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Small Molecule Inhibitors of α-Synuclein Filament Assembly†

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ABSTRACT: α-Synuclein is the major component of the filamentous inclusions that constitute defining characteristics of Parkinson's disease and other α-synucleinopathies. Here we have tested 79 compounds belonging to 12 different chemical classes for their ability to inhibit the assembly of α-synuclein into filaments in vitro. Several polyphenols, phenothiazines, porphyrins, polyene macrolides, and Congo red and its derivatives, BSB and FSB, inhibited α-synuclein filament assembly with IC_{50} values in the low micromolar range. Many compounds that inhibited α-synuclein assembly were also found to inhibit the formation of Aβ and tau filaments. Biochemical analysis revealed the formation of soluble oligomeric α-synuclein in the presence of inhibitory compounds, suggesting that this may be the mechanism by which filament formation is inhibited. Unlike α-synuclein filaments and protofibrils, these soluble oligomeric species did not reduce the viability of SH-SY5Y cells. These findings suggest that the soluble oligomers formed in the presence of inhibitory compounds may not be toxic to nerve cells and that these compounds may therefore have therapeutic potential for α-synucleinopathies and other brain amyloidoses.

Filamentous inclusions made of the protein α-synuclein in nerve cells or glial cells are the defining neuropathological feature of a group of neurodegenerative diseases which include Parkinson's disease (PD),[1] dementia with Lewy bodies (DLB), and multiple-system atrophy (MSA) (*1*). In these so-called "α-synucleinopathies", α-synuclein is deposited in a hyperphosphorylated form (*2*−*8*). Missense mutations (A30P, E46K, and A53T) in the α-synuclein gene cause familial forms of PD and DLB (*9*−*11*). Furthermore, multiplications (duplication and triplication) of a region on the long arm of chromosome 4 that encompasses the α-synuclein gene cause an inherited form of PD dementia (*12*−*14*), indicating that the simple overproduction of wild-type α-synuclein is sufficient to cause PD dementia.

α-Synuclein is a 140-amino acid protein of unknown function that is abundantly expressed in brain, where it is concentrated in presynaptic nerve terminals (*15*, *16*). The amino-terminal region of α-synuclein (residues 7−87) consists of seven imperfect repeats, each 11 amino acids in length, with the consensus sequence KTKEGV. The repeats are continuous, except for a four-amino acid stretch between repeats 4 and 5, and partially overlap with a hydrophobic region (amino acids 61−95). The carboxy-terminal region (amino acids 96−140) is negatively charged. α-Synuclein is a natively unfolded protein with little ordered secondary structure that binds to lipid membranes through its amino-terminal repeats, indicating that it may be a physiological lipid-binding protein (*17*−*21*). Upon binding to lipid membranes, α-synuclein adopts structures rich in α-helical character (*18*, *20*−*25*).

Recombinant α-synuclein readily assembles into filaments that share many of the morphological and ultrastructural characteristics of the filaments present in human brain (*26*−*29*). Assembly is a nucleation-dependent process and occurs through sequences located in the 100 amino-terminal amino acids of α-synuclein (*30*, *31*). The carboxy-terminal region, in contrast, inhibits assembly (*26*, *29*, *32*). Although α-synuclein is largely unstructured, its negatively charged C-terminus makes long-range contacts with the central

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[1] Abbreviations: PD, Parkinson's disease; DLB, dementia with Lewy bodies; MSA, multiple-system atrophy; ThS, thioflavin S; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; ThT, thioflavin T; DAPH, 4,5-dianilinophthalimide; BSB, 1-bromo-2,5-bis(3-carboxystyryl)benzene; FSB, 1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxystyryl)benzene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PET, positron emission tomography; MRI, magnetic resonance imaging; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17.

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hydrophobic region (*33*, *34*). Perturbation of these interactions is likely to expose hydrophobic surfaces, facilitating filament formation.

