

Entacapone and Tolcapone, Two Catechol O-Methyltransferase Inhibitors, Block Fibril Formation of α -Synuclein and β -Amyloid and Protect against Amyloid-induced Toxicity^{*[5]}

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Parkinson disease (PD) is the second most common neurodegenerative disorder after Alzheimer disease (AD). There is considerable consensus that the increased production and/or aggregation of α -synuclein (α -syn) plays a central role in the pathogenesis of PD and related synucleinopathies. Current therapeutic strategies for treating PD offer mainly transient symptomatic relief and aim at the restitution of dopamine levels to counterbalance the loss of dopaminergic neurons. Therefore, the identification and development of drug-like molecules that block α -synuclein aggregation and prevent the loss of dopaminergic neurons are desperately needed to treat or slow the progression of PD. Here, we show that entacapone and tolcapone are potent inhibitors of α -syn and β -amyloid (A β) oligomerization and fibrillogenesis, and they also protect against extracellular toxicity induced by the aggregation of both proteins. Comparison of the anti-aggregation properties of entacapone and tolcapone with the effect of five other catechol-containing compounds, dopamine, pyrogallol, gallic acid, caffeic acid, and quercetin on the oligomerization and fibrillization of α -syn and A β , demonstrate that the catechol moiety is essential for the anti-amyloidogenic activity. Our findings present the first characterization of the anti-amyloidogenic properties of tolcapone and entacapone against both α -synuclein and A β 42 and highlight the potential of this class of nitro-catechol compounds as anti-amyloidogenic agents. Their inhibitory properties, mode of action, and structural properties suggest that they constitute promising lead compounds for further optimization.

Parkinson disease (PD)² is the second most common neurodegenerative disorder after Alzheimer disease (AD), affecting

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² The abbreviations used are: PD, Parkinson disease; A β 42, amyloid β -(1–42); AD, Alzheimer disease; CA, caffeic acid; E, entacapone; GA, gallic acid;

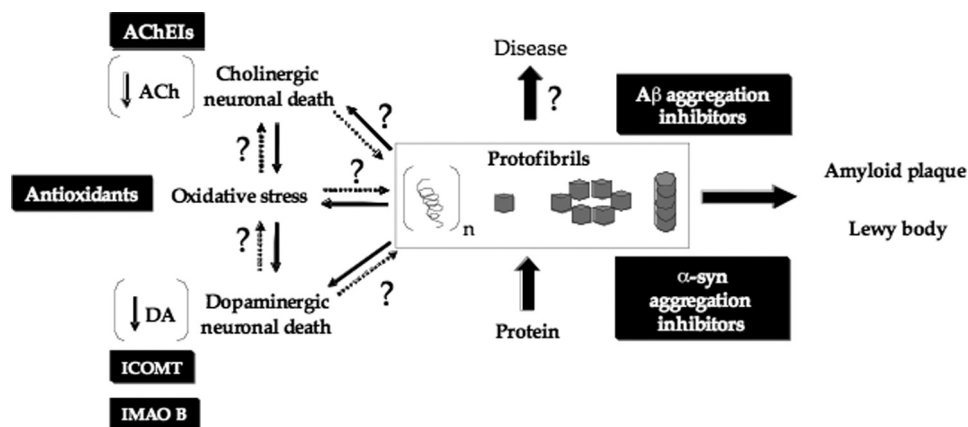
nearly 1–2% of the population 65 years and older. A characteristic early pathological change associated with PD is the selective loss of dopaminergic neurons of the substantia nigra pars compacta and other areas of the brain resulting in the degeneration of the nigro-striatal tract and loss of dopamine (DA) (1). Current therapeutic strategies for treating PD offer mainly transient symptomatic relief by aiming to restore the loss of dopamine by “dopamine replacement therapy.” This is accomplished through the administration of levodopa (L-DOPA), a direct precursor of DA and other drugs that increase the lifetime of DA by slowing its metabolism. Catechol O-methyltransferase inhibitors (COMT), monoamine oxidase B inhibitors (MAO B), and peripheral aromatic L-amino acid decarboxylase inhibitors (IAADC) are used as adjunctive medications to L-DOPA to slow DA degradation and increase the availability of brain DA (Scheme 1) (2).

Neuropathologically, PD is characterized by the formation of intraneuronal Lewy bodies and Lewy neuritis consisting primarily of fibrillar aggregates of α -synuclein (α -syn), a 14-kDa “natively unfolded” cytosolic protein (3). Accumulating evidence from genetics, animal models, and biochemical and biophysical studies suggests that α -syn aggregation is a toxic event that plays a central role in the initiation and/or progression of PD (4). Mutations or increased expression of α -syn are associated with early onset familial forms of PD (5–7). Overexpression of wild type and disease-associated mutants enhances α -syn aggregation and toxicity in several animal and cellular models of synucleinopathies (8–10). *In vitro* studies have consistently shown that disease-associated mutations accelerate and enhance α -syn oligomerization (A30P, A53T, and E46K) and/or fibrillization (A53T and E46K) (11–14).

Despite numerous advances in our understanding of the molecular mechanism of α -syn aggregation and toxicity *in vitro* and *in vivo*, we still have limited knowledge about the following:

HSQC, heteronuclear single quantum coherence; L-DOPA, levodopa; DA, dopamine; P, pyrogallol; Q, quercetin; α -syn, α -synuclein; SEC, size exclusion chromatography; TEM, transmission electron microscopy; ThT, thioflavin T; T, tolcapone; WT, wild type; COMT, catechol O-methyltransferase; PF, protofibril; LMW, low molecular weight; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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SCHEME 1. Working model illustrating that protein aggregation, *i.e.* α -syn in PD and $A\beta$ in AD, has a central role in the generation of the cascade of events that result in neurodegeneration and disease.

1) the normal physiological function of α -syn; 2) the relative contribution of α -syn aggregation to the pathogenesis of PD; 3) the identity of the toxic α -syn species; and 4) the exact mechanism by which α -syn contributes to the loss of dopaminergic neurons and PD pathology (15–17). The lack of animal models that represent the full spectrum of PD in humans and the absence of effective drugs capable of blocking α -syn aggregation at different stages on the amyloid formation pathway have contributed significantly to this knowledge gap and slow the pace of drug discovery in PD and related disorders.

The ability to block protein aggregation using small molecules provides a unique opportunity to investigate the link between α -syn aggregation and toxicity by assessing the functional and cellular consequences of blocking aggregation at different stages on the amyloid formation pathway. Initial efforts aimed at blocking or reversing protein aggregation focused on preventing amyloid formation or disruption of preformed fibrils as a means of promoting their clearance. Several classes of small molecules and short peptides have been reported as inhibitors or modulators of α -syn and amyloid- β ($A\beta$) fibrillization *in vitro*, including polyphenols such as catecholamines, DA, and other catechols (18–22). Dopamine agonists and monoamine oxidase B inhibitors, currently used as anti-parkinsonian agents, were also reported to destabilize preformed fibrils (23, 24).

The aim of this work was to evaluate the inhibitory potency of anti-Parkinsonian drugs toward the oligomerization, fibrillogenesis, and toxicity of monomeric and oligomeric forms of α -syn. These molecules include two nitrocatechols, *i.e.* entacapone (E) and tolcapone (T) (25) (Fig. 1), that have never been specifically tested for their anti-amyloidogenic properties. Here, we report that E and T are potent inhibitors of α -syn and $A\beta$ oligomerization and fibrillogenesis and protect against extracellular toxicity induced by the aggregation of both proteins in PC12 cells. To determine the relative contribution of the catechol moiety in these compounds, we compared their effect to four other natural polyphenols (quercetin (Q), caffeic acid (CA), gallic acid (GA), and pyrogallol (P)) known for their antioxidant properties (26–29) and dopamine (DA), a well known inhibitor of the fibrillization of α -syn and $A\beta$. Our studies present the first characterization of the anti-amyloidogenic

properties of T and E against both α -syn and $A\beta$ fibrillization and provide new insights into the mechanism of action of catechol-containing compounds on the amyloid formation pathway of both proteins.

EXPERIMENTAL PROCEDURES

Thioflavin T, gallic acid, quercetin, and MTT were purchased from Sigma. Dimethyl sulfoxide (DMSO) of microbiological quality, dopamine, caffeic acid, and pyrogallol were from Fluka (Buchs, Switzerland). Entacapone and tolcapone were kindly donated by Prof. Alberto Gasco (Dipartimento della

Scienza e Tecnologia del Farmaco, University of Turin, Italy). Dulbecco's modified Eagle's medium, heat-inactivated horse serum, fetal bovine serum, gentamicin, insulin, NuPAGE 4–12% BisTris gel, goat anti-mouse AlexaFluor 680, and SilverXpress silver staining kit were from Invitrogen. β -Amyloid monoclonal antibody 6E10 was from Covance and nitrocellulose membranes from Protran. All chemicals were of analytical grade, and all solutions were prepared in autoclaved distilled water. Compound solutions were prepared in 100% DMSO (10 mM). Fresh solutions were prepared by diluting stock solutions in water to achieve a final amount of co-solvent less than 1% in the reaction mixtures.

Expression and Purification of α -Synuclein—Human wild type (WT) α -syn was expressed as described previously (12). Cells were harvested, resuspended in buffer, and lysed. The supernatant was saved, concentrated, and loaded onto a Superdex 200 size exclusion column. α -Syn containing fractions were combined, lyophilized, and stored at -20°C until use.

Preparation and Characterization of $A\beta$ 42 Low Molecular Weight (LMW) and Protofibrils (PF)— $A\beta$ 42 was synthesized and purified by Dr. James I. Elliot at Yale University (New Haven, CT). Monomeric $A\beta$ 42 stock solutions were prepared by dissolving the peptide in 6 M guanidine HCl at a concentration of 1 mg/ml and centrifuged at 8600 rpm for 5 min. LMW and PF $A\beta$ 42 stock solutions were prepared by dissolving the peptide in 5% DMSO, 2 M Tris base, pH 7.6. The mixture was subjected to low speed centrifugation at 6000 rpm for 5 min. The supernatant of $A\beta$ 42 containing monomeric, LMW, and PF was loaded onto a gel filtration column (Superdex 75 HR 10/30 Amersham Biosciences) previously equilibrated with 10 mM Tris buffer, pH 7.4 (30). The fractions were analyzed by SDS-PAGE, and protein concentration was determined by UV absorbance at 280 nm in 10-mm path length cuvettes using the theoretical molar extinction coefficient at 280 nm for $A\beta$ 42 ($1490\text{ M}^{-1}\text{ cm}^{-1}$) (31). All $A\beta$ 42 stock solutions were diluted with Tris buffer, pH 7.4, to a final peptide concentration of 10 μM . Samples of $A\beta$ 42 were incubated at 37°C in 1.5-ml polypropylene sterile tubes, with and without different concentrations of inhibitors, at molar ratios of $A\beta$ 42/inhibitor of 1:0.5 and 1:2.

Preparation of α -Syn and A β 42 Seeds—The seeds were prepared by incubation of α -syn peptide solution (20 mM Tris buffer, 150 mM NaCl, pH 7.4) and A β 42 peptide solution (5% DMSO, 2 M Tris buffer, pH 7.6), at 37 °C under agitation for 3 days. The fibrils were then mechanically fragmented to yield narrow distribution of smaller fibrillar structures (100–300 nm long) by ultrasonication on ice using SONICS Vibra Cell™ equipped with a fine tip (20 5-s pulses, amplitude of 40, and output of 6 watts). The sonicated fibrils were diluted in 10 mM Tris buffer, pH 7.4. Seeds and monomeric α -syn or A β 42 were incubated for 4 h at 37 °C with continuous shaking, with and without inhibitors, in polystyrene black 384-well plates (Nunc).

