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Inhibition and disaggregation of α-synuclein oligomers by natural polyphenolic compounds

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ABSTRACT

Aggregation of alpha-synuclein (αS) into oligomers is critically involved in the pathogenesis of Parkinson's disease (PD). Using confocal single-molecule fluorescence spectroscopy, we have studied the effects of 14 naturally-occurring polyphenolic compounds and black tea extract on αS oligomer formation. We found that a selected group of polyphenols exhibited potent dose-dependent inhibitory activity on αS aggregation. Moreover, they were also capable of robustly disaggregating pre-formed αS oligomers. Based upon structure–activity analysis, we propose that the key molecular scaffold most effective in inhibiting and destabilizing self-assembly by αS requires: (i) aromatic elements for binding to the αS monomer/oligomer and (ii) vicinal hydroxyl groups present on a single phenyl ring. These findings may guide the design of novel therapeutic drugs in PD.

Structured summary of protein interactions:
Alpha-synuclein binds to **Alpha-synuclein** by biophysical (View Interaction 1, 2)

1. Introduction

Convergent genetic, biochemical and animal studies over the past decade strongly suggest that the aggregation of the 140-residue pre-synaptic alpha-synuclein (αS) protein plays a fundamental role in the etiology and pathogenesis of Parkinson's disease (PD) and related disorders [1]. These disorders are in fact collectively known as "α-synucleinopathies", with PD being the most common movement disorder [2]. Motor symptoms in PD reflect the severe degeneration of dopaminergic neurons in the *substantia nigra pars compacta*. The critical etiological link between αS aggregation and toxicity to dopaminergic neurons was recently demonstrated in vivo in a transgenic *Drosophila* model of the disorder [3]. Pathogenesis of PD thus appears to be linked to conditions that increase the propensity for αS to aggregate and form fibrils [4]; among these: an increase in the intracellular concentration of αS (e.g. by increased gene copy number) [5,6], missense mutations [7], oxidative modifications [8,9], phosphorylation [10], the presence of metal ions (e.g. Fe^{3+}) [11], and interaction with phospholipid membranes [12].

A lot of attention is therefore being directed at the development of molecular inhibitors of αS aggregation for the prevention and treatment of PD [13]. Indeed, an important class of compounds found to be protective against αS fibrillation is that of the polyphenols, which are important beneficial constituents in human diet and medicinal plants [14]. Polyphenols are abundant in a wide range of fruits, vegetables and beverages, including tea, red wine, apples, berries, and strawberries. The daily average polyphenol intake is difficult to estimate, but is supposed to average 200–500 mg per day [14]. Polyphenolic compounds identified with anti-fibrillogenic and fibril-destabilizing effects for αS include baicalein (Baic) [15], epigallocatechin gallate (EGCG) [16,17], rosmarinic acid (RA) [18], tannic acid (TA), nordihydroguaiaretic acid (NDGA), curcumin, myricetin (Myr), kaempferol, catechin, and epi-catechin [19]. Additional studies showed that several polyphenols inhibited αS filament assembly by forming soluble, non-cytotoxic, oligomeric complexes with the αS protein [16,20–22]. Even more recent findings suggest that EGCG is a powerful remodelling agent of

Abbreviations: αS, alpha-synuclein; Api, apigenin; Baic, baicalein; BTE, black tea extract; Desf, desferrioxamine; DMSO, dimethylsulfoxide; EGCG, (−)-epigallocatechin gallate; FCS, fluorescence correlation spectroscopy; FIDA, fluorescence-intensity distribution analysis; Gen, genistein; Gink, ginkgolide B; Mor, morin; Myr, myricetin; N-acetylC, *N*-acetyl-L-cysteine; NDGA, nordihydroguaiaretic acid; PD, Parkinson's disease; Purp, purpurogallin trimethyl ether; Quer, quercetin; RA, rosmarinic acid; Resv, resveratrol; Scut, scutellarein; SIFT, scanning for intensely fluorescent targets; TA, tannic acid; VitC, vitamin C

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Table 1
Description and physiochemical profiles of the 15 phenolic compounds used in this study.

Name of compound	Class/ description	MW	*c* Log *P*[*]	Sources
Apigenin (Api)	Flavone	270.2	2.00	Chamomile, celery, parsley
Baicalein (Baic)	Flavone	270.2	2.69	Isolated from the roots of *Scutellaria baicalensis*
Black tea extract (BTE)	>80% theaflavins	–	–	Black tea
(−)-Epigallocatechin gallate (EGCG)	Flavanol	458.4	2.64	White tea, green tea, black tea, grapes, wine, apple juice, cocoa, lentils, black-eyed peas
Genistein (Gen)	Isoflavone	270.2	1.94	Soy, alfalfa sprouts, red clover, chickpeas, peanuts, other legumes
Ginkgolide B (Gink)	–	424.4	-2.07	Isolated from the leaves of *Ginkgo biloba*
Morin (Mor)	Flavonol	302.2	1.88	Isolated from *Maclura pomifera*, *Maclura tinctoria* and from leaves of *Psidium guajava*
Myricetin (Myr)	Flavonol	318.2	1.50	Grapes, walnuts
Nordihydroguaiaretic acid (NDGA)	Stilbene	302.4	4.34	Isolated from the leaves of *Larrea tridentata*
Purpurogallin trimethyl ether (Purp)	Lignin	262.3	N/A	–
Quercetin (Quer)	Flavonol	338.3	1.80	Red/yellow onions, tea, wine, apples, cranberries, buckwheat, beans
Resveratrol (Resv)	Stilbene	228.3	3.11	Grape skins and seeds, wine, nuts, peanuts
Rosmarinic acid (RA)	Phenolic acid	360.3	1.64	lemon balm, rosemary, oregano, sage, thyme, peppermint
Scutellarein (Scut)	Flavone	286.2	2.39	Isolated from the roots of *Scutellaria lateriflora*
Tannic acid (TA)	Phenolic acid	1701.2	7.07	Nettles, tea, berries

The 15 phenolic compounds used in this study are listed together with their molecular weight (MW), calculated partition coefficient (*c* Log *P*) and sources. Refer to Fig. 4 for the chemical structures of the compounds.