Mutations E46K and A53T in α-synuclein have been found to accelerate the rate of filament assembly (*28*, *35*, *36*). The A30P mutation has been reported to increase the total rate of aggregation of α-synuclein (*37*−*39*) but to slow the rate of formation of mature filaments (*40*). X-ray fiber diffraction and electron diffraction analyses have shown that the transition from natively unfolded to cross-β structure underlies the assembly of α-synuclein into filaments (*29*). It is therefore appropriate to consider the α-synucleinopathies a form of brain amyloidosis.

The conversion of a small number of soluble peptides and proteins into insoluble filaments is believed to be the central event in the pathogenesis of the most common neurodegenerative diseases (*41*, *42*). Consequently, many current therapeutic strategies are aimed at inhibiting filament formation and at promoting filament clearance. They include the use of antibodies, synthetic peptides, molecular chaperones, and chemical compounds. Among these, small organic molecules have been extensively tested for their ability to inhibit filament formation in vitro, particularly in relation to Aβ deposition (*43*), the formation of protease-resistant forms of the prion protein (*44*), the aggregation of huntingtin (*45*), and, more recently, the heparin-induced formation of tau filaments (*46*, *47*). Less is known about small organic molecules that inhibit the formation of α-synuclein filaments. Several studies have described inhibition of filament formation by catecholamines (*48*−*50*), with other studies reporting inhibition by the polyphenol baicalein (*51*), the porphyrin phthalocyanine (*52*), and the anti-tuberculosis drug rifampicin (*53*).

Here we have investigated the effects of 79 compounds belonging to 12 different chemical classes on α-synuclein filament formation. The ability of these compounds to inhibit the assembly of Aβ and tau protein was investigated in parallel. Filament formation was assessed by electron microscopy, thioflavin S (ThS) fluorescence, and the formation of sarkosyl-insoluble α-synuclein. Compounds belonging to seven chemical classes (polyphenols, porphyrins, phenothiazines, polyene macrolides, rifamycins, Congo red and derivatives, and terpenoids) inhibited α-synuclein filament formation. By SDS−PAGE, inhibition of assembly was reflected in a large reduction in the level of sarkosyl-insoluble α-synuclein and the accumulation of soluble, SDS-resistant dimers and oligomers. Unlike α-synuclein filaments and protofibrils, these soluble species did not reduce the viability of human dopaminergic neuroblastoma SH-SY5Y cells.

EXPERIMENTAL PROCEDURES

Expression and Purification of α*-Synuclein Proteins.* Human α-synuclein(1−140) and α-synuclein(1−120) were expressed in *Escherichia coli* BL21(DE3) cells and purified using boiling, Q-Sepharose (for 1−140) or SP-Sepharose (for 1−120) ion exchange chromatography, and ammonium sulfate precipitation, as described previously (*54*). During the course of the analysis of dimeric α-synuclein, we noticed that a cysteine residue was incorporated at position 136 instead of a tyrosine in ~20% of bacterially expressed human α-synuclein and that mutagenesis of codon 136 (TAC to TAT) resulted in the expression of α-synuclein lacking cysteine (*55*). Therefore, the Y136-TAT construct was used in all experiments. α-Synuclein proteins were dialyzed against 30 mM Tris-HCl (pH 7.5) and cleared by centrifugation at 113000*g* for 20 min. Following separation by reverse phase high-performance liquid chromatography (HPLC) (Aquapore RP300 column), the absorbance at 215 nm was measured and compared with that of α-synuclein of known concentration, to give the concentration of the freshly purified proteins. For immunoblotting, aliquots of reaction mixtures were separated by SDS−PAGE, blotted onto a PVDF membrane, blocked with 3% gelatin/PBS, and incubated overnight at room temperature with antibody Syn102 (1:1000 dilution), which recognizes residues 131−140 of human α-synuclein, in a 10% fetal calf serum/PBS solution. Following washing, the blots were incubated for 2 h at room temperature with biotinylated secondary antibody (1:500) (Vector Laboratories). Following further washing, the blots were incubated with peroxidase-labeled avidin−biotin (Vector Laboratories) for 30 min at room temperature and developed with NiCl-enhanced diaminobenzidine (Sigma).

