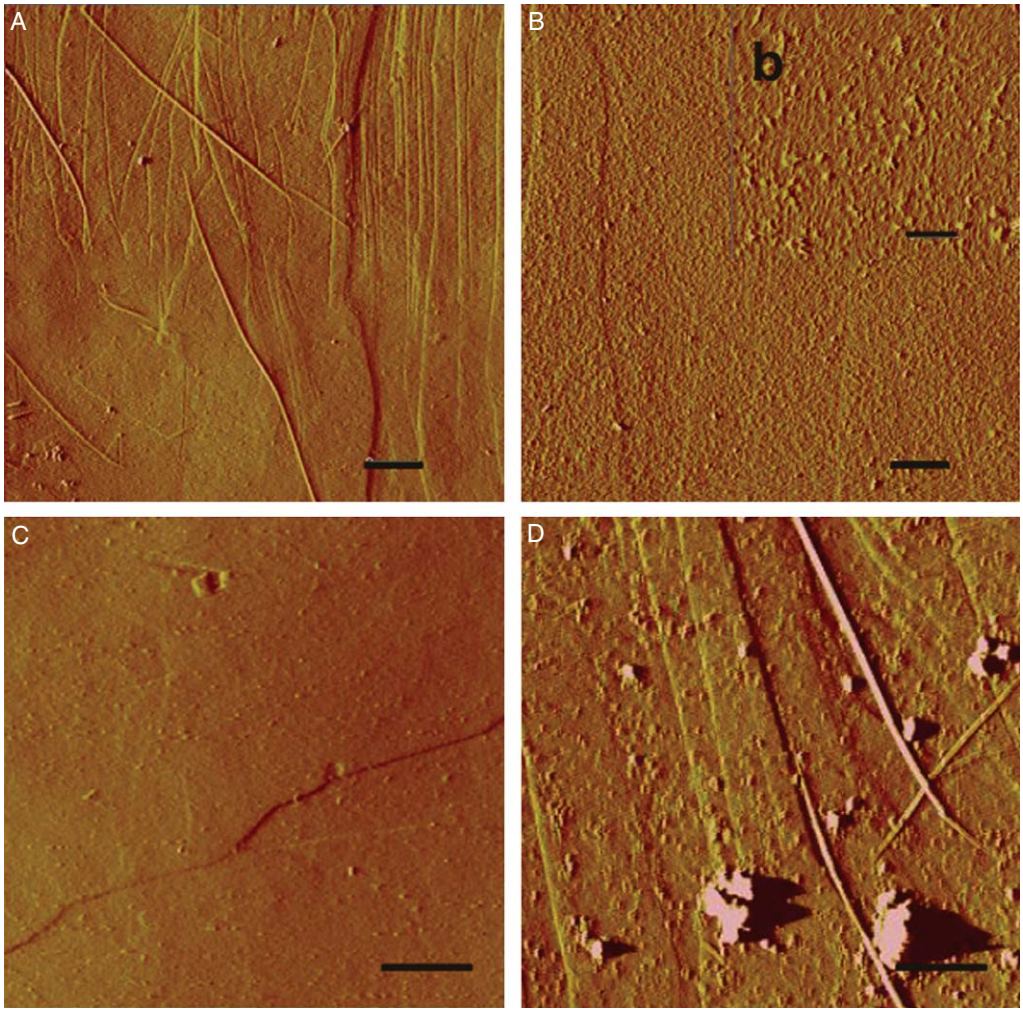

Fig. 2. The assembly of 4RMBD tau fragment is inhibited by fulvic acid. A) The aggregation of 4RMBD was induced by heparin and analyzed by atomic force microscopy over a HOPG surface (Control). Bar = 1 μm. B) The structures formed in presence of 4RMBD as analyzed by atomic force microscopy after treatment with 120 μM fulvic acid. Bar = 1 μm. Inset b) Detailed image resulting from atomic force microscopy of fibrils treated with fulvic acid. Bar = 500 nm. C) Negative control 4RMBD in absence of both heparin and fulvic acid. Bar = 1 μm. D) Internal control of the inhibition process using methylene blue at 100 μMm (instead of fulvic acid) as analyzed by atomic force microscopy. Bar = 500 nm.