[*] The calculated Log *P* value of a compound is the logarithm of its partition coefficient between *n*-octanol and water, and is therefore a measure of the molecule's hydrophobicity. Hydrophobicity influences drug absorption, bioavailability, metabolism, and toxicity of the molecule. Most drugs on the market have a *c* Log *P* of 0–5.0.

mature αS-synuclein fibrils, converting them into non-toxic, smaller, amorphous aggregates [17].

In this study, we wanted to make use of the structural diversity of natural polyphenols to define key molecular scaffolds most effective in inhibiting oligomer formation by alpha-synuclein and/or disaggregating pre-formed oligomers. Fourteen polyphenolic compounds and black tea extract (BTE) (Table 1) were systematically tested using confocal single-particle fluorescence techniques. In recent years, fluorescence correlation spectroscopy (FCS), fluorescence-intensity distribution analysis (FIDA) and scanning for intensely fluorescent targets (SIFT) have been recognised as powerful tools for highly sensitive analysis of aggregation processes in neurodegenerative diseases, including detection and characterization of synuclein oligomers [23–25]. Importantly, this technique allowed us to use nanomolar and low micromolar concentrations of αS and compounds. The fact that a diverse group of polyphenols were analyzed enabled us to gain insight into particularly important structure–activity relationships. We also looked at the controversial issue of whether the anti-amyloid effects of polyphenols are related, or not, to their antioxidant or metal-ion chelation activities.

2. Materials and methods

2.1. Expression, purification and labeling of recombinant alpha-synuclein

Expression and purification of recombinant αS was performed as described previously by Kostka et al. [25]. Briefly, pET-5a/α-Synuclein (136TAT) plasmid (wt-plasmid a kind gift by Philipp Kahle; the 136-TAC/TAT-mutation was performed by Matthias Habeck) was used to transform *Escherichia coli* BL21(DE3)pLys (Novagen, Madison, WI, USA), and expression was induced with isopropyl β-D-thiogalactopyranose (IPTG) for 4 h. Cells were harvested, resuspended in 20 mM Tris and 25 mM NaCl, pH 8.0 and lysed by boiling at 95 °C for 30 min in a water bath. The lysate was centrifuged at 17 000 g and 4 °C for 15 min and the supernatant was filtered and loaded into a HiTrap Q HP column (5 ml, GE Healthcare). After elution, it was ultra-centrifuged at 40 000 g and 4 °C for 45 min and the supernatant concentrated using Vivaspin 2 columns (MWCO 3 kDa). Afterwards the synuclein-fraction was gel filtrated over a Superdex 75 prep grade column (25 ml, GE Healthcare) with 20 mM Tris and 150 mM NaCl, pH 7.0 as running buffer to separate monomeric and oligomeric α-synuclein by size. The protein concentration was determined in a standard Bicinchoninic Acid (BCA)-solution assay. Aliquoted protein was stored at −80 °C after freezing in liquid nitrogen.

2.2. Fluorescent labeling of αS

Protein labeling by fluorophores was performed as previously described [25]. Briefly, αS was labeled with Alexa Fluor-488-O-succinimidylester (“green”) and Alexa Fluor-647-O-succinimidylester (“red”) (Invitrogen), respectively. Unbound fluorophores were separated by two filtration steps in PD10 columns (Sephadex G25; Amersham Biosciences). Quality control of labeled αS was performed by FCS measurements. To avoid repeated freeze/thawing, the purified recombinant fluorescently labeled monomeric αS stocks were divided into smaller aliquots of 5–10 μl, and stored at −80 °C.

2.3. Confocal single particle analysis

FIDA and SIFT measurements were carried out on an Insight Reader (Evotec-Technologies, Hamburg, Germany) with dual color excitation at 488 and 633 nm. Excitation power was 200 W at 488 nm and 300 μW at 633 nm. All measurements were performed at room temperature. The fluorescence signal was analyzed by FIDA using FCSPP evaluation software version 2.0 (Evotec-Technologies). For FIDA and SIFT analysis, fluorescence from the two different fluorophores was recorded simultaneously with two single-photon detectors; photons were summed over time intervals of constant length (bins) using a bin length of 40 μs. The frequency of specific combinations of “green” and “red” photon counts was recorded in two-dimensional intensity distribution histograms. Evaluation of SIFT data in two-dimensional intensity distribution histograms was performed by summing up the numbers of high intensity bins each of 18 equally sized sectors using a 2D-SIFT software module (Evotec-Technologies). For threshold setting, non-aggregated reference samples were used. This single molecule detection technology allows highly sensitive analysis of protein aggregation by changes in the brightness of individual particles. FIDA is able to distinguish between differently bright species and, as such, gives indirect information about particle sizes.

2.4. Aggregation of alpha-synuclein

A fivefold stock solution of fluorescently labeled αS was prepared by mixing αS labeled with Alexa-488 and αS labeled with Alexa-647. The concentrations of αS-Alexa-488 and αS-Alexa-647 were adjusted to approximately 10 molecules per focal volume and 15 molecules per focal volume, respectively. Aggregation was started by diluting the stock solution in 50 mM Tris–HCl buffer (pH 7.0) containing final 1% DMSO and 20–30 nM labeled αS in a total volume of 20 µl. $FeCl_3$ was used at a final concentration of 10 µM. The aggregation reaction was typically complete in 10 min, but the plate was left for another 20 min at room temperature to minimize variance in measurements. All experiments were performed in 96-well-plates with a cover slide bottom.

2.5. Compound testing

All polyphenol compounds and black tea extract (Table 1) were obtained from Sigma–Aldrich, Germany; except for scutellarein (Scut) which was purchased from Pharmasciences Laboratories, France. *N*-Acetyl-L-cysteine (N-acetylC), Vitamin C (VitC) and Desferrioxamine (Desf) were also obtained from Sigma. Compounds were dissolved in dimethylsulfoxide (DMSO) and kept as 10 mM stock solutions at −20 °C to maintain maximal stability. During the experiments they were used immediately after thawing and kept away from light. Compound screening was typically done at 10 µM (3 µg/ml for BTE) in a total assay volume of 20 µl. We initially confirmed that all compounds did not autofluoresce or quench at the diluted concentration (data not shown). To assay for inhibitory activity, compounds were added to monomeric αS in 50 mM Tris–HCl buffer (pH 7.0), before starting aggregation with 1% DMSO ± 10 µM $FeCl_3$. To test for disaggregating activity, the compounds were added to pre-formed oligomeric αS. In both cases, after initiating aggregation the plate was left for ~30 min at room temperature. The average SIFT signal from samples (in triplicate) was expressed as a percentage of the average signal from αS aggregation control wells. SIFT signals were generated by summing up the photon counts from the "green" and "red" channels for all bins above cut-off (threshold) level.