α-Synuclein Filament Assembly and Inhibitor Testing. The compounds listed in Table 1 were dissolved in dimethyl sulfoxide (DMSO) and kept as 20 mM stock solutions. Purified recombinant human α-synuclein(1−140) and α-synuclein(1−120) (2 mg/mL) were incubated with shaking (200 rpm) at 37 °C for 72 h in 50 μL of 30 mM Tris-HCl (pH 7.5) containing 0.02% sodium azide, in the presence or absence of 200 μM compound. For a quantitative assessment of filament formation, the amount of sarkosyl-insoluble α-synuclein and the level of ThS fluorescence were measured. Sarkosyl-soluble and sarkosyl-insoluble α-synuclein were prepared as follows. Aliquots (10 μL) of assembly mixtures were removed and added to 40 μL of 30 mM Tris-HCl (pH 7.5) containing 1% sarkosyl. This was followed by a 20 min centrifugation at 453000*g*. The supernatants (sarkosyl-soluble) were removed and the pellets (sarkosyl-insoluble) resuspended in 50 μL of sample buffer, followed by SDS−PAGE. Following staining with Coomassie Brilliant Blue, the intensities of the sarkosyl-insoluble α-synuclein bands were quantified by scanning densitometry, as described previously (*47*). The supernatants were stained with the silver stain MS kit (Wako). For ThS fluorescence, aliquots (10 μL) of the assembly mixtures were removed and brought to 300 μL with 5 μM ThS (Sigma-Aldrich) in 20 mM MOPS (pH 6.8). Fluorimetry was performed using a Hitachi F4000 fluorescence spectrophotometer (set at 440 nm for excitation and 521 nm for emission), as described previously (*47*). The results were expressed as the percentage of α-synuclein assembly in the absence of compound (taken to be 100%). Statistical analysis was carried out with an unpaired *t* test using Kai plot software, and the results were expressed as the means ± the standard deviation. IC_{50} values were calculated for each compound by quantifying the amounts of sarkosyl-insoluble α-synuclein. For a semiquantitative assessment of filament formation, electron microscopy was used. Aliquots of assembly mixtures were placed on collodion-coated 300-mesh copper grids, stained with 2% phosphotungstate, and micrographs were recorded at a nominal magnification of 10000× on a JEOL 1200EX electron microscope.