Fulvic acid is able to inhibit the aggregation process of full length tau protein

Even though our previous experiments have shown that fulvic acid is able to inhibit the aggregation process and to avoid the fibrils formation, it was pertinent to investigate whether the fulvic is capable to inhibit the aggregation process of full length tau protein as well. To rule this out, we induced the aggregation of hTau 40 in the absence or presence of fulvic acid. As we observed in Fig. 3A, the longest fibrils structures and oligomers were formed after heparin induction. The most abundant elements on HOPG surface were fibrils whose lengths are varying between ∼1 μm and 3 μm. In turn, Fig. 3B shows that pre-fibril and oligomeric structures are also well formed after heparin induction whose lengths range from 30 nm to 100 nm, in the case of oligomers, and 300 nm in the case of pre-fibril structure. After treatment of full length tau protein with fulvic acid (Fig. 4A), the presence of fibrils on HOPG surface was not detected over several trials since the length of oligomeric structures or pre fibrils were clearly affected. Figure 4B shows in detail the oligomeric structures that remain after the aggregation

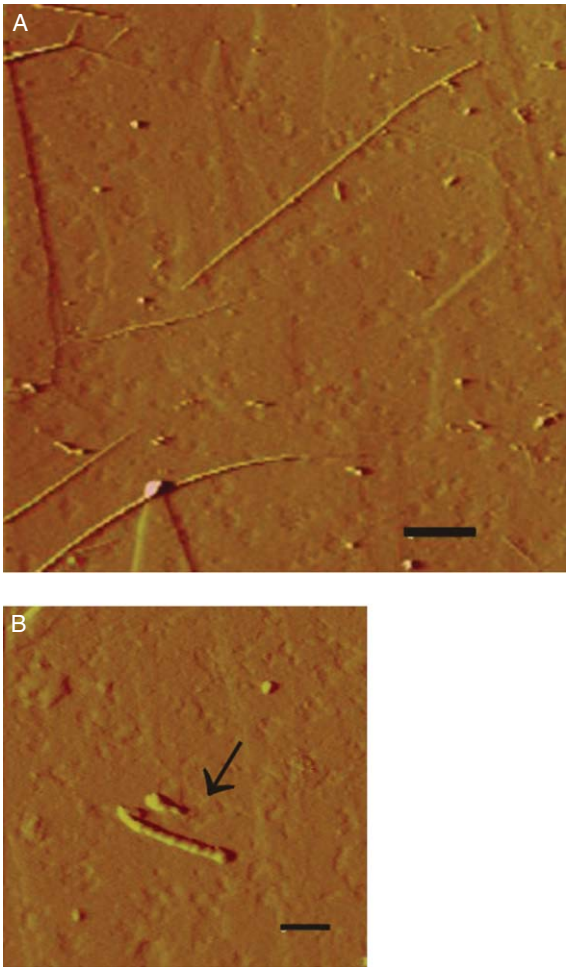

Fig. 3. Full length tau recombinant protein assembles into fibrils. A) Fibril formation of hTau 40 protein was induced by heparin and analyzed by atomic force microscopy. Several fibrils and oligomers over HOPG surface are observed. Bar = 500 nm. B) Oligomers and pre fibril structures are observed by atomic force microscopy after heparin induction of hTau 40 tau protein. Arrow is indicating an oligomeric and a pre-fibril structure. Bar = 100 nm.