2.6. Statistical analysis

Results were expressed as the means and the standard deviation (S.D.) values, with *n* as the number of experiments. Calculation of IC_{50} values from dose-dependency curves was performed using an on-line software facility (http://bsmdb.tmd.ac.jp:3000/cbdb/ic50). Differences between means were determined by unpaired Student's *t* test. In all analyses, the null hypothesis was rejected at the 0.05 level.

3. Results and discussion

3.1. Biophysical characterization of the αS aggregates

Using three independent single particle-based methods, we recently described a robust in vitro multistep aggregation model for αS [25,26]. In this model, incubation of nanomolar quantities of αS with 1% DMSO for 10 min was sufficient for maximal conversion of αS monomers into small aggregates, termed 'type-I' oligomers (Fig. 1, *upper panels*). Cross-correlation analysis indicated a size of ~20 monomers for these oligomers [25]. Addition of 10 µM Fe^{3+} ions in the aggregation assay resulted in the formation of larger (>100meres) 'type-II' oligomers (Fig. 1, *upper panels*). AFM measurements agreed with the confocal single particle fluorescence results [25]. Both type-I and type-II oligomer species are on-

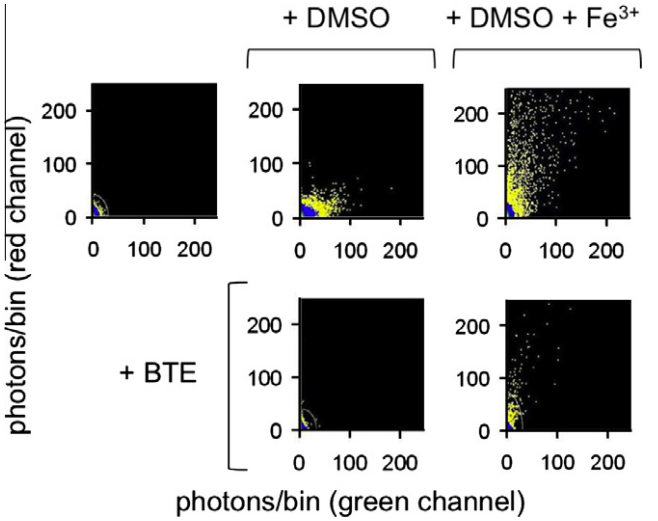

Fig. 1. *Upper panel*: SIFT two-dimensional intensity distribution histograms showing the induction of small type-I oligomers (using 1% DMSO) and larger type-II aggregates (using 1% DMSO and 10 µM Fe^{3+}) from αS monomers (control). *Lower panel*: SIFT two-dimensional intensity distribution histograms showing the inhibition of both types of oligomeric assemblies upon incubation with 3 µg/ml black tea extract (BTE).

pathway to amyloid fibrils, as indicated by Thioflavin T assays. The robust αS aggregation model thus characterized is ideally suited for application in screening of small-molecule compound libraries for aggregation inhibitors.

3.2. Select polyphenols and BTE potently inhibit formation of type-I and type-II αS oligomers

We first tested the ability of 13 polyphenols and black tea extract (Table 1) to antagonize the aggregation of alpha-synuclein into type-I oligomers (Fig. 2A). The degree of inhibition by the various compounds was wide-ranging, from very strong inhibition (e.g. BTE = 0.4% and Mor = 0.7%), to strong (e.g. Myr = 10%, Quer = 12%), moderate (e.g. RA = 45%, Resv = 47%), and relatively weak (e.g. Gen = 68.6%, Api = 65.1%); this feature would later prove useful in deducing structure–activity relationships. Nine compounds – namely, BTE (see also Fig. 1, *lower panels*), Mor, TA, Baic, NDGA, EGCG, Gink, Myr and Quer – blocked type-I αS oligomer formation to less than 20% of the control value, the latter representing aggregation in the presence of DMSO only. Dose-dependency curves were also performed on seven of these anti-aggregation compounds, using compound concentrations between 12.5 nM and 10 µM. The following IC_{50} values were obtained: TA, 61 nM; NDGA, 77 nM; EGCG, 0.79 µM; BTE, 0.81 µM; Baic, 2.03 µM; Myr, 3.57 µM; Mor, 4.24 µM (Fig. 7). Aggregation of αS occurs more strongly and results in larger type-II oligomers when ferric iron is added to the reaction mixture (Fig. 1). Thus, we selected the best ten compounds from the previous assay, and assessed their ability to antagonize αS aggregation in the presence of Fe^{3+} ions as well. A broad range of inhibition was again observed, with six compounds blocking type-II αS oligomer formation to lower than 20% of the control value (Fig. 2B): Baic (2.62 ± 0.6%), NDGA (7.96 ± 1.1%), Myr (10.12 ± 0.7%), EGCG (10.13 ± 1%), BTE (15.9 ± 4.4%), and TA (18.9 ± 1%). Hence, five polyphenols – namely, Baic, EGCG, Myr, NDGA and TA – as well as BTE could be selected as compounds that potently interfere with the assembly of alpha-synuclein into multimeric oligomers, with IC_{50} in the low micromolar range. Other studies have shown that polyphenols can induce oligomer formation. EGCG, for instance, promoted the folding of αS monomers into