Fibrillization Studies—Purified, lyophilized α -syn was dissolved in 20 mM Tris buffer, 150 mM NaCl. The samples of α -syn at a concentration of 100 μ M (as estimated by spectroscopy) were incubated at 37 °C in 1.5-ml sterile polypropylene tubes, with continuous shaking, in the absence and presence of inhibitors, at a molar ratio of α -syn/inhibitors of 1:1, 1:0.5, 1:0.1, and 1:0.01. Aliquots (10 μ l) of the α -syn incubations (final protein concentration of 10 μ M), previously incubated at 37 °C with and without compounds, were added to 80 μ l of 50 μ M glycine buffer, pH 8.5, and 10 μ l of solution of 100 μ M ThT. Aliquots of 80 μ l of 10 μ M A β 42 solutions (LMW or PF), previously incubated at 37 °C in the absence and presence of compounds, were added to 10 μ l of 100 μ M ThT and 10 μ l of 50 μ M glycine buffer, pH 8.5.

The time course of α -syn and A β 42 fibrillization was measured by the ThT fluorescence assay. Fluorescence measurements were carried out with a spectrofluorometer (Analyst™ AD 96-384, Bucher Biotec AG, Basel) at 25 °C using polystyrene black 384-well plates. The excitation wavelength was set to 450 nm, and emission was monitored at 485 nm. All measurements were done in triplicate by performing three identical experiments.

Seeding Polymerization Assay—The polymerization of soluble α -syn with or without α -syn fibrils added as seeds and A β 42 with or without A β 42 seeds was assayed as described elsewhere (32). Nunc 384 fluorescence plates were filled with monomeric and seed protein solutions. Compounds of interest (or Tris buffer containing 1% DMSO for the control) were finally added to the reaction mixture. The plate was incubated at 37 °C for 3 h under agitation. The kinetics and extent of fibrillization was monitored using the standard ThT binding assay as described above. The assay was run in triplicate by processing three identical plates.

Electron Microscopy Analysis of Fibril Formation—A β and α -syn fibril formation was monitored by transmission electron microscopy (TEM). Samples for TEM analysis were prepared by placing 10 μ l of the sample solution on Formvar-carbon copper grid for 1 min before removing the excess solution. The grid was washed with 2 drops of distilled water and 1 drop of uranyl acetate before staining with 1% of fresh uranyl acetate for 30 s. The grids were thoroughly examined to get an overall evaluation of the structures present in the sample. Specimens were inspected at 80 kV using a Philip CIME 12 electron microscope. Digitized photographs were recorded with a slow scan CCD camera (Gatan, model 679).

NMR Spectroscopy—NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer at 15 °C. Measurements were performed using 60 μ M 15 N-labeled α -syn in 20 mM Tris, 150 mM NaCl, pH 7.4. Two-dimensional 1 H- 15 N heteronuclear single quantum coherence (HSQC) spectra were recorded using 256 \times 1024 complex data points in the F1 and F2 dimensions, with a relaxation delay of 1.0 s (33). Sixty four scans per increment were recorded for each spectrum. Spectral widths were 1612.9 and 7211.5 Hz in the 15 N and 1 H dimensions, respectively.

Spectra were processed with Topspin 1.3 (Bruker Biospin) and NMRPipe (34). Visualization and manipulation were performed using the public domain graphics program Sparky 3 (34). Resonance assignments had been previously obtained (35). The addition of 1% DMSO and a small change in temperature did not interfere with transfer of resonance assignment.

Two-dimensional 1 H- 15 N HSQC spectra were recorded to monitor chemical shift changes induced by the presence of three compounds (E, Q, and T). Compounds were freshly dissolved in DMSO to high concentration and added to the α -syn sample. The concentration of DMSO did not exceed 3% (v/v). Measurements were performed for molar ratios of 1:0.5, 1:2, and 1:10 α -syn/ligand. For each titration step, a reference spectrum was obtained by addition of the same amount of DMSO only. Both intensity and chemical shift differences were analyzed with respect to the DMSO reference spectrum. Mean weighted 1 H- 15 N chemical shift differences were calculated according to $\Delta_{av} = \{((\Delta\delta_{HN}^2 + \Delta\delta_N^2)/25)\}^{1/2}$ (33, 35, 36).

PC12 Preparation and Toxicity Studies—The rat adrenal gland pheochromocytoma cell line, PC12, was grown at 37 °C in 5% of CO₂ in Dulbecco's modified Eagle's medium supplemented with 6% heat-inactivated horse serum, 6% of fetal bovine serum, and 50 μ g/ml gentamicin. Exponentially growing PC12 cells (5 \cdot 10⁴ cells per well) were plated in 96-well tissue culture plates (Falcon) in media supplemented with 2 μ M insulin and 50 μ g/ml gentamicin. The cells were co-treated with A β 42 crude preparation (40 μ M), and the compounds (40 μ M) at different concentrations (5, 10, 20, and 40 μ M) for 24 h at 37 °C and in 5% of CO₂. The peptide and the compounds were added directly to the Dulbecco's modified Eagle's medium supplement with 2 μ M insulin and 50 μ g/ml gentamicin. Given the slow rate of spontaneous α -syn fibrillization *in vitro*, the α -syn toxicity studies were performed using preaggregated proteins formed in the presence and absence of inhibitors. Briefly, the α -syn protein was incubated alone for 72 h at 37 °C under agitation conditions alone or in the presence of the inhibitors at a different molar ratio. At this point, α -syn or the α -syn/inhibitor mixtures were added directly to the cell culture medium (Dulbecco's modified Eagle's medium supplemented with 2 μ M insulin and 50 μ g/ml gentamicin) and incubated with PC12 for 24 h at 37 °C in 5% of CO₂. The viability of PC12 cells was evaluated by thiazolyl blue MTT (37) and luminescent cells assays. The MTT assay is based on the conversion of tetrazolium salt to formazan (blue compound), a reaction that only occurs in viable cells because the chemical reaction is carried only by mitochondrial dehydrogenases. MTT was added at final concentration of 0.5 mg/ml for 2 h at 37 °C, and the formed crystals were dissolved using 100 μ l of solubilization buffer

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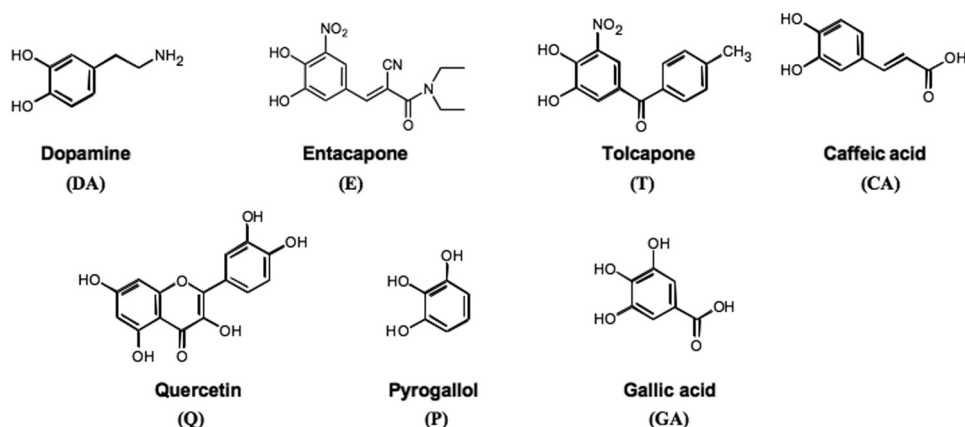


FIGURE 1. Chemical structure of compounds examined as inhibitors of human WT α -syn and A β 42 fibril formation.

containing 30% SDS and 70% isopropyl alcohol in water. The absorbance was determined at 570 nm using a microplate reader. The luminescent cell viability assay is a sensitive method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. The assay procedure involves adding the single reagent (Promega CellTiter-Glo[®] reagent) directly to cultured cells. The light emission, measured by Safire Instrument, is directly proportional to the ATP produced. All quantitative data are presented as means \pm S.E. Statistical analysis between different treatments was calculated by using one way analysis of variance followed by post hoc comparison through Bonferro-ni's test. A value of $p > 0.05$ was considered statistically significant.

RESULTS

Inhibition of α -Syn Fibrillization by Entacapone, Tolcapone, and Related Catechols— α -Syn fibril formation is a concentration-dependent process and occurs readily *in vitro* only under conditions that combine high protein concentrations (100–200 μ M) and mechanical agitation to accelerate the process (supplemental Fig. 1). We evaluated the fibrillization of human WT α -syn alone under agitation in the range of 50–200 μ M, and a 100 μ M protein concentration was chosen as the optimal concentration to assess the inhibitory activity of the compounds (Fig. 1). Under these conditions, α -syn fibrillization was complete after 72–96 h of incubation at 37 $^{\circ}$ C.

To investigate the influence of the two nitrocatechols, entacapone and tolcapone, and the four natural compounds, quercetin, CA, GA, and pyrogallol on α -syn aggregation, we monitored the fibrillization of α -syn in the absence and presence of each compound in the concentration range of 1–100 μ M using ThT fluorescence, SDS-PAGE analysis of soluble protein, and TEM as a function of time. DA was chosen as a reference compound and was tested under the same working conditions. As shown in Fig. 2, after 72 h of incubation, all compounds tested abolished α -syn fibril formation at concentrations of 50 and 100 μ M. Compounds E, GA, and Q exhibited strong inhibition of α -syn aggregation even at concentrations of 10 μ M, with compound T demonstrating the greatest inhibition potency (Fig. 2A). More than 70–80% reduction of the ThT fluorescence

signal was observed in all samples containing ≥ 10 μ M of compounds.

The ThT results were confirmed by TEM, which demonstrated the absence of significant amounts of amyloid fibrils in α -syn samples incubated with the compounds at 100 μ M concentrations (Fig. 2B). After 72 h, WT α -syn alone formed extensive fibrillar structures with an average diameter of 35 nm. In the samples containing 100 μ M of E, T, Q, CA, GA, and P, the number of fibrils was significantly reduced, and spherical oligomers and short sheared fibrils were observed instead. Aggregates formed in the

presence of the various compounds were morphologically distinct from those formed by WT α -syn.

To further confirm the inhibitory effect observed by ThT and TEM assays, we quantified the amount of remaining soluble protein by SDS-PAGE analysis of the supernatant after removal of fibrils and insoluble materials by centrifugation. The sample containing α -syn alone showed a reduction in band intensity corresponding to approximately >70–80% loss of soluble α -syn after 72 h of incubation at 37 $^{\circ}$ C, suggesting that the majority of soluble α -syn has been converted into insoluble fibrils under these conditions. In contrast, all samples containing the compounds (100 μ M) showed levels of soluble protein that correspond to the inhibitory effect reported by ThT and TEM. At 1:1 molar ratio, more than $\sim 60\%$ of the starting protein remained in solution after 72 h of incubation (Fig. 2A).