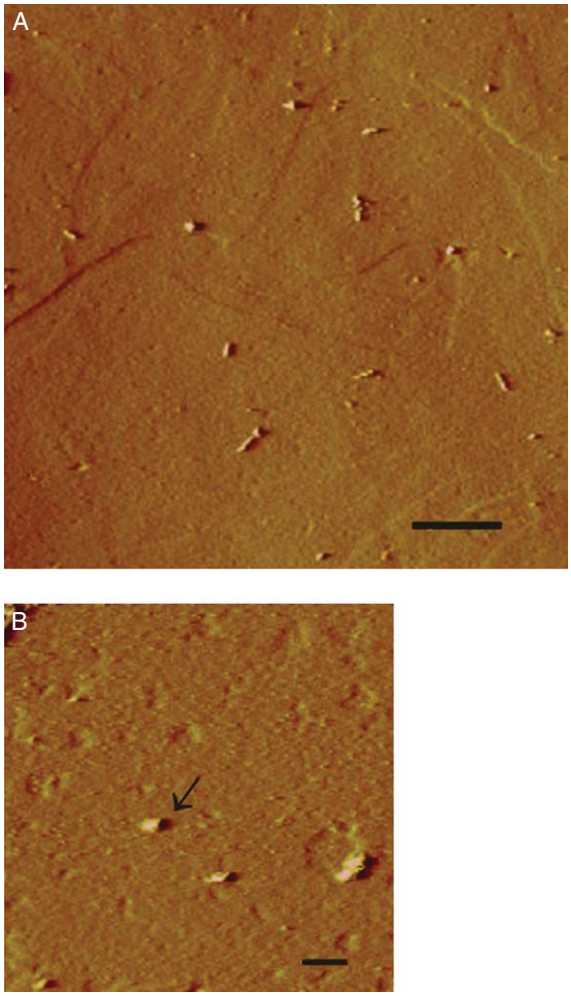

Fig. 4. Fulvic acid treatment blocks PHF-like fibrils of tau molecules. A) Oligomeric structures rather than tau fibrils are observed by atomic force microscopy in the presence of 200 μM fulvic acid. Bar = 500 nm. B) Detailed observation of few oligomeric structures present after treatment with fulvic acid. Arrows indicate an oligomeric structure. Bar = 100 nm.

process induced by heparin in the presence of fulvic acid. In order to investigate in more detail these observations, we used EM. EM images reveal the presence of several fibrils after heparin induction (Fig. 5A) and also the presence of typical PHF (Fig. 5C) as control images. Otherwise, in the case of hTau 40 treated with fulvic acid, we were not able to observe fibrils but only a few elements resembling pre fibril structures (Fig. 5B). Figure 5D shows in more detail a pre-fibril structure, but we did not observe either straight filaments or PHF as observed in control images (Fig. 5A and Fig. 5C).

On the other hand, in order to analyze differences in fibril length of tau protein control and tau protein treated with fulvic acid, we analyzed all these data with our homemade software. First, the lengths of fibrils were obtained using Veeco software of Nanoscope III and all data were normalized before analyses. Figure 6 shows the relative frequencies as a percentage of tau control (fulvic acid absence) and tau-treated (with fulvic acid). As seen in Fig. 6, the relative frequency of length in tau protein treated with fulvic acid is about to 200 nm, suggesting that the aggregation process was inhibited by fulvic acid, avoiding fibril formation. In