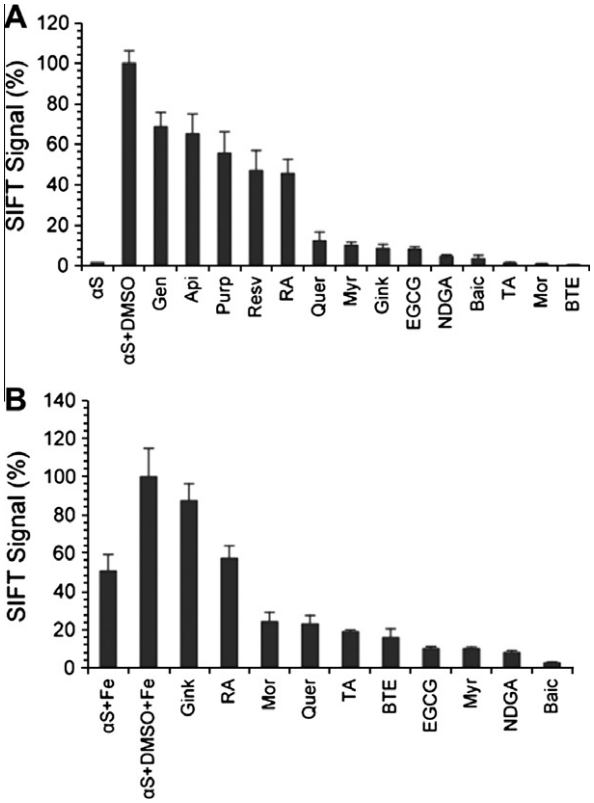

Fig. 2. Inhibition of αS aggregation by polyphenols and black tea extract. Polyphenols and BTE were tested for their ability to inhibit de novo aggregation of αS into type-I (A) or type-II (B) oligomers. Reaction mixtures containing monomeric αS and 1% DMSO in 50 mM Tris–HCl buffer (pH 7.0) (A), or 1% DMSO and 10 μM $FeCl_3$ in 50 mM Tris–HCl buffer (pH 7.0), were assayed by SIFT analysis following incubation at room temperature in the presence of 10 μM polyphenols and 3 μg/ml BTE. Average type-I oligomer formation induced by 1% DMSO alone (αS + DMSO) was regarded as 100% in (A); average type-II oligomer formation induced by 1% DMSO and 10 μM Fe^{3+} (αS + DMSO + Fe) was regarded as 100% in (B). Values represent means ± S.D. ($n \geqslant 3$).

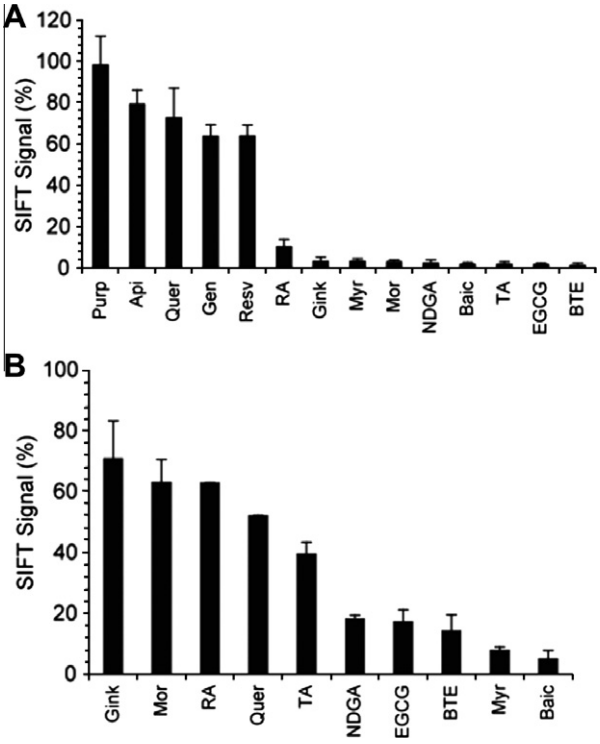

Fig. 3. Disaggregation of type-I (A) and type-II (B) αS oligomers by polyphenols and black tea extract. Complete aggregation of alpha-synuclein in each well was first confirmed by SIFT measurements (100% control), before adding polyphenols (10 μM) and BTE (3 μg/ml). SIFT analysis was conducted again after 30 min in the presence of compounds. Average type-I oligomer formation induced by 1% DMSO alone (αS + DMSO) was regarded as 100% in (A); average type-II oligomer formation induced by 1% DMSO and 10 μM Fe^{3+} (αS + DMSO + Fe) was regarded as 100% in (B). Values represent means ± S.D. ($n = 4$).

highly stable oligomers that were non-cytotoxic and off-pathway to fibrillogenesis [16]. Similarly, Baic induced the formation of, and stabilized, αS oligomers consequently preventing their fibrillization [21]. Nevertheless, very strong inhibition of αS oligomerization by the potent compounds was evident in our single-molecule approach.

3.3. Disaggregation of pre-formed αS oligomers by polyphenols and BTE

The 13 polyphenols and BTE listed in Table 1 (excluding scutellarein) were next tested for their ability to disaggregate preformed αS oligomeric structures, the latter induced either by DMSO alone (type-I; Fig. 3A), or with the inclusion of ferric iron (type-II; Fig. 3B). It was found that Baic, EGCG, Myr, NDGA and BTE significantly disrupted both types of oligomers to <20% of their initial aggregated state. We also carried out dose-dependency experiments (0.01 – 10 μM) to determine IC_{50} values for disaggregation potency: Baic, 0.85 μM; EGCG, 0.26 μM; Myr, 2.52 μM; NDGA, 20 nM; TA, 67 nM; BTE, 0.79 μM (Fig. 7). Interestingly, the two compounds having the lowest IC_{50} values for disaggregation, NDGA (IC_{50} = 20 nM) and EGCG (IC_{50} = 260 nM), show "linear" dose–response curves. This contrasts with the sigmoidal shapes of the other compound curves. An effect was observed even at the lowest concentrations of 10 nM for NDGA and 100 nM for EGCG, with a linear increase (on a logarithmic scale) to almost 100% effect at 10 μM. The implication is that disassembly of the αS aggregate structures occurred even at concentrations just above zero and increased with the concentration of the compound.

3.4. Overall compound potency correlates with number of vicinal hydroxyl groups on a phenyl ring

At this stage, we therefore concluded that Baic, EGCG, Myr, NDGA and BTE could be classified as being the best combined inhibitors and disaggregators of αS oligomers. On the other hand, compounds like purpurogallin trimethyl ether (Purp), Api, Gen and Resv, which were relatively weak inhibitors, were also poor disaggregators of DMSO-induced synuclein oligomers. With regards to Fe-induced oligomers, Gink and RA were similarly weak in both inhibition and destabilization of larger type-II synuclein species. Thus, a pattern was emerging suggesting an important structure–function relationship: the ranking of overall compound potency (i.e. inhibition and disaggregation) in our aggregation model could be fairly accurately predicted based upon the number of hydroxyl groups present on a *single* phenyl ring, as follows: trihydroxy- > dihydroxy- > monohydroxy-phenyl ring (refer to Fig. 4 for grouping of compounds according to their chemical structures). The favouring of trihydroxyphenyl rings is made especially clear when comparing Baic and Api, the latter being much weaker in inhibiting or disaggregating type-I synuclein oligomers. Structurally, both have the same flavone structure and both possess a total of three –OH groups. The difference lies in that whilst Baic has all three –OH groups attached to the same benzene ring, Api has a dihydroxyphenyl at one end of the molecule and a hydroxyphenyl ring at the other end (Fig. 4). Therefore, since both compounds