Table 1: Inhibitors of Aβ, α-Synuclein, and Tau Filament Formation

no.	compound	IC_{50} (μM) Aβ	α-synuclein	tau
polyphenols				
A1	apigenin	>40	>80	>200
A2	baicalein	4.5	8.2	2.7
A3	(+)-catechin	>40	>80	>200
A4	(-)-catechin gallate	5	21.4	>200
A5	chlorogenic acid	>40	>80	>200
A6	curcumin	1.7	>80	>200
A7	cyanidin	4	10.3	33.3
A8	daidzein	>40	>80	>200
A9	delphinidin	3	6.5	6.9
A10	2,2′-dihydroxybenzophenone	>40	>80	>200
A11	4,4′-dihydroxybenzophenone	>40	>80	>200
A12	dopamine chloride	28.6	7.1	>200
A13	(−)-epicatechin	>40	29.9	>200
A14	(−)-epicatechin 3-gallate	3	14.5	4.5
A15	epigallocatechin	7	10.6	8.4
A16	epigallocatechin gallate	2	9.8	9.6
A17	exifone	0.7	2.5	3.3
A18	(−)-gallocatechin	7	8.9	13.3
A19	(−)-gallocatechin gallate	1.5	3.6	1
A20	gingerol	25	>80	>200
A21	gossypetin	1.3	5.6	2
A22	hinokiflavone	5	8.1	>200
A23	hypericin	0.9	7.5	26.8
A24	kaempferol	8	>80	>200
A25	luteolin	3	28	>200
A26	myricetin	0.9	13.3	1.2
A27	naringenin	25	>80	>200
A28	2,3,4,2′,4′-pentahydroxybenzophenone	2.8	28.3	2.4
A29	procyanidin B1	14	7.3	27.5
A30	procyanidin B2	>40	4.3	>200
A31	purpurogallin	0.5	12.9	5.6
A32	quercetin	5	20	>200
A33	rosmarinic acid	12	4.8	16.6
A34	rutin	32	>80	>200
A35	(+)-taxifolin	>40	>80	>200
A36	2,2′,4,4′-tetrahydroxybenzophenone	>40	>80	>200
A37	theaflavine	2	5.8	7.9
A38	(+)-α-tocopherol	>40	10.9	107.7
A39	2,3,4-trihydroxybenzophenone	3.1	18.6	12.2
anthracycline				
B1	daunorubicin hydrochloride	1.4	>80	>200
benzothiazoles				
C1	2-(4-aminophenyl)-6-methyl-benzothiazole	2	>80	>200
C2	basic blue 41	1.4	>80	>200
C3	2-[4-(dimethylamino)phenyl]-6-methylbenzothiazole	2	>80	>200
C4	3,3′-dipropyl thiodicarbocyanine iodide (DTCI)	0.3	>80	>200
lignans				
D1	magnolol	>40	>80	>200
D2	sesamin	>40	>80	>200
phenothiazines				
E1	acetopromazine maleate salt	>40	>80	>200
E2	azure A	0.4	>80	2.6
E3	azure C	0.2	>80	1.9
E4	chlorpromazine hydrochloride	>40	>80	>200
E5	lacmoid	1.4	14.7	31.2
E6	methylene blue	2.3	>80	1.9
E7	perphenazine	>40	38.7	>200
E8	promazine hydrochloride	>40	>80	>200
E9	propionylpromazine hydrochloride	>40	>80	>200
E10	quinacrine	8.4	>80	79.6
E11	quinacrine mustard	1.2	>80	3.1
polyene macrolides				
F1	amphotericin B	2.2	27.1	>200
F2	filipin III	14.6	78.2	>200
porphyrins				
G1	ferric dehydroporphyrin IX	0.2	9.7	1.4
G2	hematin (from bovine blood)	0.2	15	10.4
G3	phthalocyanine tetrasulfonate	3.2	27.5	67
rifamycin				
H1	rifampicin	4.9	46.2	>200
steroids				
I1	taurochenodeoxycholic acid	>40	>80	>200
I2	taurohydroxycholic acid	>40	>80	>200
I3	taurolithocholic acid	>40	>80	>200
I4	taurolithocholic acid 3-sulfate	>40	>80	>200
I5	tauroursodeoxycholic acid	>40	>80	>200
Congo red and derivatives				
J1	Congo red	0.9	2.3	2.2
J2	chlorazol black E	0.3	16.4	>200
J3	BSB	6.4	4	18.2
J4	FSB	1.9	12.4	35.7
J5	Ponceau SS	1.2	>80	>200
terpenoids				
K1	asiatic acid	>40	45	>200
K2	ginkgolide A	>40	>80	>200
K3	ginkgolide B	11	>80	>200
K4	ginkgolide C	>40	>80	>200
others				
L1	4,5-dianilinophthalimide (DAPH)	2.9	>80	>200
L2	methyl yellow	1.5	>80	>200