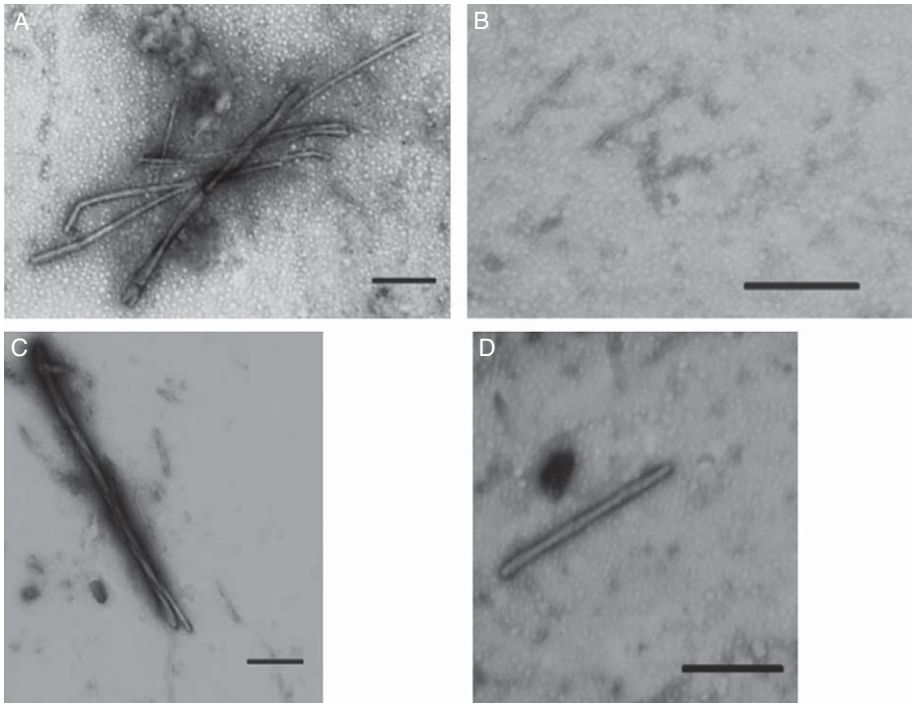

Fig. 5. Fibrils formation of heparin-induced hTau 40 and treated with fulvic acid as analyzed by electron microscopy. A) Control of fibrils of full length tau protein induced by heparin. Bar = 500 nm. 10,000 × magnification. B) Fibril formation is clearly diminished and only a small number of structures are observed in the presence of 200 μM fulvic acid. Bar = 500 nm. 15,000 × magnification. C) PHF-like structures formed with heparin, and twisted structures suggest that fibers are two strands. Bar = 500 nm. 10,000 × magnification. D) A few pre fibrils were found in heparin-induced filaments from full length tau protein after treatment with fulvic acid. The length and thickness is diminished in those filaments. Bar = 500 nm. 15,000 × magnification.

contrast, the lengths of tau protein control fibrils (red bars) were more heterogeneous.

Fulvic acid promotes the disassembly of preformed tau fibrils

So far, we demonstrated that fulvic acid is an inhibitory compound of both 4RMBD and full length tau protein. However, it is interesting to prove whether fulvic acid is capable of disassembling preformed fibrils of tau, whose structures are responsible for neuronal cell death [26].

In this context, we first induced tau fibril formation by heparin (as control) and then after this formation, the filaments were assayed with fulvic acid (200 μM). Interestingly, using fluorescence spectroscopy, we found that fulvic acid disassembles preformed fibril tau in a dose-depending manner (Fig. 7). In addition, we were able to determine the IC_{50} concentration of this compound (95 μM). After this analysis we focused on demonstrating, by AFM, which fibril structures remain after fulvic acid treatment. Figure 8A shows that several structures are formed after heparin induction of full length tau protein. Heterogeneous populations (Fig. 8B) were also observed. Analysis by Nanoscope

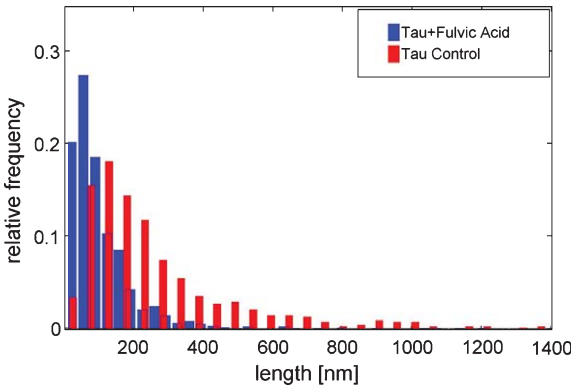

Fig. 6. Data analysis of the filaments length of hTau 40 protein treated with fulvic acid. Modal relative frequencies of the length of control tau fibrils (red clear) and lengths of fibrils of tau treated with fulvic acid (Blue darker). All data were normalized and analyzed by a homemade software for the fibers analysis produced at the University of Santiago.