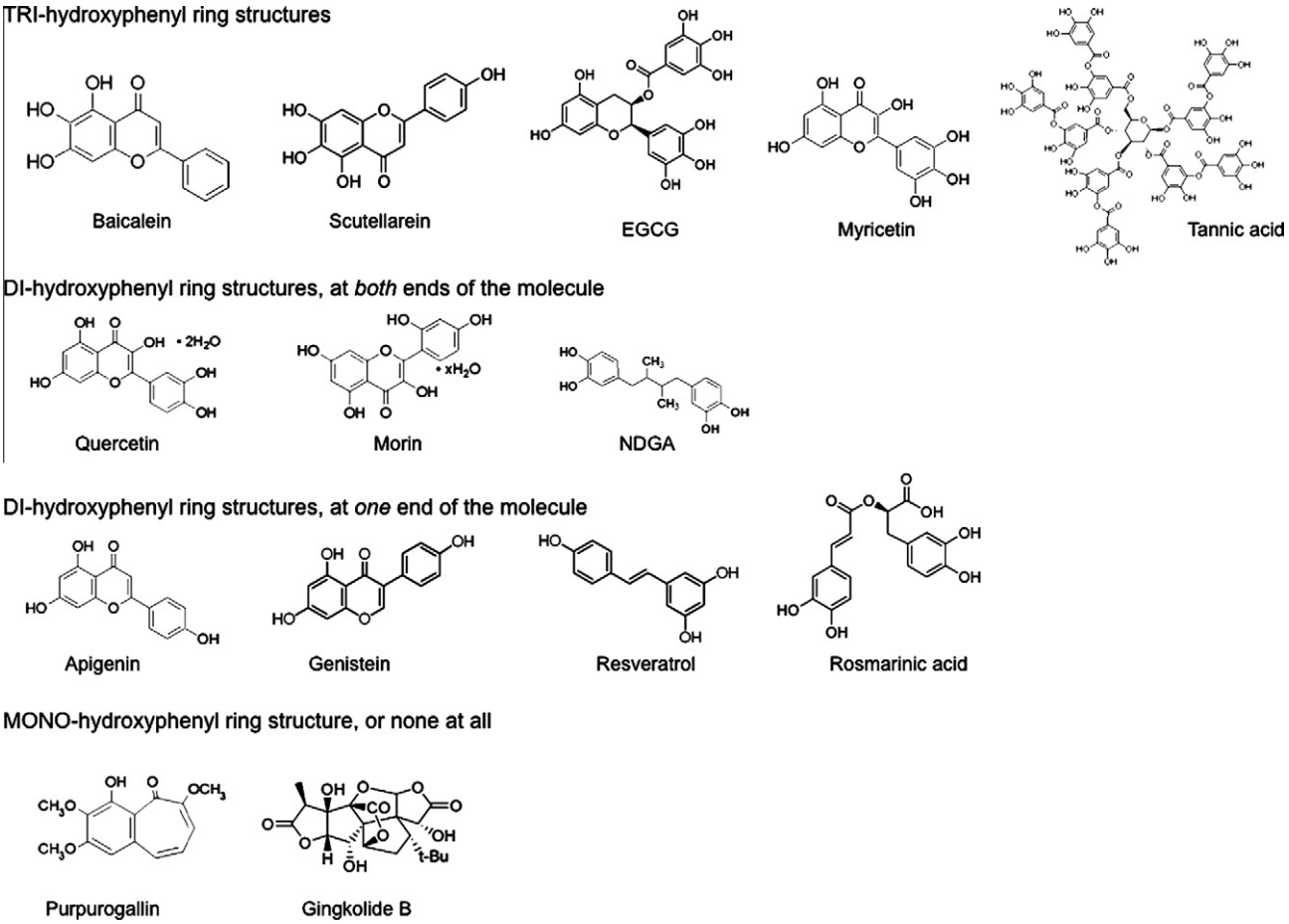

Fig. 4. Two-dimensional chemical structures of the polyphenolic compounds used in this study, grouped according to structure–activity data.

have a total of three hydroxyl groups it can be inferred that it is not the *total number* of –OH groups present in the molecule that is important for anti-aggregate activity, but rather the *distribution* of the –OH groups, most significantly the presence of three vicinal hydroxyl groups as in baicalein.

To test this hypothesis another polyphenol with a trihydroxyphenyl ring, Scutellarein (Scut; Fig. 4), was selected for direct comparison to Baic and Api. Indeed, in agreement with the structure–activity hypothesis outlined above, Scut behaved in a similar way to Baic with regards to both inhibition of type-II synuclein oligomers and disaggregation of pre-formed oligomers, whereas Api was much less effective (Fig. 5). One can point out the apparent exception of NDGA to this insight into structure–activity relationship. NDGA, which does not have three adjacent –OH groups, is nonetheless one of the most effective compounds overall. However, NDGA has a very symmetrical structure with dihydroxyphenyl rings at *both* ends of the molecule; perhaps the structural symmetry confers an advantage with regards to possible orientations when binding to the αS molecules. Our data is in agreement with a related finding that dopamine and derivative compounds that have vicinal dihydroxy groups were shown to be effective inhibitors of αS fibrillization [9].

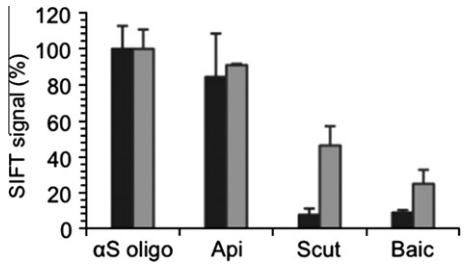

Fig. 5. The flavones apigenin (Api), scutellarein (Scut) and baicalein (Baic) inhibit formation of, and disaggregate, type-II αS oligomers. Reaction mixtures containing αS and 10 μM polyphenol in 50 mM Tris–HCl buffer (pH 7.0) were assayed by SIFT analysis following incubation at room temperature, thus determining the inhibitory activity of the compounds (black). In the disaggregation experiments, the polyphenols were added to pre-formed type-II oligomers (gray). Average type-II oligomer formation induced by 1% DMSO and 10 μM Fe^{3+} was regarded as 100% (αS oligo). Values represent means ± S.D. (n = 2).