Inhibition of the Seeding Capacity of Fibrillar α -Syn—The process of amyloid fibril formation follows a nucleation-dependent polymerization mechanism that is characterized by an initial lag time phase (nucleation phase), followed by an exponential growth phase (polymerization phase) and a final plateau (equilibrium phase) (38). The spontaneous breakage of fibrils into smaller aggregates or their disassociation by small molecules or chaperones is believed to contribute to the spreading and acceleration of amyloid formation *in vivo* via a seeding mechanism (39, 40). This is supported by *in vitro* and *in vivo* studies demonstrating that amyloid fibril formation is accelerated by the addition of preformed aggregates (41, 42), which act as seeds that nucleate fibril formation and growth. In other words, the addition of seeds eliminates the lag phase associated with fibril formation. Therefore, blocking the seeding capacity of preformed fibrils is an attractive strategy for slowing amyloid formation and disease progression in PD and related disorders. For this purpose, the ability of the compounds to inhibit the seeding capacity of α -syn fibrils was investigated.

To produce α -syn seeds, mature α -syn fibrils were mechanically disrupted by sonication to yield a narrow distribution of short fibrils. As expected, addition of small amounts of seeds abolished the lag phase and accelerated α -syn fibrillization (Fig. 3A). To determine the relative potency of the compounds toward blocking the seeding capacity of α -syn fibrils, 10 and 50 μ M of each compound was added to a solution of freshly pre-

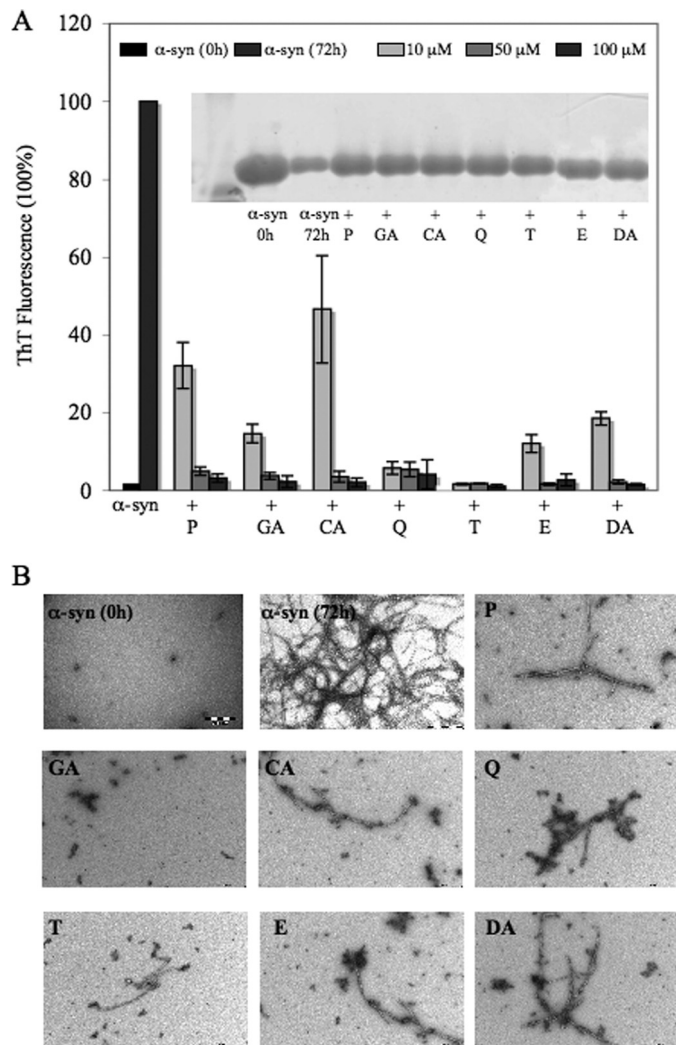


FIGURE 2. Compounds abolish $\alpha\text{-syn}$ fibril formation and increase the soluble forms of $\alpha\text{-syn}$ after incubation for 72 h. *A*, samples of 100 μM $\alpha\text{-syn}$ were incubated at 37 $^{\circ}\text{C}$, with continuous shaking, with and without compounds, at molar ratios of $\alpha\text{-syn}$ /inhibitor of 1:0.1, 1:0.5, and 1:1. The time course of protein fibrillization was measured by ThT fluorescence assay after incubation for 72 h. The bar graph represents the amount of fibril formation in absence and in presence of the compounds P, GA, CA, Q, T, E, and DA. The figure shows means of three independent experiments \pm S.D. ($n = 6$). The inhibitory effect of the compounds on $\alpha\text{-syn}$ aggregation was evaluated by SDS-PAGE. Samples of $\alpha\text{-syn}$ (100 μM) were incubated with and without compounds, at molar ratios of $\alpha\text{-syn}$ /inhibitor of 1:1. The bands represent the amount of monomeric form of $\alpha\text{-syn}$ before incubation and after incubation for 72 h in the absence and in the presence of the compounds. *B*, electron micrographs of negatively stained quaternary structures deposited from solutions of $\alpha\text{-syn}$ before incubation and after 72 h incubation at 37 $^{\circ}\text{C}$ in the absence and in the presence of 100 μM compound. Scale bar, 200 nm.

pared monomeric $\alpha\text{-syn}$. Fibrillar seeds at 2 μM final concentration were then added, and the kinetics of fibrillization was monitored by ThT fluorescence. In the absence of compounds, fibrillization proceeds immediately to yield a dense network of amyloid fibrils. The fibrillization reaction was complete within 3 h as opposed to 72–96 h in the absence of the seeds. At 50 μM concentration of E, T, Q, P, and CA (Fig. 3*A*, filled triangles), the seeding capacity of short $\alpha\text{-syn}$ fibrils was abolished by >90%. However, only Q and T exhibited a similar potency at lower concentrations (10 μM). The remaining compounds E, P, CA, GA, and DA still showed greater than 60–75% inhibition of

seeded fibril growth at this concentration. These findings were confirmed by TEM, which demonstrated the presence of predominantly spherical structures and short isolated fibrillar assemblies (Fig. 3*B*) in seeded samples containing 50 μM of the compounds compared with extensive fibril formation in samples containing only $\alpha\text{-syn}$.

Entacapone, Tolcapone, and Related Catechols Do Not Bind to Monomeric $\alpha\text{-Syn}$ —Binding of E, T, and Q to monomeric $\alpha\text{-syn}$ was probed using NMR spectroscopy. NMR signals of backbone amides constitute excellent probes of complex formation providing maps of interaction interfaces (43). We monitored the position and intensity of the NMR signals of $\alpha\text{-syn}$ in the presence of E, T, and Q for molar ratios up to 1:10 $\alpha\text{-syn}$: compound. In the case of Q, only a 1:2 ratio was reached due to its lower solubility. No significant chemical shift changes were observed for any of the compounds, with the exception of very minor chemical shift changes for His-50 and some N-terminal residues (Fig. 4, *A–C*). As the very small chemical shift changes observed for His-50 and the two to three N-terminal residues are most likely due to slight changes in pH, the NMR data suggest that there is no direct interaction of the compounds with the backbone of monomeric $\alpha\text{-syn}$.

Besides the position of NMR signals, their intensity is very sensitive to changes in the conformational properties of a protein as well as its chemical environment. For example, signal broadening indicates increased chemical exchange. When we compared the intensity of NMR signals in two-dimensional ^1H - ^{15}N HSQC spectra of $\alpha\text{-syn}$ in the free state and in the presence of the compounds E and T, residues in the C-terminal domain showed a different response than those of residues 20–105 (Fig. 4, *D–F*). NMR signal intensities in the C-terminal domain in the presence of E and T were within 10–15% of the values in the free state. In contrast, a large number of residues in the N-terminal domain of $\alpha\text{-syn}$, in particular in the non-amyloid β -component region, showed an increase of up to 30% in NMR signal intensity in the presence of E and T when compared with the DMSO control spectrum. The increased NMR signal intensities point to an increase in the backbone flexibility of these residues or to a reduced amide proton exchange. Interestingly, a similar increase in NMR signal intensities was observed for residues 22–93 of $\alpha\text{-syn}$ in the presence of polyamines, which bind to the C terminus of monomeric $\alpha\text{-syn}$ (44).

All Compounds Protect PC12 against $\alpha\text{-Syn}$ -induced Cell Death—To investigate the effect of the E and T and related compounds in Fig. 1 against $\alpha\text{-syn}$ -induced extracellular toxicity on the PC12 cells, we used a preparation of $\alpha\text{-syn}$ that was incubated in the presence and/or absence of compounds for 72 $^{\circ}\text{C}$ at 37 $^{\circ}\text{C}$ under agitating conditions. The $\alpha\text{-syn}$ /compound mixtures were then added to the cell culture media, and cell viability was evaluated using the MTT assay. The treatment with the preaggregated $\alpha\text{-syn}$ (40 μM) showed a reduction of cellular viability by \sim 40% (Fig. 5). $\alpha\text{-Syn}$ samples, which were incubated with inhibitors, showed a significant increase of cell viability in the range of 10–30%. Interestingly, E was found to be the most active compound, with a protective effect close to 100%. These results suggest a direct correlation between the effect of these compounds on the fibrillization of $\alpha\text{-syn}$ and protection against $\alpha\text{-syn}$ -induced extracellular toxicity, which

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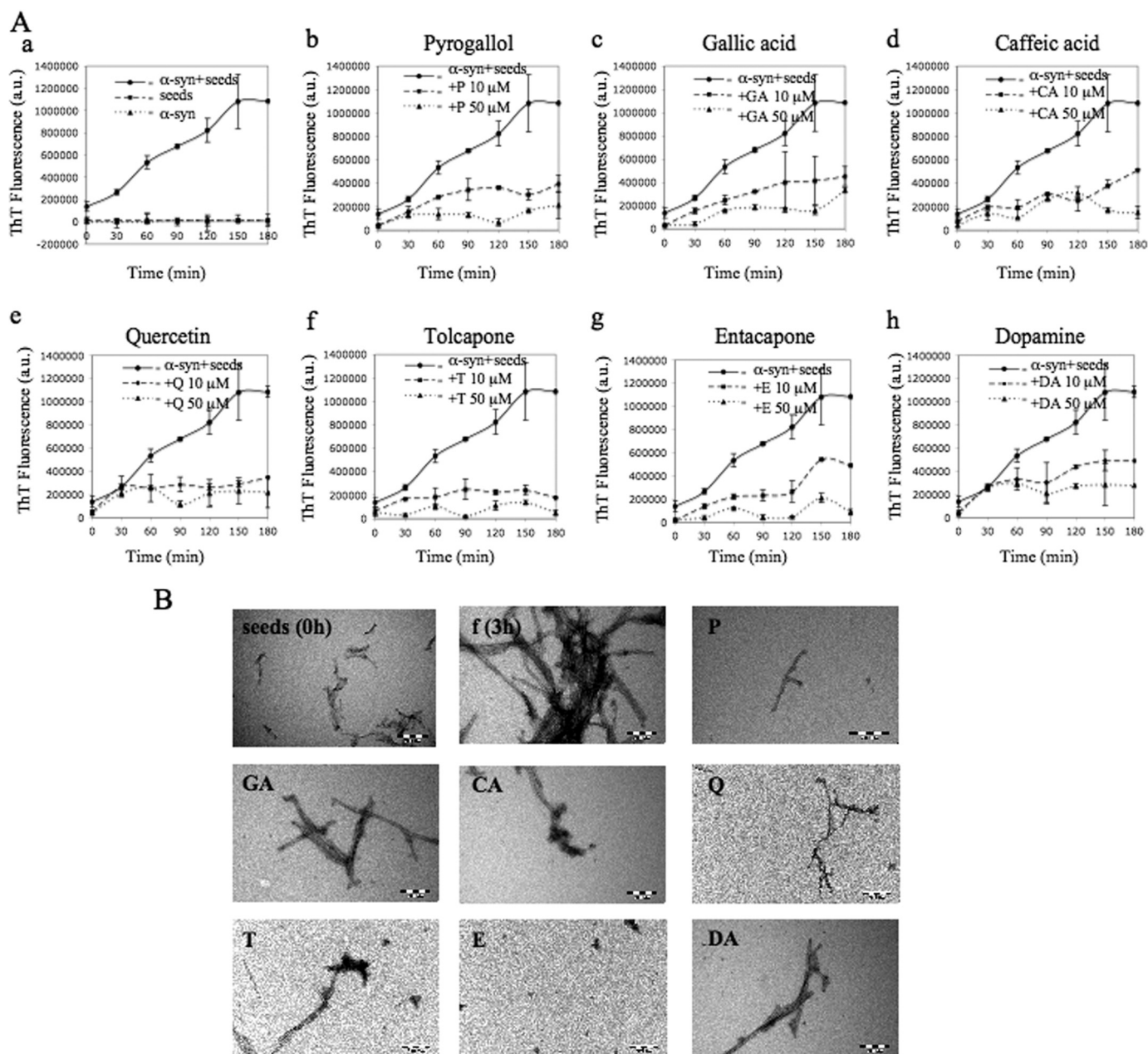