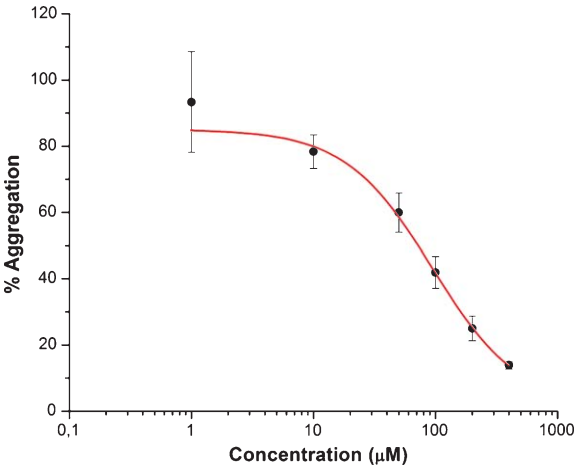

Fig. 7. Fulvic acid promotes disassembly of preformed fibrils of tau fragment 4RMBD as monitored by fluorescence spectroscopy (ThT). For the disassembly experiments, tau fragment 4RMBD was induced to aggregate into PHF-like structures for 20 h as described, and afterward the fibrils were exposed for another 20 h to fulvic acid. The process of inhibition is represented as a percentage of aggregation as a function of fulvic acid concentration. The disassembly concentration for 50% inhibition, DC_{50} value, is 95 μM.

software of fibrils allows us to determinate the height and thickness of structures observing a regular distribution of fibrils whose heights vary between 8 to 10 nm. Thicknesses of fibrils are also regular, varying between 50 to 100 nm (Fig. 8C). Typical dimensions vary depending on which structure is analyzed but clearly the structures are forming part of heterogeneous population (Fig. 8A, B).

In addition, to determine which effects are observed over tau fibrils formed after treatment with fulvic acid, we analyzed this effect by AFM. Figure 9A shows a marked effect of fulvic acid on preformed fibrils diminishing the number of structures and their lengths. In Fig. 9B we observe a clear effect of fulvic acid on the filamentous structures, with a phenomenon of shortening of these elements. Thickness of fibrils treated with fulvic acid are very similar to controls but their heights are diminished (Fig. 9C). To determine in independent and normalized experiments whether fulvic acid has a significant effect on fibril lengths, compared with untreated controls, we used the homemade software. Thus we were able to determine the relative frequencies. Figure 10A shows that preformed fibrils are effectively disassembled (blue bars) instead of controls (red bars). The controls also show a heterogeneous population of fibrils being distributed along the lengths axis. Otherwise, the highest relative frequencies coincide with fibrils having in length less than 200 nm, showing that fulvic acid treatment of preformed fibrils

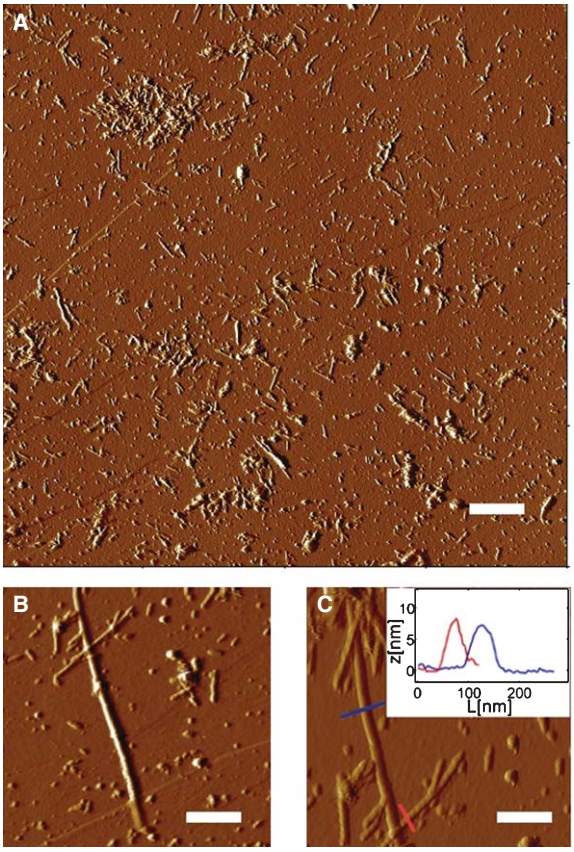

Fig. 8. Characterization of the assembly process of full length tau as monitored by atomic force microscopy. A) AF image of fibrils formation induced by heparin and analyzed by atomic force microscopy on HOPG surface. Bar = 1 μm. B) Detailed image of fibrils formation from hTau 40, co-existing also pre-fibrils and oligomeric structures. Bar = 500 nm. C) Analysis of the thickness and height (in the 3D structural analysis) of fibrils carried out with a Nanoscope III software. Red and blue represent two different fibers. Bar = 300 nm.

is a very effective compound against either aggregation or disassembly process.

