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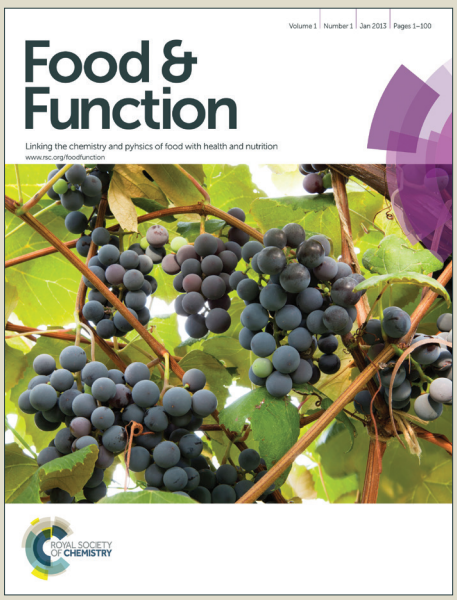

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Inhibition effects of tanshinone on the aggregation of α-synuclein

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative diseases. Lewy bodies that formed by the aggregated α-synuclein is a major pathological feature of PD. *Salvia miltiorrhiza* has been used as food and as a traditional medicine for centuries in China, with tanshinone I (TAN I) and tanshinone IIA (TAN IIA) as its major bioactive ingredients. Here, we investigated the effect of TAN I and TAN IIA on α-synuclein aggregation both *in vitro* and in a transgenic *Caenorhabditis elegans* PD model (NL5901). We demonstrated that TAN I and TAN IIA inhibited the aggregation of α-synuclein as demonstrated by the prolonged lag time and reduced thioflavin-T fluorescence intensity; TAN I and TAN IIA also disaggregated preformed mature fibrils *in vitro*. Moreover, the presence of TAN I or TAN IIA affected the secondary structural transformation of α-synuclein from unstructured coils to β-sheets, and alleviated the membrane disruption caused by aggregated α-synuclein *in vitro*. Besides, immuno-dot-blot assay indicated that TAN I and TAN IIA reduce the formation of oligomers and fibrils. We further found that TAN I and TAN IIA extended the life span of NL5901, a strain of transgenic *C. elegans* that expresses human α-synuclein, possibly through attenuating the aggregation of α-synuclein. Together, our results suggested that TAN I and TAN IIA may be further explored as potential candidates for the prevention and treatment of PD.

Keywords: Parkinson's disease; α-synuclein; aggregation; tanshinone I; tanshinone IIA; *Caenorhabditis elegans*

1. Introduction

Parkinson's disease (PD) is a major neurodegenerative disease, with the morbidity in people over 60 can reach 1%, second only to Alzheimer's disease (AD).[1] The main pathological features of PD are apoptosis of dopamine neurons and the formation of intracellular Lewy bodies in *substantia nigra*,[2] which further cause bradykinesia, muscular rigidity, resting tremor and mental abnormality.[1, 3] The composition of Lewy bodies has been found to be deposition of abnormally aggregated α-synuclein.[4] Native α-synuclein is an unstructured soluble protein that plays roles in memory, recognition, regulation of dopamine and presynaptic vesicle trafficking.[5, 6] Under pathological conditions, unfolded α-synuclein forms β-structures, leading to the formation of toxic oligomers, which further aggregate into protofibrils and mature fibrils, and cause cell dysfunction.[7, 8] Discovery of inhibitors that restrain α-synuclein abnormal aggregation has been an important investigation direction for PD treatment.[9]

Presently, many small molecular inhibitors originated from traditional herbal medicine have been demonstrated to have inhibitory effects on α-synuclein aggregation, such as curcumin, gallic acid and epigallocatechingallate (Figure 1A & D).[10-12] *Salvia miltiorrhiza* (also known as Danshen) is a medicinal food herb, which was used as food auxiliary,[13] and widely used in traditional Chinese medicine for cancer, cardiovascular and cerebrovascular diseases.[14] TAN I and TAN IIA (Figure 1B & C) are main liposoluble phenanthrenequinonic compounds from *Salvia miltiorrhiza*. We have recently shown that phenanthrenequinone can inhibit insulin aggregation.[15] In addition, TAN I and TAN IIA share similar structures with curcumin by having two ketone groups and aromatic groups, which has been reported to inhibit α-synuclein aggregation *in vitro* and in a *Drosophila* PD model.[16-18] Recently, there are reports supporting that TAN IIA can prevent the loss of nigrostriatal dopaminergic neurons based on its

anti-oxidative and anti-inflammatory properties in the MPTP model of Parkinson's disease, TAN I and TAN IIA can inhibit amyloid aggregation of amyloid-β peptide.[19, 20] All these suggest that TAN I and TAN IIA may have inhibitory effects on α-synuclein aggregation.

To test this hypothesis, we performed a set of experiments *in vitro* and *in vivo*. Our results suggested that TAN I and TAN IIA not only effectively inhibited the toxic aggregation of α-synuclein *in vitro*, but also extended the life span of a transgenic *Caenorhabditis elegans* strain NL5901 which expresses human α-synuclein.

2. Materials and methods

2.1. Materials

Plasmid expressing human α-synuclein PT7-7 was obtained from Addgene (Cambridge, MA, USA). Tanshinone I (TAN I, ≥ 98%), tanshinone IIA (TAN IIA, ≥ 98%) and (-)-epigallocatechingallate (EGCG, ≥ 98%) were purchased from Aladdin-reagent INC. (Shanghai, China). Anti-amyloid oligomer polyclonal antibody (A11, cat. # AB9234) and anti-amyloid fibrils polyclonal antibody (OC, cat. # AB2286) were obtained from Merck Millipore (Billerica, USA). Goat anti-rabbit IgG-HRP conjugate was from Bio-Rad Laboratories, Inc. (Hercules, USA). Thioflavin-T (ThT), 2-Oleoyl-1-palmitoyl-sn-glycerol-3-phospho-rac-(1-glycerol) sodium salt (POPG) and carboxyfluorescein were purchased from Sigma-Aldrich (St. Louis, USA). Transgenic *Caenorhabditis elegans* strain NL5901 [unc-54p::alphasynuclein::YFP+unc-119(+)] was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN). Other chemicals used were of the highest grade available.