FIGURE 3. Compounds have an inhibitory effect on the α -syn seeding polymerization. *A*, samples of monomeric α -syn ($100 \mu\text{M}$) were incubated with the seeds ($2 \mu\text{M}$) at 37°C , with continuous shaking, without (*panel a*) and with (*panels b–h*) 10 and $50 \mu\text{M}$ of compounds P, GA, CA, Q, T, E, and DA. The time course of protein fibrillization was measured every 30 min by ThT fluorescence assay for 3 h. *B*, electron micrographs of negatively stained quaternary structures deposited from solutions of seeds and α -syn + seeds after 3 h of incubation in the absence and in the presence of the compounds ($50 \mu\text{M}$). Scale bar represents 200 nm.

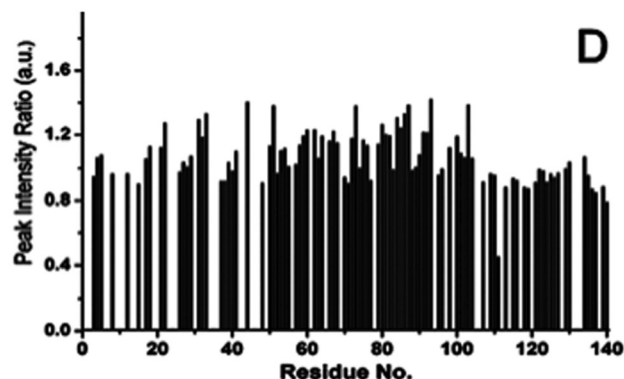
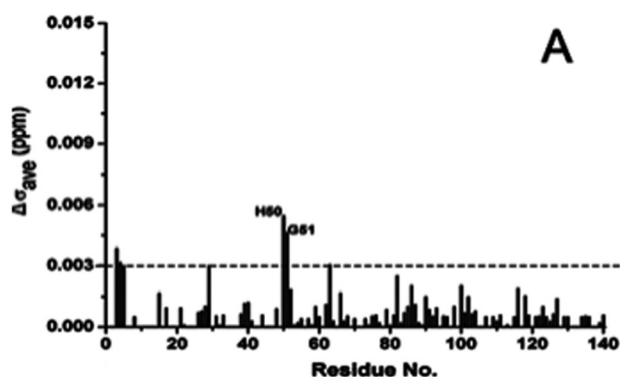
may be linked to the ability of the compounds to block the formation of the toxic entity or processes.

Entacapone, Tolcapone, and Related Catechols Inhibit the Conversion of LMW A β 42 into Fibrils—To assess the inhibitory specificity of the compounds toward α -syn fibrillation, we investigated their capacity to inhibit the fibrillization of monomeric LMW and PF β 42, which was prepared freshly by size exclusion chromatography, as described previously (30). Fresh monomeric A β 42 solutions were incubated (37°C), with and without compounds, at molar ratios compound, A β 42 of 0.5:1 and 2:1 for 24 and 48 h. In the presence of 5 (data not shown) and $20 \mu\text{M}$ compounds (Fig. 6A), A β 42 fibrillization was

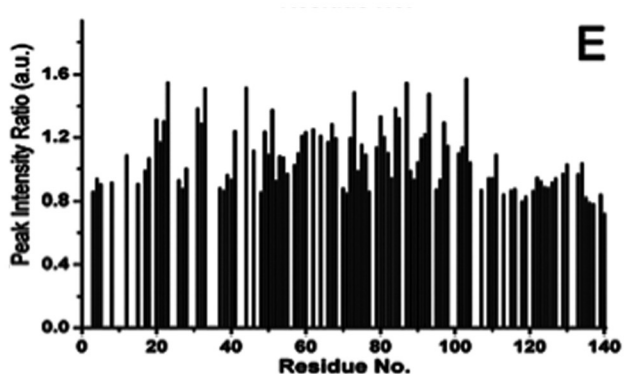
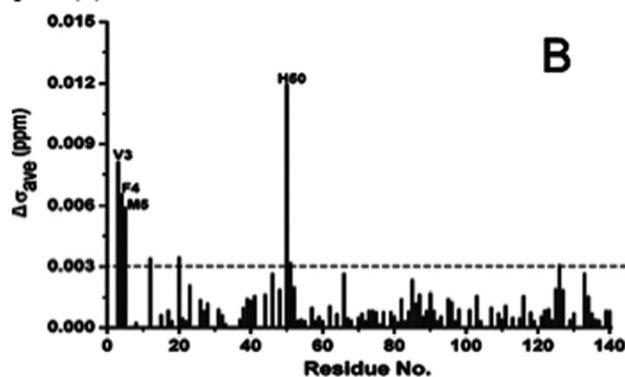
decreased by $\geq 50\%$ after 48 h of incubation. Q was found to be the most potent compound, showing greater than 80% inhibition of A β 42 fibrillization at 5– $20 \mu\text{M}$, whereas the remaining compounds showed inhibition in the range of 60–70% at higher molar ratios (2:1, compound/A β 42). These findings indicated that these compounds act by one of the following mechanisms: 1) by stabilizing monomeric A β ; 2) by kinetic stabilization aggregation intermediates that precede mature fibril formation; or 3) by altering the aggregation properties of A β 42 such that ThT negative large aggregates are formed.

To determine their mode of action, the samples were analyzed by electron microscopy. TEM images of LMW A β 42 fol-

Entacapone (E)



Tolcapone (T)



Quercetin (Q)

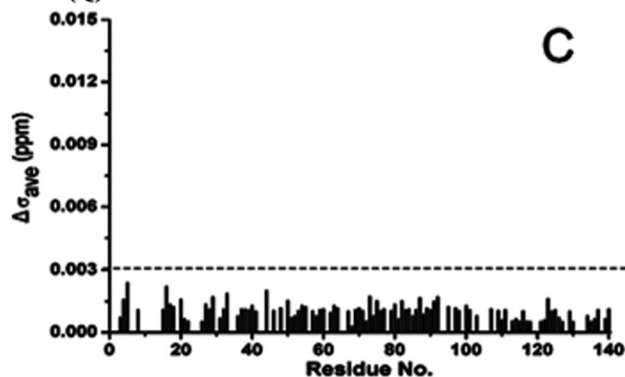


FIGURE 4. Analysis of compound binding to monomeric α -syn by NMR spectroscopy. Changes in individual cross-peak positions (A–C) and intensities (D–F) of backbone ^{15}N - ^1H resonances of α -syn ($60\ \mu\text{M}$) in two-dimensional ^1H - ^{15}N HSQC spectra in the presence of compounds E (A and D), T (B and E), and Q (C and F). For compounds E and F, molar ratios of 1:10 α -syn/compound were used. Compound Q is less soluble, and only the 1:2 α -syn/compound ratio could be measured. Horizontal lines indicate the average variation of chemical shifts observed for α -syn from sample to sample due to slightly different buffer conditions.

lowing incubation for 48 h in the presence of compounds indicate E and T stabilize distinct aggregate morphologies compared with the other compounds (Fig. 6B). Negatively stained TEM images of LMW alone revealed amyloid fibrils (diameter of 30 nm) in the sample after 48 h of incubation (Fig. 6B). A β 42 solutions containing 20 μM E and T revealed predominantly large networks of PF-like structures and the absence of mature fibrils. In comparison with E and T, the other compounds P, GA, CA, and DA appeared to exert different effects on A β fibrillization. GA and CA were shown to stabilize smaller PF structures and result in the formation network of amorphous aggregates, after 48 h of incubation at 37 $^{\circ}\text{C}$. When

Q was added to the sample containing A β 42 LMW, we observed the formation of predominantly LMW and PF species.

Entacapone, Tolcapone, and Related Catechols Inhibit the Conversion of A β 42 Protofibrils into Mature Fibrils in a Specific and Concentration-dependent Manner—To test our hypothesis and determine whether these compounds act by targeting intermediates on the amyloid pathway, we evaluated their capacity to block the conversion of PFs into mature amyloid fibrils. PFs are metastable oligomeric intermediates, which have been observed during the *in vitro* fibrillization of A β and almost all other amyloidogenic proteins (45). During the last decade, mounting evidence from *in vivo* and *in vitro* studies point

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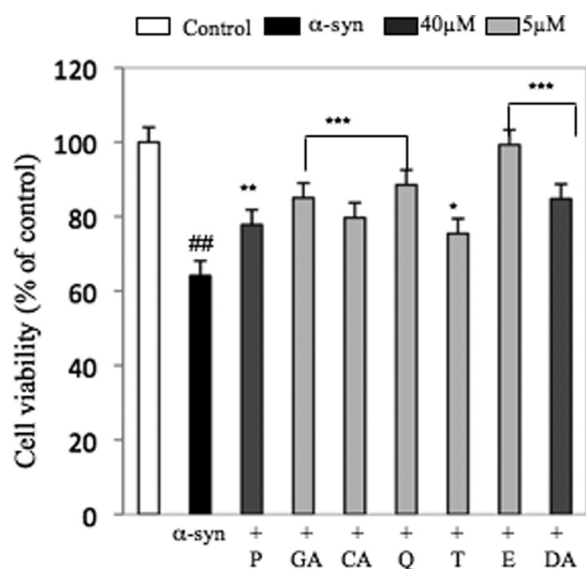


FIGURE 5. Protective effect of the compounds against α -syn-induced toxicity in PC12 cells. PC12 cells were treated with preincubated α -syn (40 μ M) alone or co-incubated with 5 μ M GA, CA, Q, T, and E and 40 μ M P and DA. The cellular viability was evaluated by MTT assay, and the data were expressed as percentage of the control (nontreated cells). The control treatment is set to 100%. Bars are means \pm S.E. We used # to compare the data to the control and * with respect to the treatments with α -syn. ##, $p < 0.001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

toward early aggregation intermediates, including PFs, as the major cytotoxic species responsible for triggering neurodegeneration in AD, PD, prion diseases, and other related diseases (46). Freshly isolated PFs were co-incubated with 5 and 20 μ M of compounds, and the aggregation was monitored by ThT fluorescence and TEM after 24 h (data not shown) and 48 h at 37 $^{\circ}$ C. After 48 h, the samples containing PFs alone showed an increase in ThT signal consistent with a conversion of the PFs into mature fibrils (Fig. 7A). When PFs were incubated with 20 μ M GA and CA the increase in ThT fluorescence observed with PFs alone was reduced by \sim 50%. More than 70% reduction in the ThT signal was observed in the samples containing 20 μ M P, E, and DA suggesting that these compounds are more effective at inhibiting the PF to fibril conversion. However, co-incubation of PF with 20 μ M Q or T resulted in >80 –90% inhibition, with Q being the most potent inhibitor of PF growth and fibrillization in the series. As expected, TEM analysis of the samples containing PFs alone after 48 h of aggregation showed dense networks of fibrils. In the presence of Q, T, E, and DA, mainly PF-like structures, similar to those present at the starting conditions, were observed, in addition to only some isolated short fibrils (Fig. 7B). Short fibrils were observed in the presence of GA and CA after 48 h (Fig. 7B), which is consistent with the higher ThT signal in these samples (Fig. 7A).