DISCUSSION

Neurodegenerative disorders such Alzheimer's disease (AD) are characterized by misfolding and/or aggregation processes affecting specific brain regions. One interesting aspect of these misfolding and/or aggregation processes is that it could be handled by efficient processes of clearance such autophagy, proteolysis, and proteosome system. Instead, in aging and disease the accelerated process of oligomerization and subsequent fibril formation in general, can increase the stress levels and also appears to reduce the ability of cells to avoid the protein aggregate burden [30–32].

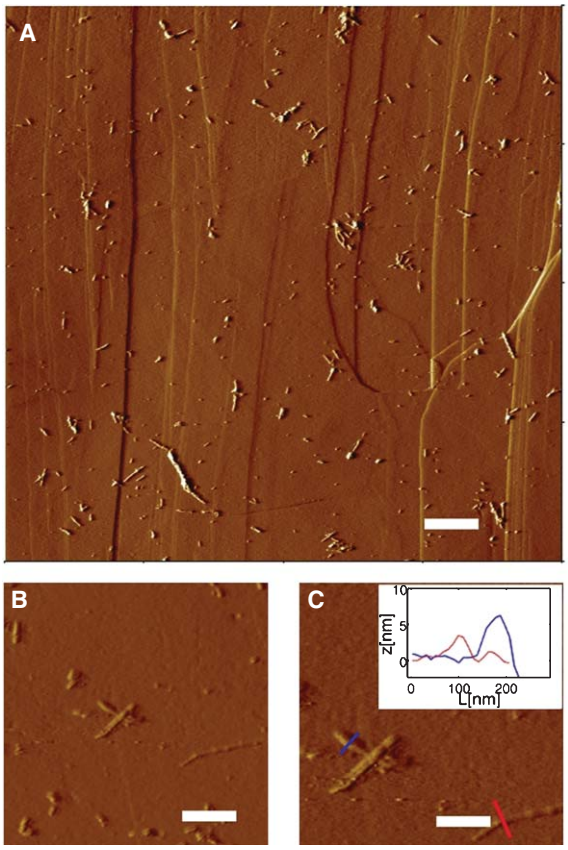

Fig. 9. Fulvic acid induced disassembly of full length tau fibrils as analyzed by atomic force microscopy. A) Images of filaments resulting from disassembly of tau aggregates by fulvic acid treatment on HOPG surface. Bar = 1 μm. B) Detailed image of disassembly and shortening process of fibrils after fulvic acid treatment. Bar = 500 nm. C) Analyses of thickness and height of fibrils after fulvic acid treatment, as analyzed with Nanoscope III software (Veeco, digital Instrument Metrology group). Bar = 300 nm.

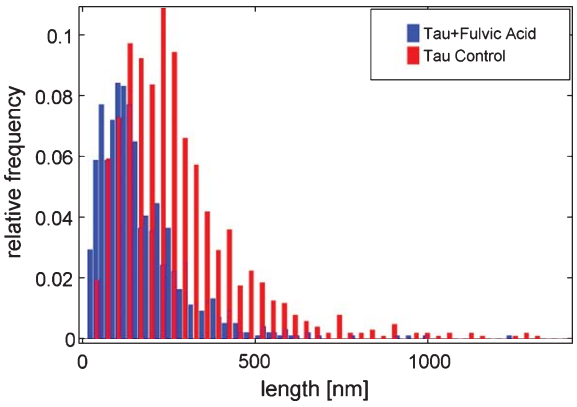

Fig. 10. Data analysis of the disassembly of hTau 40. Modal relative frequencies of the lengths of tau fibrils induced by heparin (red clear) and preformed fibrils treated with fulvic acid (blue darker) are represented as percentage of aggregation as a function of fulvic acid concentration. All data were normalized and analyzed by our home-made software for morphometric parameters of fibers.