2.2. Expression and purification of human α-synuclein

Human α-synuclein was expressed in *E. coli* BL21 (DE3) and induced by 0.7 mM IPTG for 4 h. For one purification batch, 12 L induced *E. coli* was centrifuged at 4 ℃ (10000 rpm, 15 min) and the pellet was resuspended in 200 mL TEN buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) and boiled for 10 min. Then the solution was centrifuged, followed by adding 136 μL 10% (w/v) streptomycin sulfate and 222 μL glacial acetic acid per 1 mL supernatant. The solution was then centrifuged, and the supernatant was mixed with the same volume of saturated ammonium sulfate, followed by sitting at 4 ℃ for 30 min. After centrifugation at 4 ℃ (10000 rpm, 15 min), the precipitate was washed with 1:1 (v/v) saturated ammonium sulfate and H_2O and then centrifuge again. The obtained precipitate was then washed with 1:1 (v/v) 100 mM ammonium acetate and ethanol for three times. A Hitachi L-2000 high performance liquid chromatography system (Tokyo, Japan) was then applied with a semi-preparative Apollo C18 column (Grace, USA) to purify the α-synuclein as we previously described.[21] The mobile phases contain 0.1% TFA, with a 10% to 30% acetonitril gradient for the first 5 min, and then a 30% to 80% gradient in the next 22 min, a flow rate of 4 mL/min and a detection wavelength of 280 nm. The collected peak was lyophilized, and quantified by a U-2900 UV spectrophotometer (Hitachi, Tokyo, Japan). α-synuclein expression was verified by SDS-PAGE and coomassie blue staining. After semi-preparative HPLC purification, 5.42 mg α-synuclein (> 95% pure, determined by HPLC) was obtained per 1 L *E. coli* culture.

2.3. α-synuclein aggregation by protein misfolding cyclic amplification

The amyloid formation of α-synuclein was performed with a protein misfolding cyclic amplification (PMCA) approach as previously reported.[22, 23] Briefly, α-synuclein was dissolved in 50 mM PBS buffer (containing 100 mM NaCl, pH 7.4) to a final concentration of 50 μM. The solution was sonicated for 2 min with a SB25-12DTD sonicator (Scientz Biotechnology, China). TAN I and TAN IIA were freshly dissolved in DMSO to saturated (2.67 and 6.85 mM, respectively, as determined by their UV-absorptions at 270 nm), and EGCG was dissolved in DMSO to 6 mM as stock solutions. 100 μL α-synuclein (50 μM) in the presence or absence of different compounds were incubated in eppendorf tubes at 37 ℃ with three 1.0 mm Zirconia/Silica beads (37.9 ± 0.7 mg; Gong Tao INC., Shanghai, China) inside. Samples were sonicated for 30 s per 1 h with an output power setting at 90%. For α-synuclein aggregation, 35 cycles of PMCA were needed and aliquot samples were removed at the time points of 0 h, 6 h, 11 h, 14 h, 17 h, 20 h, 22 h, 29 h, 35 h.

2.4. Thioflavin-T (ThT) fluorescence assay

During incubation, 3 μL of samples were removed at designated points for ThT fluorescence assay on a Hitachi FL-2700 fluorometer (Tokyo, Japan) as we previously reported.[24] The assay solution includes 50 mM PBS (containing 100 mM NaCl, pH 7.4) and ThT with a final concentration of 20 μM, with excitation and emission wavelength set at 450 nm and 482 nm, respectively. Each experiment was repeated for at least three times. 1% DMSO was presented in every group (blank control and TAN I, TAN IIA, EGCG treated groups), and control study suggested at this concentration, DMSO only showed minor effect on α-synuclein aggregation (data not shown).

2.4. Disaggregation of α-synuclein fibrils

Mature α-synuclein fibrils were prepared (50 μM and 24 h incubation) as described above. Mature fibrils were further incubated at 37 ℃ for another 9 h in the presence or absence of TAN I, TAN IIA, or EGCG. Aliquots were removed during incubation for ThT fluorescence assay.[25] The experiment was repeated for at least three times.

2.5. Transmission electron microscopy (TEM)

As we previously described,[26] 5 μL sample for TEM was dropped onto the center of 300-mesh Formvar-carbon coated copper grid (Shanghai, China) for 5 min, followed by staining with 1% uranyl formate for another 5 min, air dried followed by analysis under an H-8100 transmission electron microscope (Hitachi, Tokyo, Japan).

2.6. Far-UV circular dichroism (CD) and data analysis

Far-UV CD was performed to study the secondary structure. Samples were detected by a JASCO-810 circular dichroism spectropolarimeter (JASCO, Japan) under continuous N_2 flow. The data were recorded from 190 nm to 260 nm with a 2 nm bandwidth, and a scanning speed of 50 nm/min. All results were repeated for three times. The spectra of different compounds alone were subtracted from the respective spectra of α-synuclein in the presence of different compounds. The data obtained were converted into mean residue ellipticity [θ].[27]

2.7. Immuno-dot-blot assay

50 μM α-synuclein was incubated in the presence or absence of TAN I, TAN IIA or EGCG as described above. 2 μL sample removed at designed interval was dropped on a nitrocellulose membrane (Biorad, USA) and air dried. Then the membrane was blocked by 5% skimmed milk (Becton Dickinson, NJ, USA) for 2 h at room temperature, followed by incubating at 4℃ overnight with A11 or OC antibody (1:2500, Millipore, Billerica, USA), which specifically recognize oligomers and fibrils, respectively.[28] After washed with TBST, anti-rabbit IgG (1:5000) was added and incubated for 2 h at room temperature. The membrane was washed again, and developed with an ECL chemiluminescence kit (Millipore, Billerica, USA) for further analysis.[15] Densitometry analysis was performed by software Quantity One 4.6.2 (Bio-Rad, Hercules, USA).