Only Entacapone and Tolcapone Are Effective in Inhibiting the Seeding Capacity of A β 42—To determine whether E, T, and related compounds can block A β 42 aggregation at a later stage of the fibrillization process, *i.e.* fibril growth, the capacity of the compounds to interfere with fibril elongation and the seeding capacity of A β 42 fibrils was evaluated using the seeding polymerization assay described above. Fibrillar seeds of A β 42 were prepared by fragmenting preformed and purified fibrils by sonication. The freshly prepared fibril seeds were added to a fresh

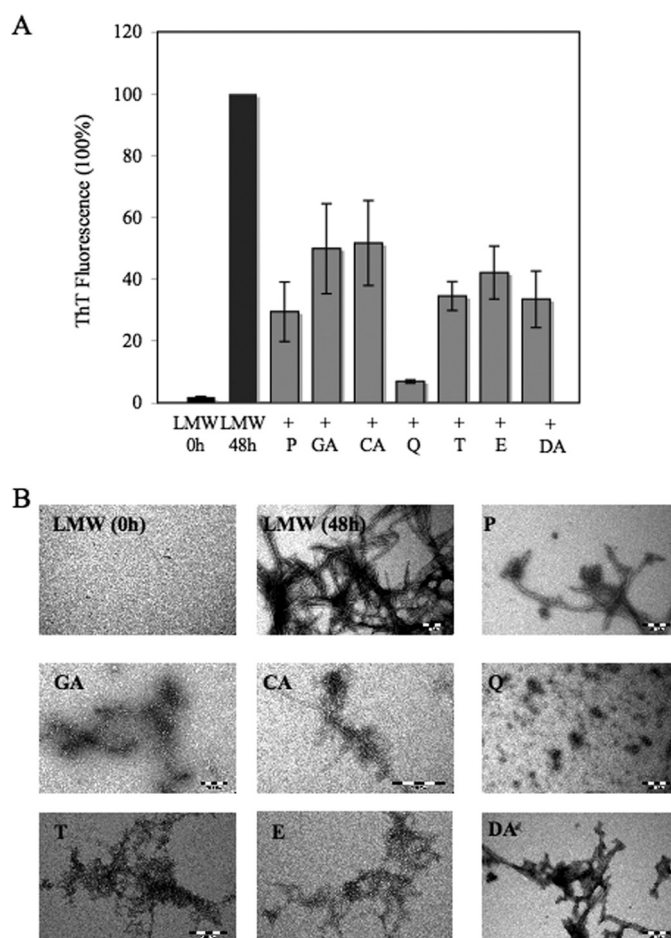


FIGURE 6. Compounds inhibit LMW A β 42 fibril formation. A, samples of LMW A β 42 were incubated at 37 $^{\circ}$ C with and without compounds, at molar ratios of A β 42/inhibitor of 1:2. The time course of protein fibrillization was measured by ThT fluorescence assay. The bar graph represents the amount of fibril formation in the absence and presence of the compounds P, GA, CA, Q, T, E, and DA. The samples containing the compounds showed a decrease of the ThT fluorescence signal after 48 h of incubation. The figure shows means of three independent experiments \pm S.D. ($n = 6$). B, electron micrographs of negatively stained quaternary structures deposited from solutions of LMW A β 42 (10 μ M) before and after 48 h of incubation at 37 $^{\circ}$ C in the absence and in the presence of 20 μ M of the compounds listed. Scale bar, 200 nm.

monomeric solution of A β 42 and co-incubated with each of the compounds (20 μ M). The kinetics of fibrillization was followed over a period of 3 h with ThT, and the structures of the final aggregates were characterized by TEM. Among all the compounds, only E, T, and Q showed significant reduction in fibril growth and inhibition of the seeding capacity by A β 42 fragmented fibrils (Fig. 8). Analysis of these samples by TEM revealed predominantly short fibrillar structures resembling the fibrillar seeds, consistent with the ThT results (data not shown). These results further confirm that E, T, and Q interfere with the elongation and growth of A β 42 fibrils possibly via direct interactions with either A β 42 seeds or monomers or both. After 3 h of incubation, no inhibition was observed in the samples containing P and CA. Moreover, addition of GA and DA to the mixtures containing A β 42 monomers and seeds did not affect the rate of fibrillization and resulted in only slight reduction of the ThT fluorescence signal (Fig. 8).

Protection against A β 42-induced Toxicity in PC12 Cells—Previous studies reported that natural polyphenols like Q, GA,

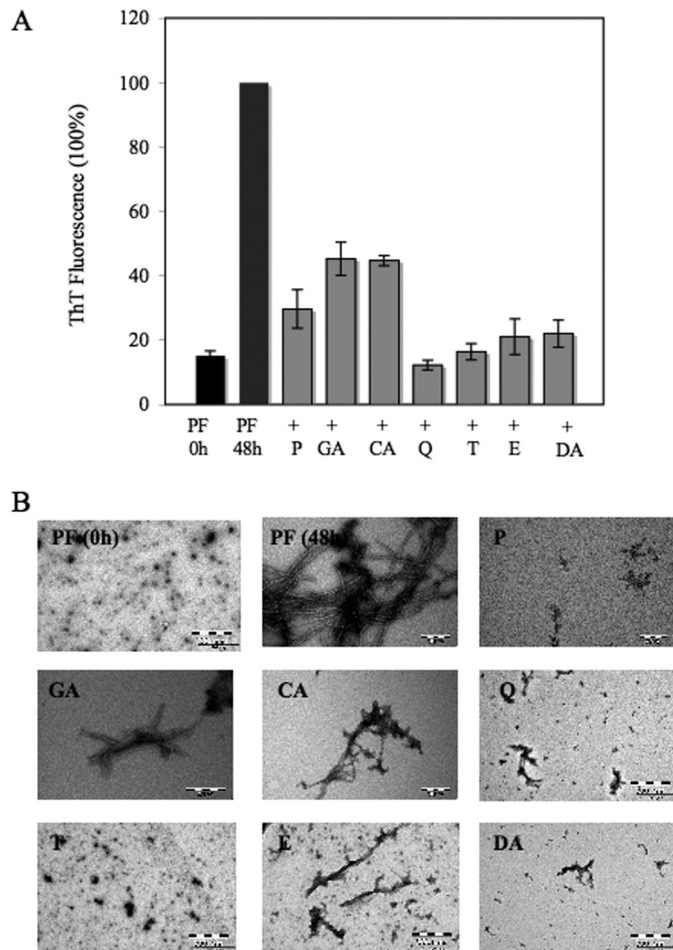


FIGURE 7. Compounds prevent the conversion of A β 42 protofibrils into mature fibrils in a specific and concentration-dependent manner. *A*, samples of PF A β 42 stock solutions were prepared by dissolving the peptide in 5% DMSO, 2 M Tris base, pH 7.6. Samples of A β 42 were incubated at 37 °C with and without compounds, at molar ratios of A β 42/inhibitor of 1:2. The time course of protein fibrillization was measured by ThT fluorescence assay. The bar graph represents the amount of fibril formation in the absence and presence of the compounds P, GA, CA, Q, T, E, and DA. The samples containing the compounds showed a decrease of the ThT fluorescence signal after 48 h of incubation. The figure shows means of three independent experiments \pm S.D. ($n = 6$). *B*, electron micrographs of negatively stained quaternary structures deposited from solutions of PF A β 42 (10 μ M) before incubation and after 48 h of incubation at 37 °C in the absence and presence of 20 μ M of the compounds listed. Scale bar, 200 nm.

and CA are neuroprotective against A β toxicity. The effects of Q and GA have been studied against A β 42 in primary cultures (47–49) and *in vivo* models of AD (50). Recently, the protective role of CA on A β -induced toxicity in PC12 cells was also described (51). We sought to determine whether there is a correlation between the inhibitory potency of the polyphenolic compounds on A β aggregation and their effect on A β -induced toxicity in PC12 cells. The compounds were preincubated at concentrations in the range of 5–40 μ M with crude preparation of A β 42, *i.e.* preparations containing predominantly LMW and PF A β 42. These conditions were chosen to mimic the pathological situation *in vivo* where both the LMW and PF species are populated in the diseased AD brain. After incubation with A β 42 for 20–30 min, the A β /compound mixtures were then added to PC12 in cell culture media. The cellular viability was assessed after 24 h using MTT and luminescent assays (30). All

the compounds tested did not increase the mortality of PC12 cells in the absence of A β 42 even at the highest concentration of inhibitors (40 μ M, see supplemental Fig. 2), demonstrating that the compounds do not enhance cell viability on their own over a concentration range of 10–40 μ M. The cells were then treated for 24 h either with 40 μ M A β 42 alone or in the presence of 5, 10, 20, and 40 μ M of the compounds (supplemental Fig. 2). Exposure of PC12 cells to 40 μ M A β 42 reduced the cellular viability by \sim 40 and 50% (Fig. 9) as determined by the MTT assay and ATP release, respectively (supplemental Fig. 3), with respect to the control (untreated cells). At higher concentrations (20 and 40 μ M), polyphenols were found to be protective against the A β 42 toxicity, but the attenuation of the toxicity was less evident when compared with the effect observed at low concentration. However, P and DA showed a strong protective effect at a concentration of 40 μ M. At a low concentration (5 μ M), E and T induced a significant (\sim 30%) protection against A β -induced toxicity, and at higher concentrations (10, 20, and 40 μ M), we found an increase of cellular viability and ATP release of \sim 20%. These observations indicate that the protection in the co-treatment of the PC12 cells could be related to a dual activity of the compounds, *i.e.* their inhibitory activity against A β aggregation and protection against A β -induced cell toxicity.

DISCUSSION

Converging evidence from various sources, including pathology, genetics, biochemistry, cell biology, and animal models suggests that the aggregation of α -syn in PD and A β in AD plays a critical role in the pathogenesis of these complex disorders. Hence, strategies aimed at inhibiting and/or reducing the aggregation and amyloid fibril formation of these proteins represent a viable therapeutic strategy to combat and/or prevent the progression of neurodegeneration in both diseases. Toward identifying potent drug-like aggregation inhibitors, E and T, two catechol *O*-methyltransferase inhibitors currently approved as adjuncts in the therapy of PD, and five other catechol-containing small molecules (GA, CA, Q, P, and DA) were selected, and their effect on the oligomerization and fibrillization of α -syn and A β 42 was investigated using ThT fluorescence, TEM, and SDS-PAGE analysis.

Our results demonstrate that all catechol-containing compounds shown in Fig. 1 inhibited α -syn and A β fibrillization *in vitro* and showed protective effects against α -syn and A β 42-induced toxicity in PC12 cells. At 1:1 protein to compound ratio, all compounds tested showed $>$ 90% inhibition of α -syn fibrillization and blocked the growth and seeding capacity of α -syn fibrils. However, at lower protein to compound ratios (10:5 and 10:1), only Q, T, and E showed the strongest inhibition. CA and P were the least effective at lower molar ratios and showed only 40–50% inhibition of α -syn fibril formation.