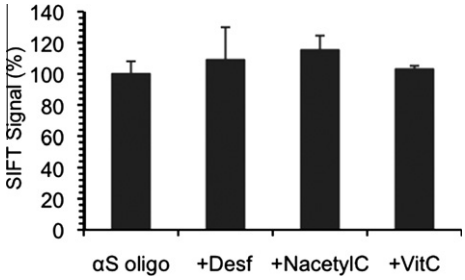

Fig. 6. The iron chelator desferrrioxamine (Desf), and the antioxidants *N*-acetylcysteine (N-acetylC) and vitamin C (VitC) do not affect αS aggregation into type-II oligomers. Reaction mixtures containing monomeric αS, 1% DMSO and 10 μM $FeCl_3$ in 50 mM Tris–HCl buffer (pH 7.0) were assayed by SIFT analysis following incubation at room temperature in the presence of 100 μM compounds. Average type-II oligomer formation induced by 1% DMSO and 10 M μFe^{3+} was regarded as 100% (αS oligo). Values represent means ± S.D. (n = 2).

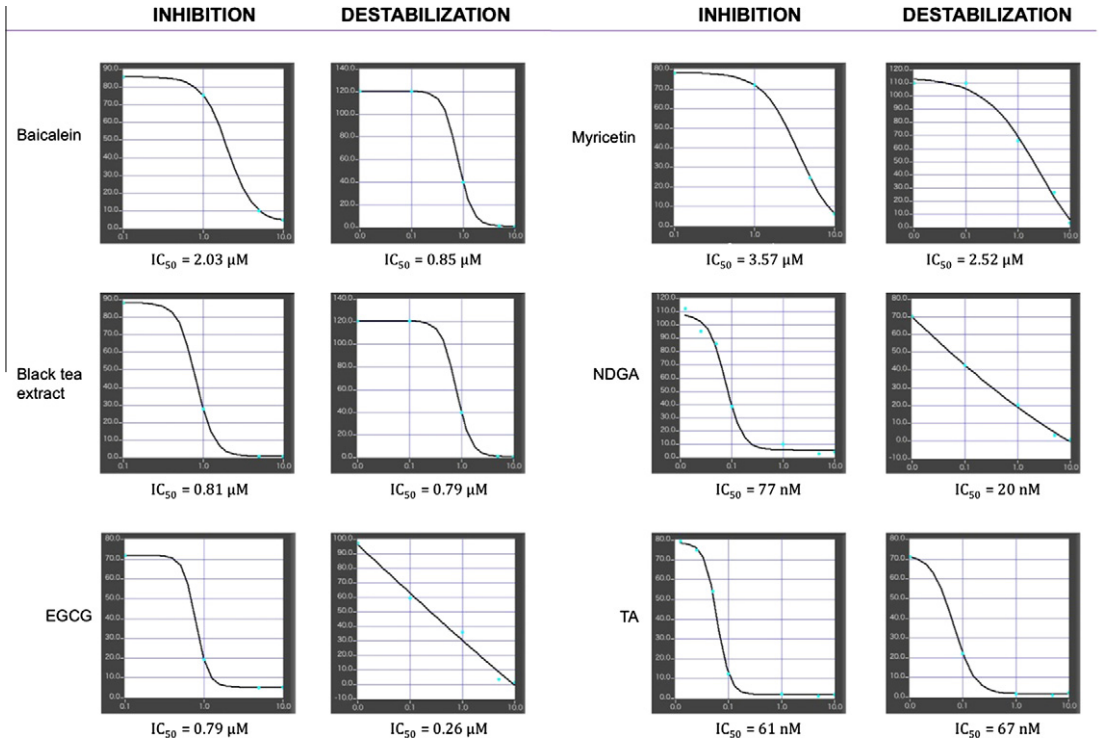

Fig. 7. Dose-dependent inhibition and disaggregation of αS oligomer formation, with determination of IC_{50} values. The reaction mixtures containing monomeric αS, 1% DMSO in 50 mM Tris–HCl buffer (pH 7.0), and 0.0125, 0.025, 0.05, 0.01, 0.1, 1, 5 or 10 μM compound were incubated at room temperature for 10 min. Average oligomer formation induced by DMSO, and without compounds, was regarded as 100%. Each point represents the mean of three independent experiments. The graphs and calculated IC_{50} values were obtained as described in Section 2.

3.5. Anti-aggregation potency is not dependent upon antioxidant and iron chelation mechanisms

An issue we wanted to address was whether the well-known antioxidant, and even metal-ion chelating, activities of polyphenolic compounds could be contributing to their observed anti-aggregation effects. We found no detectable inhibitory activity by the classical antioxidants N-acetylC (100 μM) and VitC (100 μM), or by the powerful iron chelator Desf (100 μM) on αS aggregation induced by DMSO and Fe^{3+} ions (Fig. 6). Similarly, disassembly of preformed type-II oligomers was not evident by 100 μM N-acetylC (173 ± 27%) or VitC (100 ± 15%); rather, N-acetylC appeared to actually enhance the aggregation. In conclusion, it appears unlikely that antioxidant or metal-chelating mechanisms are relevant for the antagonistic effects of polyphenols (all used at only 10 μM) on αS oligomerization. Moreover, there is no trend between our compound classification of anti-amyloid effects and published data on the anti-oxidant potencies of the studied phenols [27,28].