2.8. Dye leakage assay

50 μM α-synuclein incubated alone with DMSO or co-incubated for 35 h with TAN I, TAN IIA, or EGCG was prepared as above. The POPG vesicles were prepared as we previously described.[29] Briefly, 20 mg 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) was dissolved in 1 mL $CHCl_3$, dried with nitrogen into a lipid film and lyophilized overnight. 15 mg carboxyfluorescein dissolved in 1 mL 50 mM PBS buffer (contain 100 mM NaCl, pH 7.4) was added into POPG to form POPG vesicles, and then purified with a PD-10 column (Sangon Biotech., Shanghai). Obtained POPG vesicles was diluted with 50 mM PBS buffer (containing 100 mM NaCl, pH 7.4), then the samples were added to a final concentration of 5 μM α-synuclein for fluorescence measurement. The excitation and emission were set at 493 nm and 518 nm, separately. The scanning time for each sample was 60 s and

the mean value of fluorescence intensity during 60 s was shown. The POPG vesicles treated with 0.2% (v/v) Triton X-100 were used as the control of complete leakage, while POPG vesicles alone were used as the blank control.[30]

2.9. Analysis of *C. elegans* NL5901 life span

TAN I, TAN IIA and EGCG were diluted in DMSO to 30 mM (supersaturated solution) as stock solutions. 100 μL OP50 containing 1% (v/v) DMSO or respective compound stock solution was added onto nematode growth medium (NGM) spread in a 35 mm dish. Transgenic *C. elegans* NL5901 (expressing a fusion α-synuclein with yellow fluorescence protein, YFP), were synchronized with a bleach buffer (1 M NaOH, 1.2 M NaClO) for three minutes with gentle shaking.[31] After three days, L3 larvae were washed with M9 buffer (22 mM KH_2PO_4, 42.3 mM Na_2HPO_4, 85.5 mM NaCl, 1 mM $MgSO_4$) and centrifuged (3.2×10^3 rpm), followed by transferred onto NGM. Each group contains 100 larvae in five plates and incubated at 20 ℃. The *C. elegans* were transferred onto newly treated NGM plates every two or three days, at the same time, the number of *C. elegans* were counted under a ZSA 302 digital stereo microscope (COOC, Chongqing, China) and analyzed by software GraphPad Prism.

2.10. Analysis of α-synuclein expression in *C. elegans*

99 μL suspension of OP50 *E. coli* together with 1 μL DMSO or stock solution of compound per well was added into 96-wells plate. 20 synchronized L3 larvae per well were added into 96-well plate, and incubated at 20 ℃ for three days. Nematodes were then washed with M9 buffer, dropped onto slides and fixed with 25% (v/v) glycerin, followed by covered with coverslips for image analysis with an Olympus

IX71 inverted fluorescence microscope (Tokyo, Japan).[32, 33]

2.11. Dot-blot assay of protein extracted from *C. elegans*

Synchronized L3 *C. elegans* were transferred into *E. coli* OP50 in 96-wells plate, which contained 1% (v/v) DMSO or compound stock solution, at a density of about 20 larvae per well. After three days incubation at 20 ℃, *C. elegans* were washed for three times with M9 buffer and centrifuged. Then lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 0.5% (v/v) Triton X-100, pH 8.5) was added and sonicated. The obtained solutions were quantified by a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China), equal amount of protein was blotted onto nitrocellulose membrane for dot-blot assay as described above. Because the number of *C. elegans* needed for success immune-dot blot detection was large, we had to pool five parallel experimental groups (100 worms) to obtain enough sample for *C. elegans* immuno-dot-blot analysis, and thus the results represents the average intensity.

2.12. Statistical analysis

All data (except for the analysis of *C. elegans* life span) were shown as mean ± SD, and analyzed by the nonparametric Kruskal-Wallis test and Mann-Whitney test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. TAN I and TAN IIA inhibit the amyloid formation of α-synuclein and depolymerize preformed α-synuclein fibrils

To study the effects of TAN I and TAN IIA on the amyloid formation of α-synuclein, ThT fluorescence assay was performed. In a control study, we found that DMSO (1% v/v), TAN I, TAN IIA and EGCG alone showed no interference on the blank ThT fluorescence intensity (data not shown). α-synuclein (50 μM) showed a short lag time of 13.2 ± 2.9 h (Figure 2A & Table 1). In the presence of 26.7 μM TAN I (1% of saturated TAN I stock solution), the lag time was prolonged to 24.4 ± 3.3 h ($p < 0.05$, Figure 2A & Table 1) with the relative intensity reduced to 48.0 ± 2.6% of that of α-synuclein ($p < 0.01$). On the other hand, the presence of 68.5 μM TAN IIA (1% of saturated TAN IIA solution), a lag time of 25.8 ± 2.6 h ($p < 0.01$) with a decreased relative fluorescence intensity of 29.0 ± 1.7% of that of control was observed ($p < 0.01$, Figure 2A & Table 1). Consistent with a previous report,[11] the presence of 60 μM EGCG almost abolished the amyloid formation (Figure 2A & Table 1). Then we tested these compounds at lower concentrations, and found that in the presence of 25 μM TAN I, TAN IIA or EGCG, the lag time of α-synuclein aggregation were 18.2 ± 0.9, 18.5 ± 1.2 and 22.4 ± 1.0 h, with the relative fluorescence intensity of 64.6 ± 5.9%, 76.8 ± 12.4%, 65.0 ± 10.1%, respectively (Table 1).

TEM was performed to study the morphology of α-synuclein incubated in the presence or absence of TAN I, TAN IIA or EGCG. Consistent with previous reports,[34] TEM image of α-synuclein incubated alone exhibited extensive mature fibrils. In contrast, the TEM images of α-synuclein co-incubated with TAN I, TAN IIA or EGCG showed no obvious fibrils, which is consistent with the ThT fluorescence results (Figure 2A, 2C-F).[35]

We further tested whether TAN I and TAN IIA can depolymerize preformed α-synuclein fibrils. In the presence of TAN I, TAN IIA or EGCG, after 6 h incubation, the relative fluorescence intensities were

decreased by 19.9%, 56.2%, and 50.9%, respectively (Figure 2B).