To probe the specificity of these compounds toward α -syn, we determined their capacity to inhibit the fibrillization of A β 42 monomers, PFs, and fibrils. All the compounds exhibited reduced (55–75%) inhibitory activity against the fibrillization of LMW and PF A β 42, with the exception of Q, which resulted in $>$ 90% reduction in the fibrillization of A β 42 fibril formation. When we examined the effect of these compounds on the fibril-

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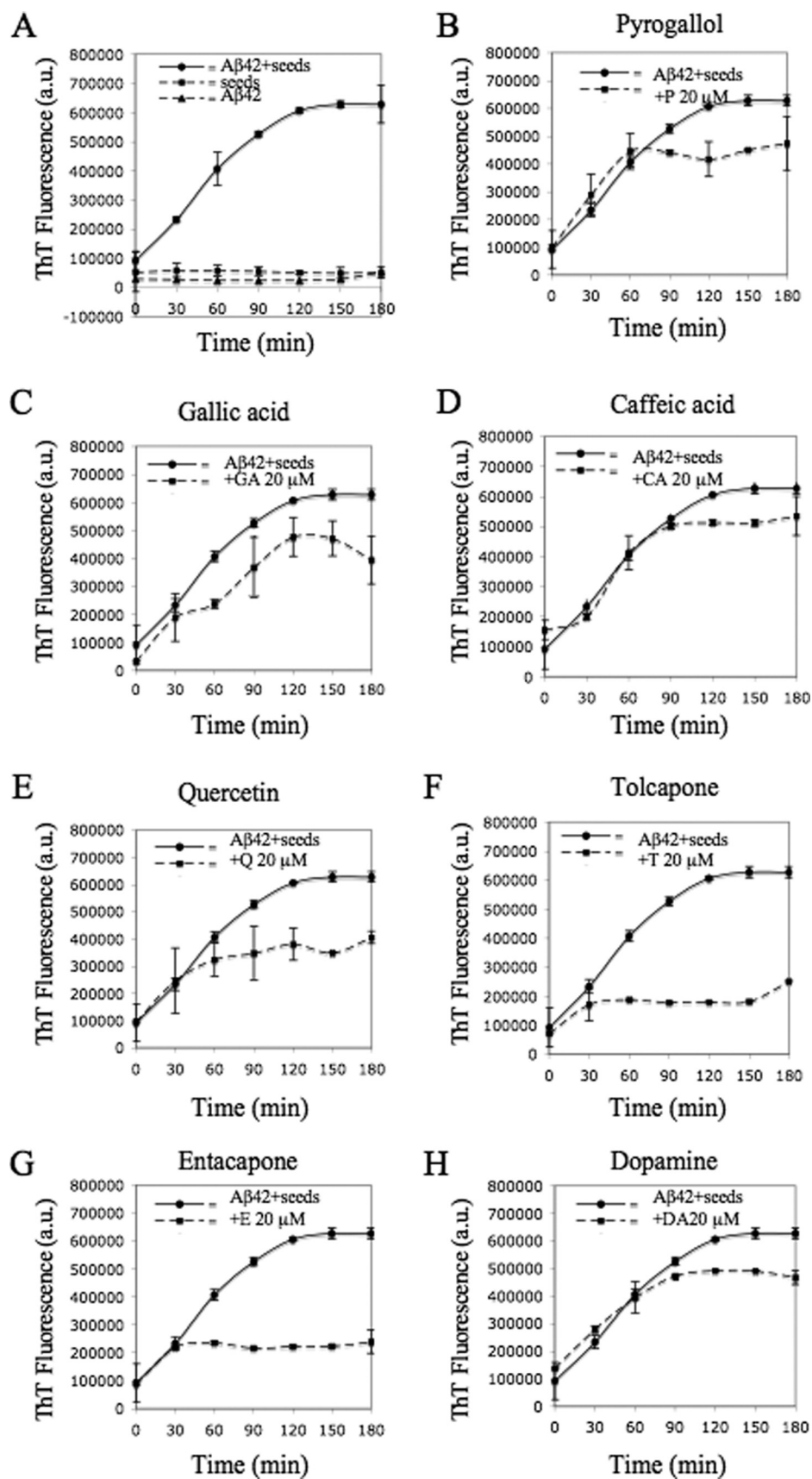


FIGURE 8. $A\beta_{42}$ monomeric seeding polymerization assays revealed that Q, E, and T have an inhibitory effect on the kinetics. Samples of monomeric $A\beta_{42}$ ($10 \mu\text{M}$) were incubated with the seeds ($2 \mu\text{M}$) at 37°C , with continuous shaking, without (A) and with (B–H) $20 \mu\text{M}$ of compounds P, GA, CA, Q, T, E, and DA. The time course of protein fibrillization was measured every 30 min by ThT fluorescence assay for 3 h.

lization of $A\beta_{42}$ PFs, we observed that T, E, and DA showed greater inhibition (80–90%), with Q being the most effective compound. By TEM analysis, we did not observe significant differences in the structural properties of the aggregates and fibrils formed among the various compounds tested.

There are several mechanisms by which these compounds could inhibit amyloid formation as follows: 1) their ability to stabilize the native monomeric state of amyloid; 2) target different intermediates on the amyloid pathway and block their conversion to fibrils; and 3) alter the aggregation pathway in favor of nonamyloidogenic aggregates. In the case of α -syn, both SDS-PAGE and TEM analyses demonstrate that the presence of these compounds enhances the solubility of α -syn (Fig. 2). On the other hand, none of the compounds tested were shown to stabilize monomeric $A\beta_{42}$, instead the efficacy of these compounds appears to be linked to their ability to interfere with $A\beta_{42}$ aggregation at different intermediate stages on the amyloid pathway (Figs. 6A and 7A). All compounds tested were shown to promote the formation of large nonfibrillar aggregates and/or protofibrillar species as discerned by TEM (Figs. 6B and 7B) and the reduced solubility of $A\beta_{42}$ in the presence of some compounds, despite the fact that incubation with such compounds results in significant reduction in the ThT signals. Consistent with the TEM data, we failed to detect significant accumulation of monomeric $A\beta_{42}$ after 96 h of incubation in the presence and absence of compounds. Interestingly, each of the compounds appears to exert very specific effects and shows preference for targeting a different aggregation state on the amyloid pathway. For example, although T and E were equally effective in blocking the fibrillization of $A\beta_{42}$ LMW and PF and showed strong inhibition of $A\beta_{42}$ fibril growth and seeding capacity, DA and Q were most effective against the fibrillization of

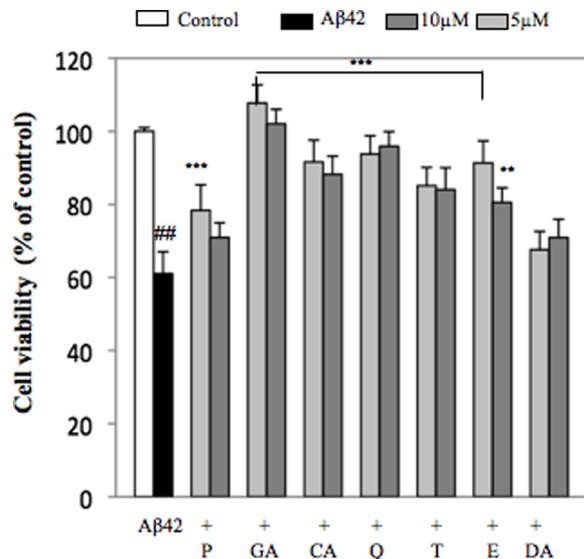


FIGURE 9. Protective effect of the compounds against A β 42-induced toxicity in PC12 cells. PC12 cells were treated with A β 42 (40 μ M) or co-treated in presence of the compounds P, GA, CA, Q, T, E, and DA. The cells were treated with two different compound concentrations, 5 and 20 μ M for 24 h. The cellular viability was evaluated by MTT assay, and the data were expressed as percentage of control (nontreated cells). The control treatment is set to 100%. Error bars are means \pm S.E. We used # to compare the data to the control and * with respect to the treatments with A β 42 crude preparation. ##, $p < 0.001$; ***, $p < 0.001$; **, $p < 0.01$.

A β 42 LMW and PF but did not show significant inhibition of A β 42 fibril growth and seeding capacity. Furthermore, incubation of each compound with A β 42 resulted in the accumulation of A β 42 aggregates of distinct size and morphological properties (Figs. 6B and 7B).

Together, these results suggest that the presence of the catechol moiety in these compounds is sufficient to impart on them an anti-amyloidogenic activity against α -syn and A β 42. However, the TEM and solubility studies demonstrate that the compounds interfere with the fibrillization of A β 42 and α -syn via distinct mechanisms. This hypothesis is further supported by our findings that all the compounds showed strong inhibition of α -syn fibril growth and seeding capacity, whereas only T and E were effective in blocking the growth and seeding capacity of A β 42 fibrils. The remaining (P, CA, GA, Q, and DA) compounds showed minimal effect even at 1:1 molar ratio, in contrast to their ability to block the seeding capacity of α -syn under similar conditions. To determine whether the specificity and potency of these compounds are mediated by their interaction with specific sequences and/or structural motifs within these two proteins, we sought to determine which residues interact with the most potent compounds, Q, T, and E, using NMR. These studies did not show any significant chemical shift changes for any of the compounds, with the exception of very minor chemical shift changes for His-50 and some N-terminal residues, suggesting that there is no direct binding of the compounds to the backbone of α -syn in its monomeric state. However, an increase in NMR signal intensity was observed in the presence of E and T for a large number of residues in the N-terminal domain of α -syn, in particular in the non-amyloid β -component region. This is in clear contrast to the action of the polyphenol (-)-epigallocatechin gallate, which binds to the

backbone of monomeric α -syn, and A β 40, which decreases the NMR signal intensity of monomeric α -syn and redirects both α -syn and A β 40 into unstructured off-pathway oligomers (52). Epigallocatechin gallate targets the polypeptide main chain that is identical in all proteins and easily accessible under unfolded conditions (52). In contrast, ThT fluorescence, SDS-PAGE, and TEM measurements indicate that the COMT inhibitors appear to target a conformational feature of oligomers. Currently, it is not known what this conformational feature is but hydrophobic patches formed in oligomers in a rather unspecific manner could be a potential target. Thus, the COMT inhibitors or their scaffold are potentially more useful lead compounds than epigallocatechin gallate, as they preferentially bind to protein aggregates and not to unfolded polypeptide backbones.

Entacapone, Tolcapone, and Related Catechols Protect against Extracellular α -Syn and A β 42-induced Toxicity in PC12 Cells— To determine whether the ability of these compounds to block and/or alter the fibrillization pathway of A β 42 and α -syn cellular toxicity, we evaluated their protective effect in the cell culture using different assays. At concentrations that showed significant inhibition of A β 42 and α -syn fibrillization, all the compounds were shown to protect PC12 cells from A β 42 and α -syn-induced cytotoxicity, by mechanisms that are directly linked to their ability to modulate the fibrillization of both proteins. These results are in agreement with previous studies. Several small polyphenol molecules were shown to exhibit strong anti-amyloidogenic and neuroprotective properties *in vitro* and *in vivo*. Bastianetto *et al.* (49) reported on the neuroprotective effects of GA and other green and black tea catechin gallate against A β 40 in neuronal cell cultures. Other groups have also demonstrated that catechol and polyphenol compounds are potent anti-amyloid agents and investigated the chemical and structural properties underlying their potency. However, to the best of our knowledge, the anti-amyloidogenic properties of the two nitrocatechols E and T, already known for other biological activities, *i.e.* antioxidants and catechol O-methyltransferase, have never been described in literature.