4. Conclusions

Using confocal single-molecule fluorescence spectroscopy, we have studied the effects of 14 phenolic compounds and black tea extract specifically on αS oligomerization induced by organic solvent. We establish that a select group of small-molecule polyphenols having three vicinal hydroxyl groups (Baic, Scut, Myr, EGCG), together with NDGA and black tea extract, constitute a set of compounds that can potently hinder αS aggregation into oligomers as well as disaggregate pre-formed αS oligomers, with IC_{50} in the submicromolar (<1 μM) range. The physiological concentration of αS is estimated to be ~1 μM in normal human brain and ~70 pM in cerebrospinal fluid [29]. Similarly, it is not expected that polyphenols or their metabolites can attain levels in the brain higher than 10 μM [30]. For these reasons, we believe that the conclusions from our study, as they stand, are already potentially relevant to the in vivo scenario. Moreover, by structure–activity analysis, we propose that the physicochemical profiles of potent compounds require: (i) aromatic recognition elements that would allow non-covalent binding to the amyloidogenic core of the αS monomer/oligomer [31] and (ii) hydroxyl groups (especially the presence of three > two > one –OH groups on the *same* ring structure) that would hinder the progress of the self-assembly process and/or destabilize its structure. For instance, molecular dynamics simulations recently showed that Myr destabilized ordered amyloid oligomers by weakening interstrand hydrogen bonds [32]. Notwithstanding, it cannot be excluded that the polyphenols can also bind to other sites of the αS protein. For example, Zhou et al. [33] have suggested that the polyphenol DOPAC (3,4-dihydroxyphenylacetic acid), a product of dopamine metabolism, inhibits fibrillation of αS by binding non-covalently to the N-terminal region.

Regarding the general usefulness of polyphenolic compounds in terms of drug development, it has to be first pointed out that polyphenols commonly display reduced oral bioavailability, increased metabolic turnover, and lower permeability through the blood–brain barrier [34]. The maintenance of a high concentration in plasma normally requires repeated ingestion of the polyphenols over time, whilst the biological activities of the metabolites may differ from the parent compounds [35]. Notwithstanding, in a large epidemiological study conducted in Asia, black tea showed an inverse association with Parkinson's disease risk that was not confounded by other factors, like total caffeine intake or tobacco smoking [36]. Oral administration of the phenolic compounds myricetin and NDGA, both of which were found to have potent activity in our assays, was successful in modulating amyloid aggregation pathways

in vivo and prevent the development of AD neuropathology in mice [37]. Another potent polyphenol singled out in our study, EGCG, has been administered orally in drinking water to AD transgenic mice resulting in a reduced plaque burden and cognitive benefit [38]. Hence, it cannot be excluded a priori that dietary supplementation with polyphenols provides an effective treatment modality for neurodegenerative disorders of the amyloid type. Moreover, systemic oral bioavailability of EGCG in rats can be increased more than twofold by formation and administration of nanolipidic EGCG particles [39]. Such strategies allow EGCG, and possibly other polyphenols, to achieve therapeutically effective concentrations in clinical settings.

Certainly, the conclusions from our study, together with others from previous reports, put forward a strong case for testing the efficacy of a select group of lead polyphenols in transgenic mouse models of PD. Thus, baicalein, scutellarein, myricetin, EGCG, NDGA and black tea extract are all valid candidates for further assessment with regard to inhibition of αS oligomer formation in vivo, the latter representing the most likely toxic molecular species of αS [40]. Finally, detailed investigations into the structure–activity relationships of natural products outlined above may guide the design of novel therapeutic drugs in Parkinson's disease which possess enhanced properties in vivo (e.g. ability to penetrate the blood–brain barrier), but which retain the bioactivity characteristic of the natural product scaffold.