3.2. TAN I and TAN IIA delay the secondary structural transition of α-synuclein

To study the secondary structural transition of α-synuclein during incubation, far-UV circular dichroism was performed. At the beginning of incubation, the spectrum of α-synuclein showed characteristics of random coil, as manifested by the negative peak at 205 nm (Figure 3). After 17 h incubation, the control group showed significant increase at 200 nm and formation of a negative peak at 215~220 nm, suggesting conversion from random coil to α-helix and β-sheet, respectively (Figure 3A). In the presence of TAN I, TAN IIA or EGCG, the native-like structure remained in random coil at 17 h, whereas notable β-sheet structures were observed at 35 h in all sample groups (Figure 3B & C).[35]

3.3. TAN I and TAN IIA alleviate the oligomerization and fibrillation of α-synuclein

The contents of oligomers and fibrils at different incubation points were assessed by immuno-dot-blot and relative densitometry analysis. All groups showed no oligomers or fibrils at 0 h, which is consistent with results of ThT fluorescence and CD assay (Figure 4A). A11- and OC-positive blots were visible in α-synuclein incubated for 17 h, suggesting the emergence of oligomers and fibrils, while TAN I, TAN IIA and EGCG treated groups showed no such positive blots, which is complementarily proven by densitometry analysis (Figure 4B). Although there were oligomers and fibrils observed in experimental groups after 35 h incubation, the blots of treated groups were obviously lighter compared with the control, especially for the EGCG group (Figure 4A).

3.4. TAN I and TAN IIA attenuate α-synuclein amyloid formation induced membrane damage

We next studied the effects of TAN I and TAN IIA on membrane damage induced by aggregated α-synuclein. POPG vesicles were damaged by 62.9 ± 6.9% ($p < 0.05$) when treated with pre-incubated α-synuclein, compared to Triton X-100, which was set as 100% (Figure 5). However, when treated with α-synuclein co-incubated with TAN I or TAN IIA, the damage was decreased to 42.6 ± 4.2% ($p < 0.05$) and 41.8 ± 2.3% ($p < 0.05$), respectively. Whereas the control EGCG group almost completely inhibited membrane damage (Figure 5). In a control study, we found TAN I, TAN IIA or EGCG alone in the concentration we tested showed no damage to the membrane (data not shown).

3.5. TAN I and TAN IIA extend the life span of *C. elegans*

The effects of TAN I and TAN IIA on the life span of transgenic *C. elegans* (NL5901, α-synuclein co-expressed with YFP) were measured with EGCG as a positive control. The T_{50} (time of percent survival reaching 50%) of *C. elegans* NL5901 treated with DMSO was 14.0 d, while the T_{50} of TAN I, TAN IIA and EGCG treated NL5901 were 16.2 d, 15.5 d and 14.9 d, respectively (Figure 6 A).

3.6. TAN I and TAN IIA reduce aggregation of α-synuclein in a *C. elegans* NL5901

The apparent intensities of fluorescence were similar among groups of un-treated and treated *C. elegans*, indicating similar expression level of α-synuclein (Figure 6B). We then detected the degree of α-synuclein aggregation with immuno-dot-blot. For the group treated with DMSO, it exhibited strong A11- and OC-positive dots (oligomers and fibrils, respectively); when treated with TAN I, TAN IIA or EGCG, the amount of both oligomers and fibrils were reduced significantly; interestingly, no detectable

differences were observed among TAN I, TAN IIA and EGCG treatments (Figure 6C).

4. Discussion

Dopamine apoptosis and α-synuclein aggregation both exist in patients undergoing PD.[36] In pathological conditions, the native non-toxic α-synuclein turned into toxic aggregated α-synuclein, and induced a series of cytotoxicity.[37]

Here, we found that TAN I and TAN IIA not only inhibited the aggregation of α-synuclein by significantly prolonging the lag time of α-synuclein aggregation, with an obvious decrease in the fluorescence intensity, which was further morphologically confirmed by TEM (Figure 2A, C-F), but also disaggregated the preformed α-synuclein fibrils (Figure 2B). We further found that TAN I and TAN IIA delayed the secondary structural change to β-sheet and reduced oligomerization, which was demonstrated by CD and immuno-dot-blot assay, respectively (Figures 3 & 4). Aggregated α-synuclein, especially oligomers, has been reported to interact with cell membrane and lead to membrane depolarization, intracellular oxidative stress and even cell apoptosis.[7, 38] Dye leakage assay results suggested that TAN I and TAN IIA could protect POPG vesicles from the damage caused by α-synuclein aggregation (Figure 5), so they may have protection effect on cell membrane against α-synuclein aggregation. TAN I and TAN IIA treatment prolonged transgenic *C. elegans* NL5901 life span by reducing the aggregation of α-synuclein without affecting its expression level (Figure 6). Although EGCG showed higher efficacy on inhibiting α-synuclein aggregation than TAN I and TAN IIA *in vitro*, no obvious difference in life span and immuno-dot-blot assays were observed in transgenic *C. elegans* NL5901, suggesting TAN I, TAN IIA and EGCG may have comparable effects on amyloid aggregation

in vivo. The possible mechanism of TAN I and TAN IIA on inhibiting α-synuclein aggregation may similar to their effects on Aβ,[19] which may involve binding to α-synuclein monomers and oligomers via hydrophobic interaction with β-structures, which leads to the delay of nucleation process and the further assembly of oligomers (Figure 7).

TAN I and TAN IIA have been reported to have neuroprotective effects in a rat model, based on their anti-oxidative and anti-inflammatory properties,[20, 39] which are closely associated with PD.[40, 41] Moreover, water-soluble sodium TAN IIA sulfonate that derived from TAN IIA, has been used in clinical for unstable angina.[42, 43] TAN I and TAN IIA are liposoluble compounds, so their molar ratios for the assays performed aqueous solution here are limited. It has been reported that the concentration of α-synuclein in cerebrospinal fluid of normal people and PD falls in the range of $2.7\sim2.9\times10^{-5}$ μM,[44] which is orders lower than that used in this study (50 μM), whereas the concentration of TAN IIA could reach 10^{-1} μM in plasma and 10^{-4} μmol/μg in brain at 6 h after intravenous injection in rat.[45] Furthermore, advanced delivery measures such as lipid nanoparticles or PEGylated nanoparticles can also be used to increase their effective concentrations in brain, which have been proven to prolong circulation of TAN IIA and increase its accumulation in brain,[45, 46] for example, a study performed on Sprague-Dawley rats showed that the concentration of TAN IIA loaded PEGylated nanoparticles in brain was *ca.* three folds of free TAN IIA at 6 h after intravenous administration.[46]

In summary, our results suggest the potential of TAN I and TAN IIA as anti-PD candidates, however, their exact working mechanism still awaits further investigation.