Although there is strong evidence in support of the protofibril hypothesis, the exact mechanisms by which protofibrils cause toxicity and the identity of the toxic species remain unknown. Protofibrils represent a heterogeneous mixture of aggregates of various size and morphologies, some of which are likely to contribute to toxicity. It is noteworthy that all the fibrillization inhibitors reported in the literature, including those that protect against A β and α -syn toxicity, exert their effect by acting at an intermediate step along the amyloid pathway, *i.e.* there are no known small molecule inhibitors that stabilize monomeric A β and α -syn. As shown in Figs. 6 and 7, the different compounds we tested appear to stabilize or induce the formation of prefibrillar aggregates of distinct morphologies. Together, these results suggest that these molecules act by altering the structure of the aggregates and diverting toxic intermediates toward off-pathway nontoxic species. Studies from several groups have shown that small molecules, including inositol stereoisomers (53), (-)-epigallocatechin gallate (52), as well as A β 42-derived peptides (54), were shown to alter the toxic properties of A β by stabilizing and/or inducing struc-

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tural remodeling of protofibrillar and fibrillar aggregates. Resveratrol blocks A β toxicity without inhibiting oligomer formation (55). Moreover, recent studies from our group (30) and others (56) suggest that amyloid toxicity requires an on-going fibrillization process, *i.e.* the presence of protofibrils or fibril is not sufficient to cause toxicity unless these species are undergoing an on-going fibrillization process.

Previous studies with DA and other catecholamines, *e.g.* apomorphine (57), linked their anti-amyloidogenic properties to their ability to undergo rapid autoxidation in aqueous solution, suggesting that one or more oxidation products is responsible for their inhibitor properties (21). However, the two major products of the catecholamine oxidation, *i.e.* quinines and aminochromes, are relatively unstable and difficult to isolate. To verify this hypothesis, the 3-methoxytyramine, the major metabolite of dopamine, was tested in the same working conditions and in the presence of the two proteins, *i.e.* α -syn and A β 42. As expected, the results obtained confirmed that the methylated derivative of dopamine had no effect on the protein fibrillization process (data not shown). The mechanism by which catechol-containing compounds block protein fibrillization remains controversial. Conway *et al.* (20) reported that DA stabilizes α -syn PFs by forming a DA- α -syn adduct. More recently, Norris *et al.* (21) suggested a novel mechanism of action in which the dopaminochrome, the oxidized product of DA, inhibits α -syn fibrillization by interacting with the specific amino acid motif in the C terminus and non-amyloid β -component region of α -syn (23, 40, 58, 59). Given the structural similarity among E, T, and other known anti-amyloidogenic catechol derivatives such as DA and Q, it is plausible to speculate that a shared mechanism may underlie the effectiveness of all these compounds. All seven molecules shown in Fig. 1 have in common the fact that each possesses at least one aromatic ring with catechol moiety.

Relevance to α -Syn Toxicity and Parkinson Disease—Although predominantly a cytosolic protein, several lines of evidence suggest a potential role of extracellular α -syn in mediating α -syn toxicity, Lewy body formation, and the pathogenesis of PD and related synucleinopathies. These lines of evidence include the following: 1) recent studies from different laboratories have shown that some monomeric and/or soluble aggregated forms of α -syn are secreted and can be detected in the blood plasma and cerebrospinal fluids of patients suffering from PD and related synucleinopathies (60–63); 2) the fact that exogenous aggregated forms of α -syn have been shown to induce microglial activation and stimulate the production of reactive oxygen species (64) and pro-inflammatory factors (65) and are toxic to mammalian cells and primary neurons (66–72); 3) studies that have shown that the cellular uptake of extracellular α -syn occurs by passive diffusion (monomers) or via receptor-mediated endocytic pathways (73) (oligomers and protofibrils), depending on the aggregation state of α -syn (74); and 4) the fact that small amounts of extracellular α -syn aggregates can efficiently catalyze the aggregation of intracellular α -syn inclusions. Specifically, Luk *et al.* (75) demonstrated that α -syn fibrils, prepared from recombinant full-length or truncated α -syn, were uptaken by cells within cultures and act as seeds that catalyzed the aggregation and conversion of soluble

intracellular α -syn into Lewy body-like inclusions. Finally, 5) Desplats *et al.* (76) demonstrated neuron-to-neuron and neuron-to-glia transmission of monomeric and aggregated α -syn species *in vivo* and in cell cultures. These studies suggest that the release and uptake of monomeric and soluble aggregates of α -syn play a central role in inclusion formation, neuronal cell death, and spreading of α -syn pathology in PD. Therefore, targeting the aggregation of extracellular α -syn and/or promoting their clearance have emerged as viable therapeutic strategies for PD and related synucleinopathies. In this regard, the identification of small molecules that block or reverse the aggregation of extracellular α -syn is desirable. Here, we demonstrated that entacapone, tolcapone, and related catechols inhibit α -syn fibrillization *in vitro* and prevent the formation of toxic aggregates as discerned by their protection against α -syn induced extracellular toxicity in PC12 cells.

Currently, the most effective treatment for PD continues to be the administration of L-DOPA together with a peripheral L-amino acid decarboxylase inhibitor that is unable to enter the central nervous system. However, the amount of L-DOPA reaching the brain after oral administration is very low (about 5–10%). Furthermore, the subsequent metabolism of L-DOPA by COMT clearly limits its availability in the brain. The two COMT inhibitors E and T, approved as adjuncts in the therapy of PD, increase the availability of L-DOPA for conversion to dopamine in the brain (25) mainly by preventing the extensive metabolism of L-DOPA through O-methylation in the periphery (E and T) and partly in brain (T) (77). However, the potential for the use of T as neuroprotective drug in AD or PD is limited due to the fact that it has been shown to cause severe hepatotoxicity resulting in its withdrawal from the market in many countries leaving entacapone as the only COMT inhibitor presently available in the clinic for the treatment of PD (78). However, recent studies demonstrate that tolcapone can be used with benefit when the liver function is actively monitored (79, 80). Although these two compounds share the same pharmacophore, their pharmacokinetic profiles are remarkably different (81). Indeed, studies with rats have shown that tolcapone has a longer duration of action than entacapone and is both a central and peripheral COMT inhibitor, whereas entacapone is essentially a peripheral inhibitor (82–85). However, the benefits of both entacapone and tolcapone in the L-DOPA treatment of patients suffering of Parkinson disease were proved (86–88).

Conclusions—In summary, our study showed that entacapone and tolcapone are potent inhibitors of α -syn and A β oligomerization and fibrillogenesis and protect against extracellular toxicity induced by the aggregation of both proteins. Our results provide additional evidence for the potential of catechols as anti-amyloidogenic agents and demonstrate that entacapone and tolcapone belong to the classes of multifunctional drugs (89) because they can inhibit COMT and act as good antioxidants and as effective inhibitors of protein aggregation (90). Whether the anti-amyloidogenic property of entacapone and tolcapone and the protection against α -syn extracellular toxicity contribute to their clinical benefits and enhanced symptomatic treatment of PD or not remains to be determined. Nonetheless, our findings suggest that the structure of entacapone and tolcapone constitute molecular scaffolds

folds that could guide the development of more potent inhibitors of amyloid formation and toxicity. Chemical modifications of entacapone and tolcapone can be envisaged to optimize its pharmacokinetic profile especially by avoiding hepatotoxicity (91), modeling its peripheral metabolism and increasing its blood-brain barrier permeation.