The Aβ cascade is placed upstream of tau, a notion supported by an AD mouse model [32]. As such, it is very interesting that preventing tau depletion in mice can avoid Aβ pathology [33].

Recently, it has been demonstrated that a peculiar grape seed polyphenolic extract (GSPE) may attenuate tau protein misfolding leading to form either aggregates or fibrils, a critical step for the progression of the disease [34]. In addition, interesting studies have shown that vitamin A and β carotene have inhibitory properties that disassemble Aβ preformed fibrils [30]. Moreover, all-trans retinoic acid has strong antioxidant properties able to reduce the burden of oxidative stress associated with AD [31]. In the context of the present work, humic substances such as fulvic acid have shown some effects on protease K resistance and cell internalization of human prion protein [25]. Despite all these promising results, only a few compounds are available to treat or prevent symptoms of AD generated by the malfunction of tau protein. Thus, the present work aimed to test the action of fulvic acid, a molecule which has been extensively characterized in previous studies [22]. We used aggregation model 4RMBD and full length tau protein to assess the potential role of fulvic acid in preventing aggregation and/or disassembly of tau protein. First, we described anti aggregation properties of fulvic acid against 4RMBD by fluorescence spectroscopy suggesting that aggregation process is strongly inhibit and as well the β-sheet conformation suggesting that stability of β-conformers are diminished. Using AFM, we were able to show that 4RMBD is diminished on HOPG surface and that the structures remaining on HOPG surface resembled unstructured oligomers. Interestingly, when we decided to test methylene blue as an internal control of our experiments by AFM, we were able to observe the presence of oligomeric structures as well as some fibrils, showing that a diminishing of ThT fluorescence intensity does not reflect an inhibitory effect of methylene blue on tau filaments over HOPG surface. Otherwise, although the IC_{50} of fulvic acid is a little higher, we did not observe fibrils formation on AFM assay suggesting that a different pathway of inhibition is involved.

While our experiments have shown that tau aggregation is inhibited by fulvic acid, it was pertinent to find out whether the same effect could be observed on full length tau protein. Interestingly our observations

reveal a marked effect on fibril formation given that only oligomeric or pre fibril structures were seen. Consistently, in control images typical PHFs were observed by EM instead of full length tau protein treated with fulvic acid. In addition, the lengths analyzed in control and full length tau protein experiments treated with fulvic acid revealed a marked difference. All data suggest that fulvic acid effectively acts by affecting the aggregation process of either 4RMBD or full length tau protein.

On the other hand, it is well known that fibril formation is a crucial event in the pathogenesis of AD. Therefore, we were interested in observing which effects could be exerting fulvic acid on preformed tau filaments. Interestingly, we found that fulvic acid is able to disassemble preformed fibrils of tau protein. It is well known that forces of interaction between HOPG and proteins involved hydrophobic interactions. In this regard it is interesting to point out data arising from AFM images of tau protein treated with fulvic acid, where we observed a decrease in tau protein interactions as compared with HOPG. This was observed despite forces being applied between 80 to 100 pN, in order to avoid disassembly of fibrils, a fact that might suggest that hydrophobic interactions are diminished by fulvic acid. The inhibitory effect of fulvic acid can occur by association between fulvic and tau monomers or oligomers, therefore avoiding the final fibril formation process. In this respect, it seems important that fulvic acid is able to disassemble preformed fibrils despite that several minor structures are seen on HOPG resembling unstructured oligomers or pre-fibrils. However, it has been demonstrated with other polyphenols that these structures can be part of other non-toxic pathways [35]. Finally we concluded that fulvic acid is a promising molecule able to inhibit and disassembly tau fibrils. Subsequent studies are necessary in order to determinate its activity *in vivo.*

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