Abbreviations Used

PD, Parkinson's disease; TAN I, tanshinone I; TAN IIA, tanshinone IIA; EGCG, (-)-epigallocatechin gallate; AD, Alzheimer's disease; ThT, Thioflavin-T; CD, circular dichroism; TEM, transmission electron microscopy; POPG, 2-Oleoyl-1-palmitoyl-sn-glycerol-3-phospho-rac-(1-glycerol) sodium salt; HPLC, high performance liquid chromatography.

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Conflict of Interest: The authors declare no conflict of interest associate with this work.

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Figure Legends

Figure 1. Chemical structures of curcumin and compounds studied.

Figure 2. TAN I and TAN IIA inhibited the formation of α-synuclein fibril. (A) Relative thioflavin-T fluorescence intensity of α-synuclein incubated with or without TAN I or TAN IIA. (B) Relative thioflavin-T fluorescence intensity of pre-incubated α-synuclein in the presence or absence of TAN I or TAN IIA. (C) TEM image of α-synuclein incubated alone for 17 h. (D) TEM image of α-synuclein incubated with TAN I for 17 h. (E) TEM image of α-synuclein incubated with TAN IIA for 17 h. (F) TEM image of α-synuclein incubated with EGCG for 17 h.

Figure 3. Secondary structures of α-synuclein in the presence or absence of TAN I and TAN IIA. (A) Far-UV circular dichroism spectra of α-synuclein alone. (B) Far-UV circular dichroism spectra of α-synuclein incubated with TAN I. (C) Far-UV circular dichroism spectra of α-synuclein incubated with TAN IIA. (D) Far-UV circular dichroism spectra of α-synuclein incubated with EGCG.

Figure 4. Immuno-dot-blot of α-synuclein incubated with or without TAN I and TAN IIA and corresponding densitometry analysis. (A) Immuno-dot-blot of α-synuclein incubated with or without TAN I and TAN IIA at different time points. (B) Densitometry analysis of immune-dot-blot. Data was compared between the treated groups and the DMSO-only control groups at the same time points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

Figure 5. Effects of TAN I and TAN IIA on membrane damage caused by α-synuclein. Triton X-100 was used as positive control with the relative intensity of dye leakage saved as 100%. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

Figure 6. TAN I and TAN IIA protect NL5901 *C. elegans* worms from α-synuclein aggregation. (A)

The life span of NL5901 *C. elegans* treated with or without TAN I, TAN IIA or EGCG. (B) The autofluorescence images of the head of NL5901 *C. elegans* treated with or without TAN I, TAN IIA or EGCG for three days. (C)Dot-blot of the lysates of NL5901 *C. elegans* treated with or without TAN I, TAN IIA or EGCG for three days.

Figure 7. Possible protective mechanisms of TAN I and TAN IIA against α-synuclein aggregation.

Table 1. Effects of TAN I and TAN IIA on the amyloidogenic properties of α-synuclein [a, b]

	Fibril formation [c]			Amyloidogenic [e]
	Lag time (h)	T_{50} (h) [d]	Relative intensity (%)	
DMSO	13.2 ± 2.9	18.2 ± 0.6	100.0 ± 6.8	+++
TAN I (25 μM)	18.2 ± 0.9	20.1 ± 0.6	64.6 ± 5.9	++
TAN I (26.7 μM)	24.4 ± 3.3	26.7 ± 2.9	48.0 ± 2.6	+
TAN IIA (25 μM)	18.5 ± 1.2	21.8 ± 1.5	76.8 ± 12.4	+++
TAN IIA (68.5 μM)	25.8 ± 2.6	29.0 ± 1.7	33.1 ± 6.6	+
EGCG (25 μM)	22.4 ± 1.0	23.9 ± 1.2	65.0 ± 10.1	+
EGCG (60 μM)	N [f]	N	7.1 ± 4.7	-

[a.] Compounds were prepared for stock solutions, and added 1% (v/v) into reaction solutions.

[b.] Each assay was repeated for at least three times.

[c.] All compounds were incubated with 50 μM α-synuclein.

[d.] T_{50} means the time of reaching the half of maximum intensity.

[e.] The relative fluorescence of α-synuclein incubated with DMSO alone was recorded as 100%. The degree of aggregation was showed as following: "+++" stood for the relative fluorescence above 75%, "++" stood for the relative fluorescence between 50% and 75%, "+" stood for the relative fluorescence above 10% and below 50%, "-" mean that the relative fluorescence was below 10%.

[f.] "N" means the lag time and T_{50} are too long for calculation.