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REFERENCES

- Recchia, A., Debetto, P., Negro, A., Guidolin, D., Skaper, S. D., and Giusti, P. (2004) *FASEB J.* **18**, 617–626
- Sommer, D. B., and Stacy, M. A. (2008) *Expert Rev. Neurother.* **8**, 1829–1839
- Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) *Nature* **388**, 839–840
- Cookson, M. R. (2009) *Mol. Neurodegener.* **4**, 9
- Chung, K. K., Dawson, V. L., and Dawson, T. M. (2003) *J. Neurol.* **250**, Suppl. 3, III15–24
- Li, J., Uversky, V. N., and Fink, A. L. (2001) *Biochemistry* **40**, 11604–11613
- Zarranz, J. J., Alegre, J., Gómez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Muñoz, D. G., and de Yebenes, J. G. (2004) *Ann. Neurol.* **55**, 164–173
- Chen, L., and Feany, M. B. (2005) *Nat. Neurosci.* **8**, 657–663
- Lo Bianco, C., Shorter, J., Régulier, E., Lashuel, H., Iwatsubo, T., Lindquist, S., and Aebischer, P. (2008) *J. Clin. Invest.* **118**, 3087–3097
- Masliyah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000) *Science* **287**, 1265–1269
- El-Agnaf, O. M., Curran, M. D., Wallace, A., Middleton, D., Murgatroyd, C., Curtis, A., Perry, R., and Jaros, E. (1998) *Neuroreport* **9**, 3925–3927
- Paleologou, K. E., Schmid, A. W., Rospigliosi, C. C., Kim, H. Y., Lambert, G. R., Fredenburg, R. A., Lansbury, P. T., Jr., Fernandez, C. O., Eliezer, D., Zweckstetter, M., and Lashuel, H. A. (2008) *J. Biol. Chem.* **283**, 16895–16905
- Conway, K. A., Harper, J. D., and Lansbury, P. T., Jr. (2000) *Biochemistry* **39**, 2552–2563
- Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Harper, J. D., Williamson, R. E., and Lansbury, P. T., Jr. (2000) *Ann. N.Y. Acad. Sci.* **920**, 42–45
- Horwich, A. (2002) *J. Clin. Invest.* **110**, 1221–1232
- Losic, D., Martin, L. L., Mechler, A., Aguilar, M. I., and Small, D. H. (2006) *J. Struct. Biol.* **155**, 104–110
- Morgan, C., Colombres, M., Nuñez, M. T., and Inestrosa, N. C. (2004) *Prog. Neurobiol.* **74**, 323–349
- Cappai, R., Leck, S. L., Tew, D. J., Williamson, N. A., Smith, D. P., Galatis, D., Sharples, R. A., Curtain, C. C., Ali, F. E., Cherny, R. A., Culvenor, J. G., Bottomley, S. P., Masters, C. L., Barnham, K. J., and Hill, A. F. (2005) *FASEB J.* **19**, 1377–1379
- Li, H. T., Lin, D. H., Luo, X. Y., Zhang, F., Ji, L. N., Du, H. N., Song, G. Q., Hu, J., Zhou, J. W., and Hu, H. Y. (2005) *FEBS J.* **272**, 3661–3672
- Conway, K. A., Rochet, J. C., Bieganski, R. M., and Lansbury, P. T., Jr. (2001) *Science* **294**, 1346–1349
- Norris, E. H., Giasson, B. I., Hodara, R., Xu, S., Trojanowski, J. Q., Ischiropoulos, H., and Lee, V. M. (2005) *J. Biol. Chem.* **280**, 21212–21219
- Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F., and Fink, A. L. (2004) *J. Biol. Chem.* **279**, 26846–26857
- Li, J., Zhu, M., Manning-Bog, A. B., Di Monte, D. A., and Fink, A. L. (2004) *FASEB J.* **18**, 962–964
- Ono, K., Hirohata, M., and Yamada, M. (2007) *J. Neurosci. Res.* **85**, 1547–1557
- Bonifácio, M. J., Palma, P. N., Almeida, L., and Soares-da-Silva, P. (2007) *CNS Drug Rev.* **13**, 352–379
- Johnson, K. S. (2005) *J. Agric. Food Chem.* **53**, 10120–10126
- Han, Y. H., Kim, S. Z., Kim, S. H., and Park, W. H. (2008) *Int. J. Mol. Med.* **21**, 721–730
- Dodo, K., Minato, T., Noguchi-Yachide, T., Suganuma, M., and Hashimoto, Y. (2008) *Bioorg. Med. Chem.* **16**, 7975–7982
- Armagan, A., Uzar, E., Uz, E., Yilmaz, H. R., Kutluhan, S., Koyuncuoglu, H. R., Soyupek, S., Cam, H., and Serel, T. A. (2008) *Hum. Exp. Toxicol.* **27**, 547–552
- Jan, A., Gokce, O., Luthi-Carter, R., and Lashuel, H. A. (2008) *J. Biol. Chem.* **283**, 28176–28189
- Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci.* **4**, 2411–2423
- Naiki, H., and Nakakuki, K. (1996) *Lab. Invest.* **74**, 374–383
- Grzesiek, S., Stahl, S. J., Wingfield, P. T., and Bax, A. (1996) *Biochemistry* **35**, 10256–10261
- Goddard, T. D., and Kneller, D. G. (2003) *SPARKY 3*, University of California, San Francisco
- Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) *J. Biomol. NMR* **6**, 277–293
- Bertoncini, C. W., Jung, Y. S., Fernandez, C. O., Hoyer, W., Griesinger, C., Jovin, T. M., and Zweckstetter, M. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 1430–1435
- Doré, S., Kar, S., and Quirion, R. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4772–4777
- Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T., Jr. (1999) *Biochemistry* **38**, 8972–8980
- Grimminger-Marquardt, V., and Lashuel, H. A. (2009) *Biopolymers* **93**, 252–276
- Jarrett, J. T., and Lansbury, P. T., Jr. (1993) *Cell* **73**, 1055–1058
- Harper, J. D., and Lansbury, P. T., Jr. (1997) *Annu. Rev. Biochem.* **66**, 385–407
- Harper, J. D., Lieber, C. M., and Lansbury, P. T., Jr. (1997) *Chem. Biol.* **4**, 951–959
- Craik, D. J., and Wilce, J. A. (1997) *Methods Mol. Biol.* **60**, 195–232
- Fernández, C. O., Hoyer, W., Zweckstetter, M., Jares-Erijman, E. A., Subramaniam, V., Griesinger, C., and Jovin, T. M. (2004) *EMBO J.* **23**, 2039–2046
- Lansbury, P. T., and Lashuel, H. A. (2006) *Nature* **443**, 774–779
- Caughey, B., and Lansbury, P. T. (2003) *Annu. Rev. Neurosci.* **26**, 267–298
- Ansari, M. A., Abdul, H. M., Joshi, G., Opii, W. O., and Butterfield, D. A. (2009) *J. Nutr. Biochem.* **20**, 269–275
- Ban, J. Y., Nguyen, H. T., Lee, H. J., Cho, S. O., Ju, H. S., Kim, J. Y., Bae, K., Song, K. S., and Seong, Y. H. (2008) *Biol. Pharm. Bull.* **31**, 149–153
- Bastianetto, S., Yao, Z. X., Papadopoulos, V., and Quirion, R. (2006) *Eur. J. Neurosci.* **23**, 55–64
- Wang, Y. J., Thomas, P., Zhong, J. H., Bi, F. F., Kosaraju, S., Pollard, A., Fenech, M., and Zhou, X. F. (2009) *Neurotox. Res.* **15**, 3–14
- Sul, D., Kim, H. S., Lee, D., Joo, S. S., Hwang, K. W., and Park, S. Y. (2009) *Life Sci.* **84**, 257–262
- Ehrnhoefer, D. E., Bieschke, J., Boeddrich, A., Herbst, M., Masino, L., Lurz, R., Engemann, S., Pastore, A., and Wanker, E. E. (2008) *Nat. Struct. Mol. Biol.* **15**, 558–566
- McLaurin, J., Golomb, R., Jurewicz, A., Antel, J. P., and Fraser, P. E. (2000) *J. Biol. Chem.* **275**, 18495–18502
- Fradinger, E. A., Monien, B. H., Urbanc, B., Lomakin, A., Tan, M., Li, H., Spring, S. M., Condrón, M. M., Cruz, L., Xie, C. W., Benedek, G. B., and Bitan, G. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 14175–14180
- Feng, Y., Wang, X. P., Yang, S. G., Wang, Y. J., Zhang, X., Du, X. T., Sun, X. X., Zhao, M., Huang, L., and Liu, R. T. (2009) *Neurotoxicology* **30**, 986–995
- Wogulis, M., Wright, S., Cunningham, D., Chilcote, T., Powell, K., and Rydel, R. E. (2005) *J. Neurosci.* **25**, 1071–1080
- Lashuel, H. A., Hartley, D. M., Balakhaneh, D., Aggarwal, A., Teichberg, S., and Callaway, D. J. (2002) *J. Biol. Chem.* **277**, 42881–42890
- Herrera, F. E., Chesi, A., Paleologou, K. E., Schmid, A., Munoz, A., Vendruscolo, M., Gustincich, S., Lashuel, H. A., and Carloni, P. (2008) *PLoS One*

- 3, e3394
59. Yamin, G., Munishkina, L. A., Karymov, M. A., Lyubchenko, Y. L., Uversky, V. N., and Fink, A. L. (2005) *Biochemistry* **44**, 9096–9107
60. Borghi, R., Marchese, R., Negro, A., Marinelli, L., Forloni, G., Zaccheo, D., Abbruzzese, G., and Tabaton, M. (2000) *Neurosci. Lett.* **287**, 65–67
61. El-Agnaf, O. M., Salem, S. A., Paleologou, K. E., Curran, M. D., Gibson, M. J., Court, J. A., Schlossmacher, M. G., and Allsop, D. (2006) *FASEB J.* **20**, 419–425
62. Lee, H. J., Suk, J. E., Bae, E. J., Lee, J. H., Paik, S. R., and Lee, S. J. (2008) *Int. J. Biochem. Cell Biol.* **40**, 1835–1849
63. Tokuda, T., Salem, S. A., Allsop, D., Mizuno, T., Nakagawa, M., Qureshi, M. M., Locascio, J. J., Schlossmacher, M. G., and El-Agnaf, O. M. (2006) *Biochem. Biophys. Res. Commun.* **349**, 162–166
64. Zhang, W., Wang, T., Pei, Z., Miller, D. S., Wu, X., Block, M. L., Wilson, B., Zhang, W., Zhou, Y., Hong, J. S., and Zhang, J. (2005) *FASEB J.* **19**, 533–542
65. Klegeris, A., Pelech, S., Giasson, B. I., Maguire, J., Zhang, H., McGeer, E. G., and McGeer, P. L. (2008) *Neurobiol. Aging* **29**, 739–752
66. El-Agnaf, O. M., Jakes, R., Curran, M. D., Middleton, D., Ingenito, R., Bianchi, E., Pessi, A., Neill, D., and Wallace, A. (1998) *FEBS Lett.* **440**, 71–75
67. Albani, D., Peverelli, E., Rametta, R., Batelli, S., Veschini, L., Negro, A., and Forloni, G. (2004) *FASEB J.* **18**, 1713–1715
68. Du, H. N., Tang, L., Luo, X. Y., Li, H. T., Hu, J., Zhou, J. W., and Hu, H. Y. (2003) *Biochemistry* **42**, 8870–8878
69. Forloni, G., Bertani, I., Calella, A. M., Thaler, F., and Invernizzi, R. (2000) *Ann. Neurol.* **47**, 632–640
70. Lee, E. N., Cho, H. J., Lee, C. H., Lee, D., Chung, K. C., and Paik, S. R. (2004) *Biochemistry* **43**, 3704–3715
71. Seo, J. H., Rah, J. C., Choi, S. H., Shin, J. K., Min, K., Kim, H. S., Park, C. H., Kim, S., Kim, E. M., Lee, S. H., Lee, S., Suh, S. W., and Suh, Y. H. (2002) *FASEB J.* **16**, 1826–1828
72. Sung, J. Y., Kim, J., Paik, S. R., Park, J. H., Ahn, Y. S., and Chung, K. C. (2001) *J. Biol. Chem.* **276**, 27441–27448
73. Ahn, K. J., Paik, S. R., Chung, K. C., and Kim, J. (2006) *J. Neurochem.* **97**, 265–279
74. Lee, H. J., Suk, J. E., Bae, E. J., and Lee, S. J. (2008) *Biochem. Biophys. Res. Commun.* **372**, 423–428
75. Luk, K. C., Song, C., O'Brien, P., Stieber, A., Branch, J. R., Brunden, K. R., Trojanowski, J. Q., and Lee, V. M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 20051–20056
76. Desplats, P., Lee, H. J., Bae, E. J., Patrick, C., Rockenstein, E., Crews, L., Spencer, B., Masliah, E., and Lee, S. J. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13010–13015
77. Lapish, C. C., Ahn, S., Evangelista, L. M., So, K., Seamans, J. K., and Phillips, A. G. (2009) *Psychopharmacology* **202**, 521–530
78. Assal, F., Spahr, L., Hadengue, A., Rubbia-Brandt, L., and Burkhard, P. R. (1998) *Lancet* **352**, 958
79. Antonini, A., Abbruzzese, G., Barone, P., Bonuccelli, U., Lopiano, L., Onofri, M., Zappia, M., and Quattrone, A. (2008) *Neuropsychiatr. Dis. Treat.* **4**, 1–9
80. Truong, D. D. (2009) *Clin. Interv. Aging* **4**, 109–113
81. Novaroli, L., Bouchard Doulakas, G., Reist, M., Rolando, B., Fruttero, R., Gasco, A., and Carrupt, P. A. (2006) *Helv. Chim. Acta* **89**, 144–152
82. Di Stefano, A., Sozio, P., Iannitelli, A., and Cerasa, L. S. (2009) *Expert Opin. Drug Delivery* **6**, 389–404
83. Forsberg, M., Lehtonen, M., Heikkinen, M., Savolainen, J., Järvinen, T., and Männistö, P. T. (2003) *J. Pharmacol. Exp. Ther.* **304**, 498–506
84. Learmonth, D. A., Palma, P. N., Vieira-Coelho, M. A., and Soares-da-Silva, P. (2004) *J. Med. Chem.* **47**, 6207–6217
85. Nissinen, E., Lindén, I. B., Schultz, E., and Pohto, P. (1992) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **346**, 262–266
86. Haefeli, W. (2007) *J. Neurol.* **254**, 29–36
87. Lees, A. J., Ratziu, V., Tolosa, E., and Oertel, W. H. (2007) *J. Neurolog. Neurosurg. Psychiat.* **78**, 944–948
88. Damier, P., Viallet, F., Ziegler, M., Bourdeix, I., and Rerat, K. (2008) *Eur. J. Neurol.* **15**, 643–648
89. Novaroli, L., Daina, A., Bertolini, F., Di Giovanni, S., Bravo, J., Reist, M., and Carrupt, P. A. (2005) *Chimia* **59**, 315–320
90. Bertolini, F., Novaroli, L., Carrupt, P. A., and Reist, M. (2007) *J. Pharmacol. Sci.* **96**, 2931–2944
91. Boelsterli, U. A., Ho, H. K., Zhou, S., and Leow, K. Y. (2006) *Curr. Drug Metab.* **7**, 715–727