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References

[1] Fink, A.L. (2006) The aggregation and fibrillation of alpha-synuclein. Acc. Chem. Res. 39, 628–634.
[2] Lees, A.J., Hardy, J. and Revesz, T. (2009) Parkinson's disease. Lancet 373, 2055–2066.
[3] Periquet, M., Fulga, T., Myllykangas, L., Schlossmacher, M.G. and Feany, M.B. (2007) Aggregated alpha-synuclein mediates dopaminergic neurotoxicity in vivo. J. Neurosci. 27, 3338–3346.
[4] Lundvig, D., Lindersson, E. and Jensen, P.H. (2005) Pathogenic effects of alpha-synuclein aggregation. Brain Res. Mol. Brain Res. 134, 3–17.
[5] Farrer, M., Kachergus, J., Forno, L., Lincoln, S., Wang, D.S., Hulihan, M., Maraganore, D., Gwinn-Hardy, K., Wszolek, Z., Dickson, D. and Langston, J.W. (2004) Comparison of kindreds with Parkinsonism and alpha-synuclein genomic multiplications. Ann. Neurol. 55, 174–179.
[6] Uversky, V.N., Cooper, M., Bower, K.S., Li, J. and Fink, A.L. (2002) Accelerated alpha-synuclein fibrillation in crowded milieu. FEBS Lett. 515, 99–103.
[7] Conway, K.A., Harper, J.D. and Lansbury, P.T. (2000) Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. Biochemistry 39, 2552–2563.
[8] Giasson, B.I., Duda, J.E., Murray, I.V., Chen, Q., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q. and Lee, V.M. (2000) Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. Science 290, 985–989.
[9] Conway, K.A., Rochet, J.C., Bieganski, R.M. and Lansbury, P.T. (2001) Kinetic stabilization of the alpha-synuclein protofibril by a dopamine–alpha-synuclein adduct. Science 294, 1346–1349.
[10] Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M.S., Shen, J., Takio, K. and Iwatsubo, T. (2002) Alpha-synuclein is phosphorylated in synucleinopathy lesions. Nat. Cell Biol. 4, 160–164.
[11] Galvin, J.E., Giasson, B., Hurtig, H.I., Lee, V.M. and Trojanowski, J.Q. (2000) Neurodegeneration with brain iron accumulation, type 1 is characterized by alpha-, beta-, and gamma-synuclein neuropathology. Am. J. Pathol. 157, 361–368.
[12] Sharon, R., Bar-Joseph, I., Frosch, M.P., Walsh, D.M., Hamilton, J.A. and Selkoe, D.J. (2003) The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. Neuron 37, 583–595.
[13] Amer, D.A., Irvine, G.B. and El-Agnaf, O.M. (2006) Inhibitors of alpha-synuclein oligomerization and toxicity: a future therapeutic strategy for Parkinson's disease and related disorders. Exp. Brain Res. 173, 223–233.
[14] Vassallo, N. (2008) Polyphenols and Health: New and Recent Advances, Nova Science Publishers, Inc., New York.
[15] Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F. and Fink, A.L. (2004) The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils. J. Biol. Chem. 279, 26846–26857.
[16] Ehrnhoefer, D.E., Bieschke, J., Boeddrich, A., Herbst, M., Masino, L., Lurz, R., Engemann, S., Pastore, A. and Wanker, E.E. (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. Nat. Struct. Biol. 15, 558–566.
[17] Bieschke, J., Russ, J., Friedrich, R.P., Ehrnhoefer, D.E., Wobst, H., Neugebauer, K. and Wanker, E.E. (2010) EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. Proc. Natl. Acad. Sci. USA 107, 7710–7715.
[18] Rao, J.N., Dua, V. and Ulmer, T.S. (2008) Characterization of alpha-synuclein interactions with selected aggregation-inhibiting small molecules. Biochemistry 47, 4651–4656.
[19] Ono, K. and Yamada, M. (2006) Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro. J. Neurochem. 97, 105–115.
[20] Masuda, M., Suzuki, N., Taniguchi, S., Oikawa, T., Nonaka, T., Iwatsubo, T., Hisanaga, S., Goedert, M. and Hasegawa, M. (2006) Small molecule inhibitors of alpha-synuclein filament assembly. Biochemistry 45, 6085–6094.
[21] Hong, D.P., Fink, A.L. and Uversky, V.N. (2008) Structural characteristics of alpha-synuclein oligomers stabilized by the flavonoid baicalein. J. Mol. Biol. 383, 214–223.
[22] Meng, X., Munishkina, L.A., Fink, A.L. and Uversky, V.N. (2009) Molecular mechanisms underlying the flavonoid-induced inhibition of alpha-synuclein fibrillation. Biochemistry 48, 8206–8224.
[23] Giese, A., Bader, B., Bieschke, J., Schaffar, G., Odoy, S., Kahle, P.J., Haass, C. and Kretzschmar, H. (2005) Single particle detection and characterization of synuclein co-aggregation. Biochem. Biophys. Res. Commun. 333, 1202–1210.
[24] Danzer, K.M., Haasen, D., Karow, A.R., Moussaud, S., Habeck, M., Giese, A., Kretzschmar, H., Hengerer, B. and Kostka, M. (2007) Different species of alpha-synuclein oligomers induce calcium influx and seeding. J. Neurosci. 27, 9220–9232.
[25] Kostka, M., Högen, T., Danzer, K.M., Levin, J., Habeck, M., Wirth, A., Wagner, R., Glabe, C.G., Finger, S., Heinzelmann, U., Garidel, P., Duan, W., Ross, C.A., Kretzschmar, H. and Giese, A. (2008) Single particle characterization of iron-induced pore-forming alpha-synuclein oligomers. J. Biol. Chem. 283, 10992–11003.
[26] Hillmer, A.S., Putcha, P., Levin, J., Högen, T., Hyman, B.T., Kretzschmar, H., McLean, P.J. and Giese, A. (2010) Converse modulation of toxic alpha-synuclein oligomers in living cells by *N*′-benzylidene-benzohydrazide derivates and ferric iron. Biochem. Biophys. Res. Commun. 391, 461–466.
[27] Aaby, K., Hvattum, E. and Skrede, G. (2004) Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: relationship to antioxidant activity. J. Agric. Food Chem. 52, 4595–4603.
[28] Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20, 933–956.
[29] Borghi, R., Marchese, R., Negro, A., Marinelli, L., Forloni, G., Zaccheo, D., Abbruzzese, G. and Tabaton, M. (2000) Full length alpha-synuclein is present in cerebrospinal fluid from Parkinson's disease and normal subjects. Neurosci. Lett. 287, 65–67.
[30] Baum, L. and Ng, A. (2004) Curcumin interaction with copper and iron suggests one possible mechanism of action in Alzheimer's disease animal models. J. Alzheimers Dis. 6, 367–377.
[31] Porat, Y., Abramowitz, A. and Gazit, E. (2006) Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. Chem. Biol. Drug Des. 67, 27–37.
[32] Berhanu, W.M. and Masunov, A.E. (2010) Natural polyphenols as inhibitors of amyloid aggregation. Molecular dynamics study of GNNQQNY heptapeptide decamer. Biophys. Chem. 149, 12–21.
[33] Zhou, W., Gallagher, A., Hong, D.P., Long, C., Fink, A.L. and Uversky, V.N. (2009) At low concentrations, 3,4-dihydroxyphenylacetic acid (DOPAC) binds non-covalently to alpha-synuclein and prevents its fibrillation. J. Mol. Biol. 388, 597–610.
[34] Youdim, K.A., Qaiser, M.Z., Begley, D.J., Rice-Evans, C.A. and Abbott, N.J. (2004) Flavonoid permeability across an in situ model of the blood–brain barrier. Free Radic. Biol. Med. 36, 592–604.
[35] Singh, M., Arseneault, M., Sanderson, T., Murthy, V. and Ramassamy, C. (2008) Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. J. Agric. Food Chem. 56, 4855–4873.
[36] Tan, L.C., Koh, W.P., Yuan, J.M., Wang, R., Au, W.L., Tan, J.H., Tan, E.K. and Yu, M.C. (2008) Differential effects of black versus green tea on risk of Parkinson's disease in the Singapore Chinese Health Study. Am. J. Epidemiol. 167, 553–560.
[37] Hamaguchi, T., Ono, K., Murase, A. and Yamada, M. (2009) Phenolic compounds prevent Alzheimer's pathology through different effects on the amyloid-beta aggregation pathway. Am. J. Pathol. 175, 2557–2565.
[38] Rezai-Zadeh, K., Arendash, G.W., Hou, H., Fernandez, F., Jensen, M., Runfeldt, M., Shytle, R.D. and Tan, J. (2008) Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice. Brain Res. 1214, 177–187.

[39] Smith, A., Giunta, B., Bickford, P.C., Fountain, M., Tan, J. and Shytle, R.D. (2010) Nanolipidic particles improve the bioavailability and alpha-secretase inducing ability of epigallocatechin-3-gallate (EGCG) for the treatment of Alzheimer's disease. Int. J. Pharm. 389, 207–212.
[40] Karpinar, D.P., Balija, M.B., Kügler, S., Opazo, F., Rezaei-Ghaleh, N., Wender, N., Kim, H.Y., Taschenberger, G., Falkenburger, B.H., Heise, H., Kumar, A., Riedel, D., Fichtner, L., Voigt, A., Braus, G.H., Giller, K., Becker, S., Herzig, A., Baldus, M., Jäckle, H., Eimer, S., Schulz, J.B., Griesinger, C. and Zweckstetter, M. (2009) Pre-fibrillar alpha-synuclein variants with impaired beta-structure increase neurotoxicity in Parkinson's disease models. EMBO J. 28, 3256–3268.