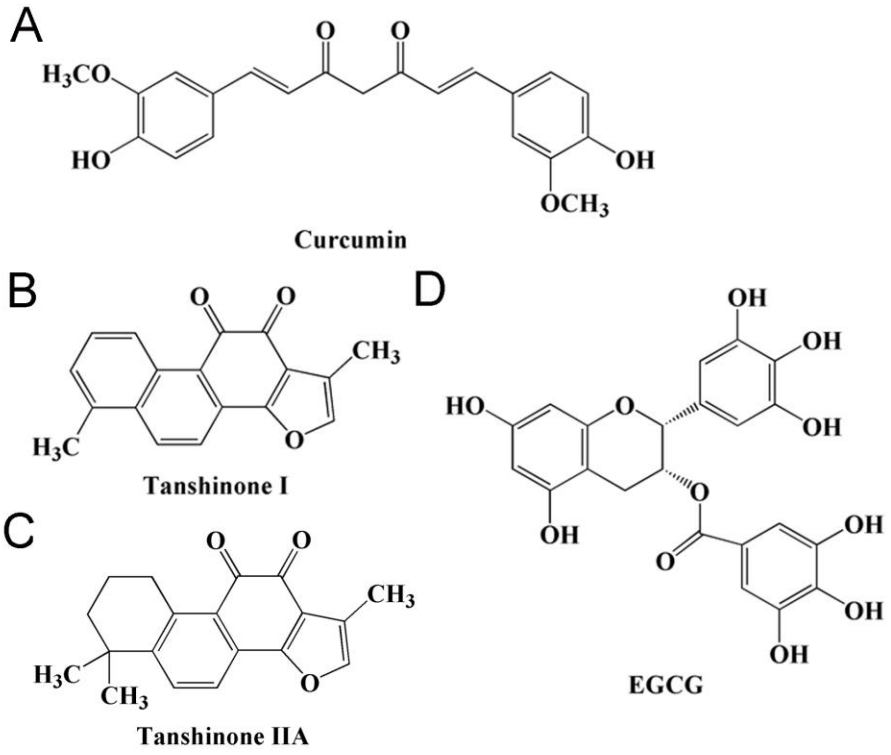

Figure 1

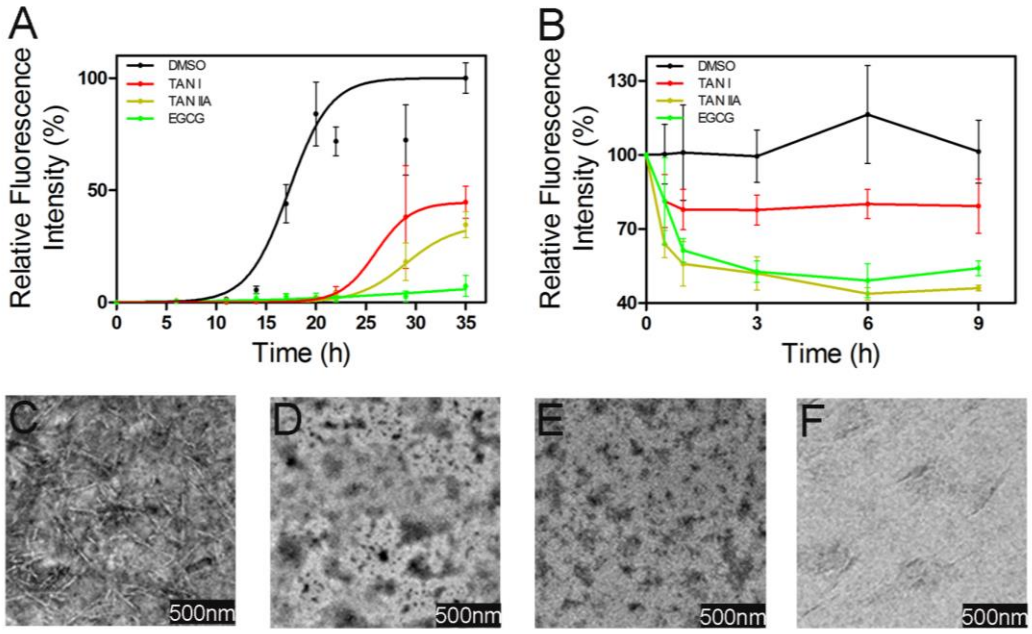

Figure 2

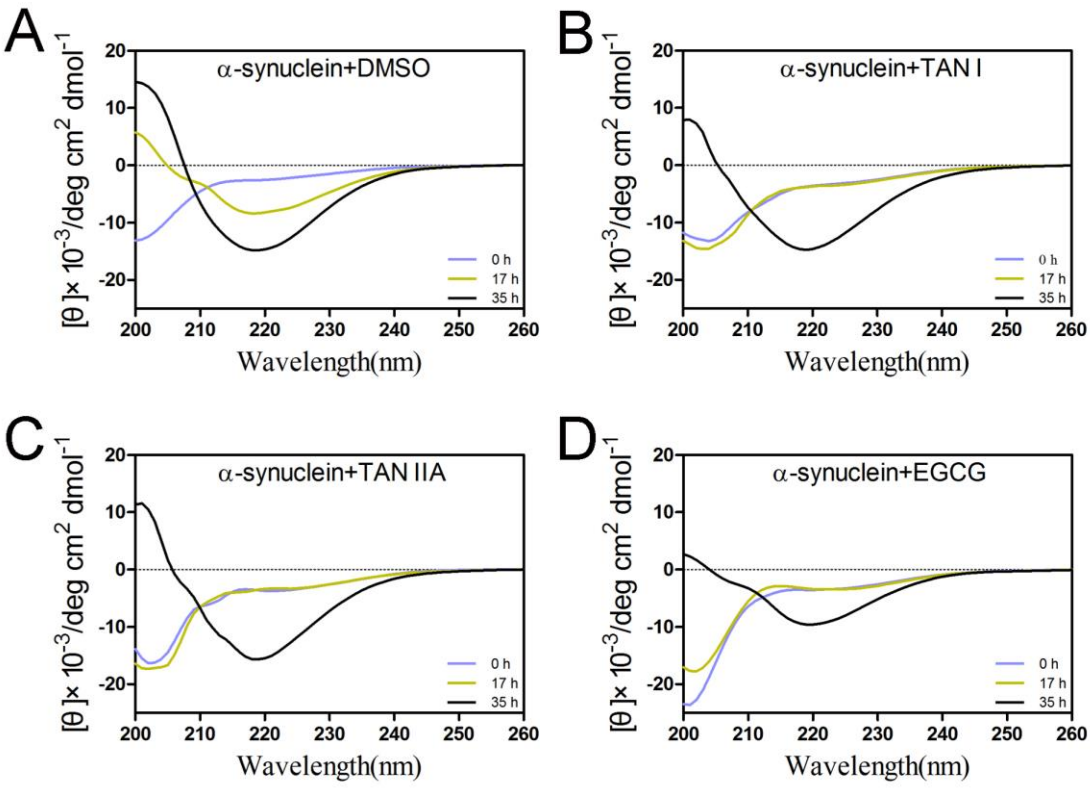

Figure 3

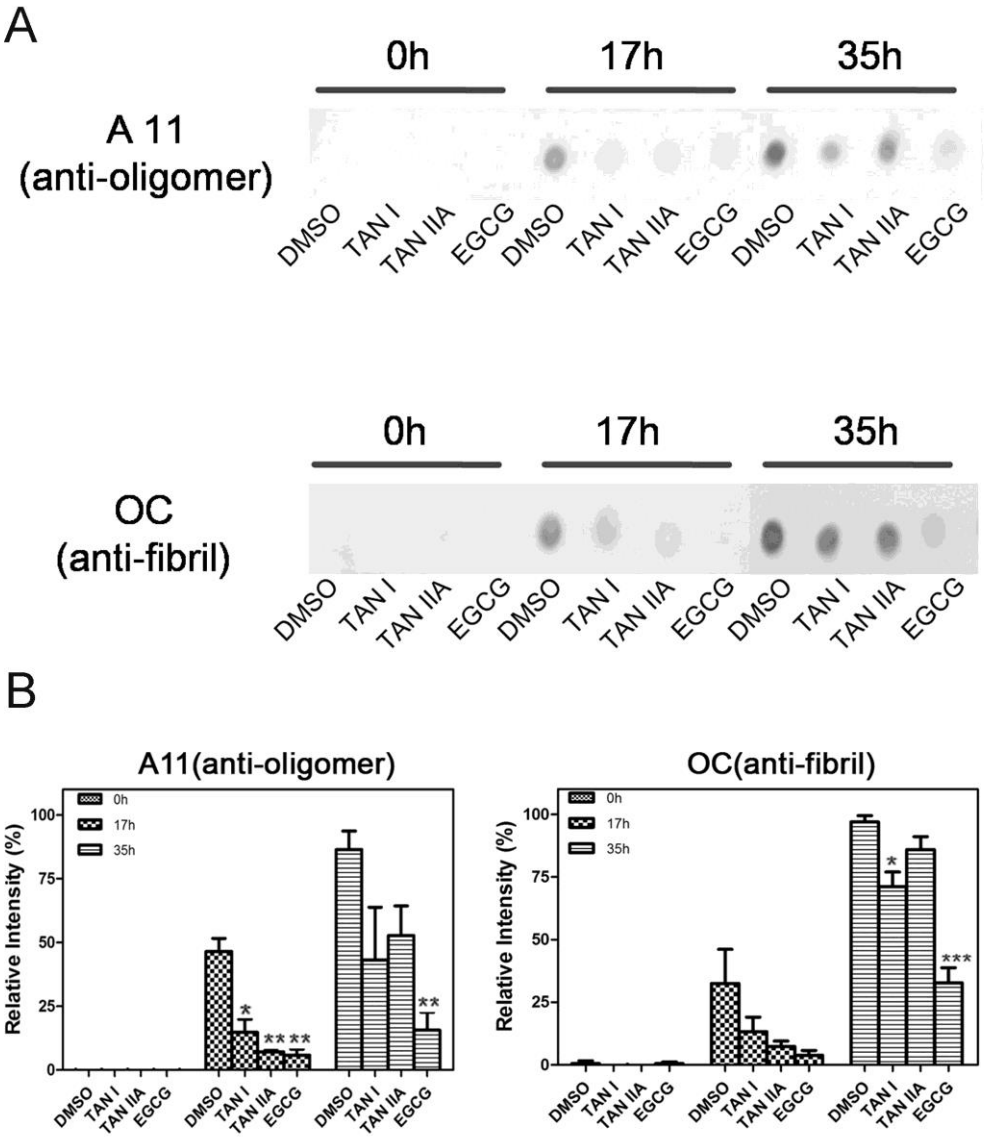

Figure 4

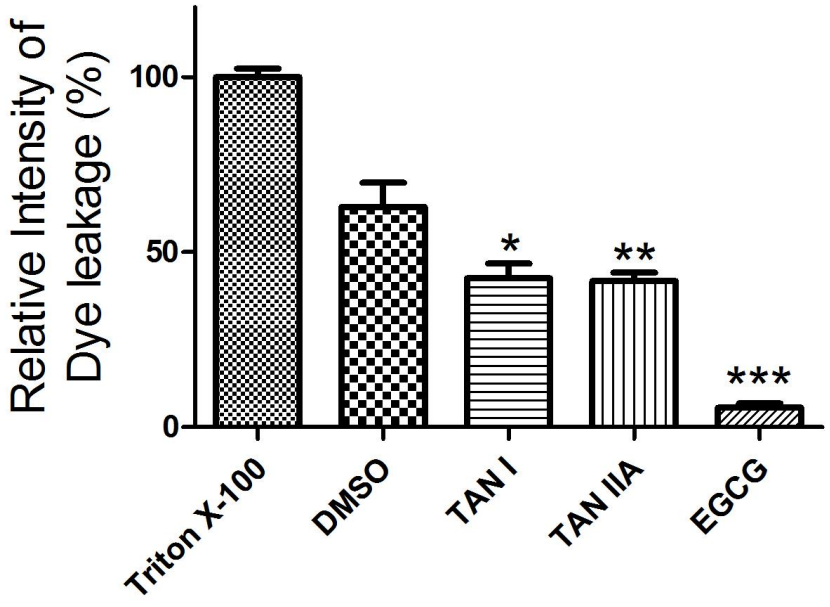

Figure 5

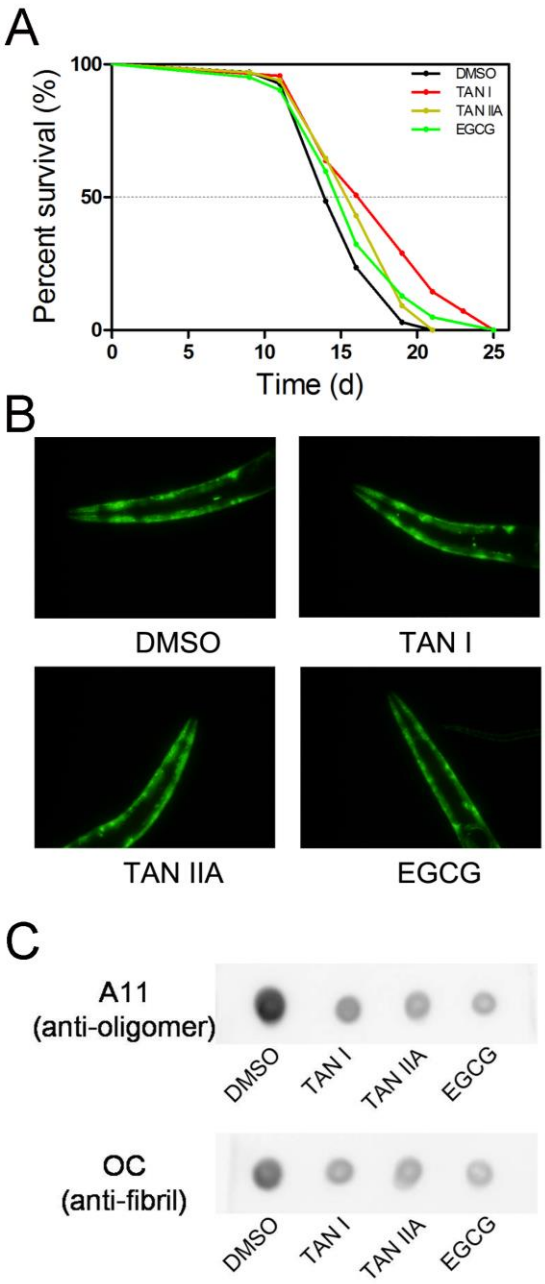

Figure 6

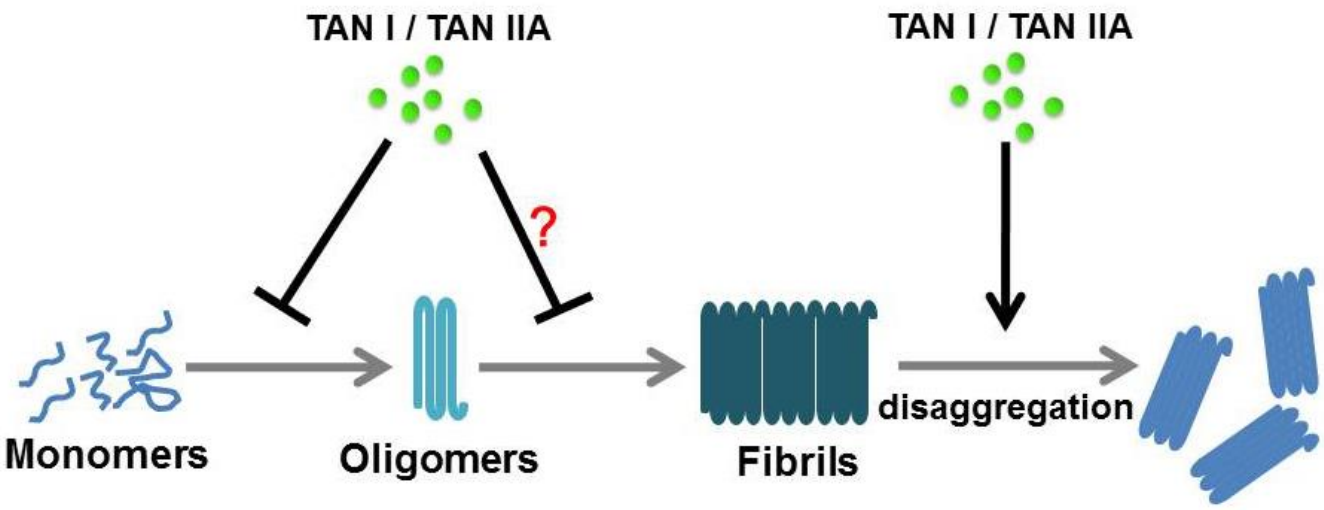

Figure 7

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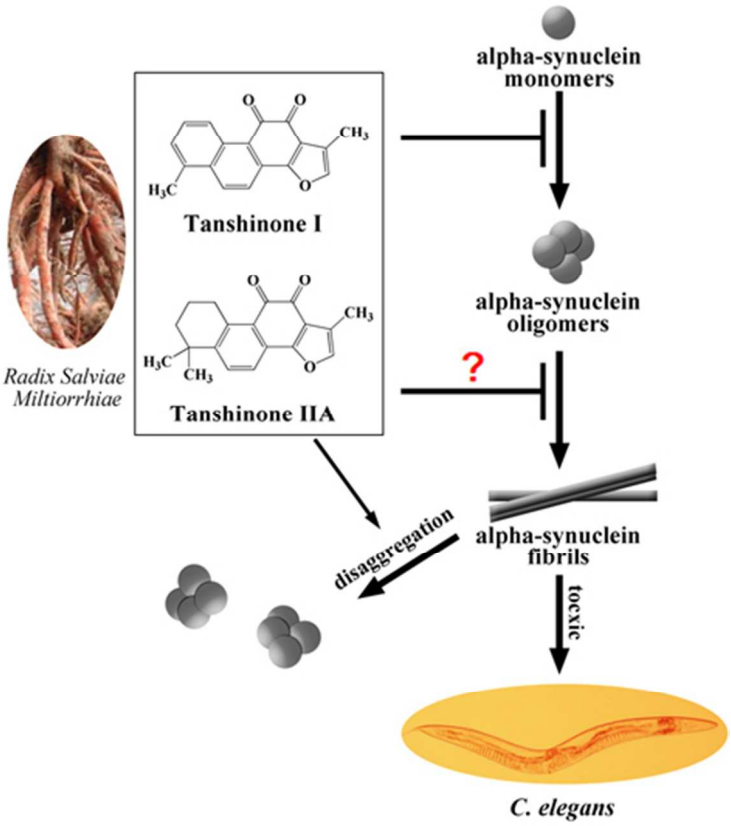

Bioactive tanshinone compounds inhibit the aggregation of α-synuclein and extended the life span of *C. elegans* model of Parkinson's disease.