Aβ and Tau Filament Assemblies and Inhibitor Testing. Monomeric Aβ(1−40) (1 mM, Peptide Institute Inc., Osaka, Japan) in distilled water was diluted with phosphate-buffered saline (PBS, pH 7.5) to a final concentration of 15 μM and incubated at 37 °C for 24 h in 40 μL of PBS, containing 5 μM thioflavin T (ThT), in the presence or absence of compounds (0.3−40 μM). Fluorimetry was performed using a Biolumin960 fluorescence spectrophotometer (set at 450 nm for excitation and 485 nm for emission, Amersham Biosciences) (*56*), and IC_{50} values were calculated. For the analysis of oligomeric Aβ, Aβ(1−40) was incubated at 37 °C for 4 days in 40 μL of PBS at a concentration of 75 μM in the presence or absence of 200 μM compound. Aliquots (10 μL) of mixtures were removed and added to 40 μL of 30 mM Tris-HCl (pH 7.5) containing 1% sarkosyl and the mixtures centrifuged at 25 °C for 20 min at 453000*g*. The supernatants (sarkosyl-soluble) were removed and the pellets (sarkosyl-insoluble) resuspended in 50 μL of sample buffer, run on a SDS−PAGE gel, and visualized by silver staining. The 412-amino acid isoform of human brain tau (*57*) was expressed from cDNA clone htau46 and purified, as described previously (*47*, *58*). The effect of inhibitory compounds on the heparin-induced filament formation of tau was measured by quantifying the levels of Sarkosyl-insoluble tau (*47*).

Preparation of α-Synuclein Filaments, Protofibrils, and Soluble Oligomers. α-Synuclein filaments were obtained by pelleting the assembly mixtures at 113000*g* for 20 min. Protofibrils were prepared as described previously (*59*). Briefly, purified α-synuclein was dialyzed against 20 mM NH_4HCO_3, lyophilized, and resuspended in PBS at 20 mg/mL. Following a 5 min centrifugation at 20000*g*, the supernatants were loaded onto a Superdex 200 gel filtration column (1 cm × 30 cm), eluted in 10 mM Tris-HCl (pH

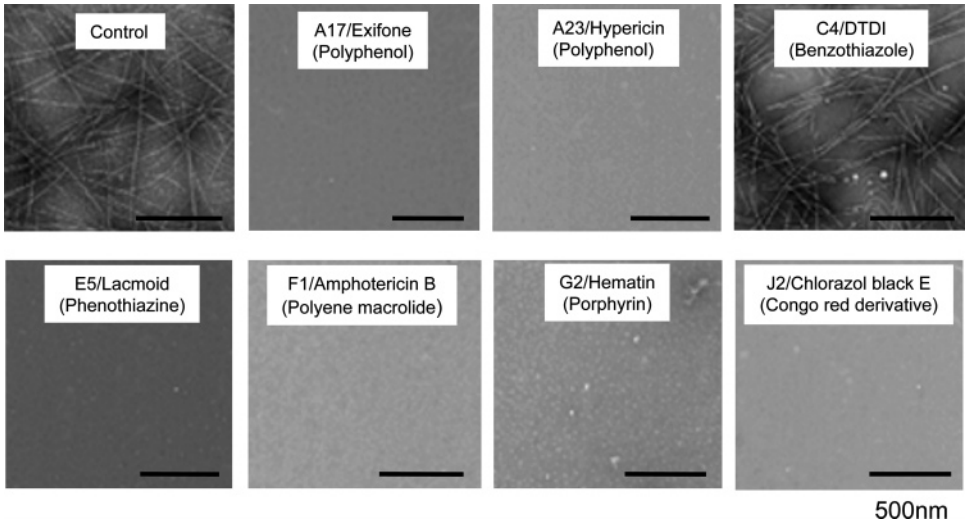

FIGURE 1: Semiquantitative assessment by electron microscopy of α-synuclein filament formation in the absence (Control) or presence of compounds. Small numbers of filaments or no filaments were seen in the presence of exifone (A17), hypericin (A23), lacmoid (E5), amphotericin B (F1), hematin (G2), and chlorazol black E (J2). Filament numbers similar to controls were observed in the presence of 3,3′-dipropylthiocarbocyanine iodide (DTCI) (C4). A typical experiment is shown. Similar results were obtained in three separate experiments.

7.5) containing 150 mM NaCl, and monitored at 215 nm. The material eluting in the void volume was defined as protofibrils. To prepare soluble oligomers, purified α-synuclein (9 mg/mL) was incubated with 2 mM inhibitory compound for 30 days at 37 °C in 30 mM Tris-HCl containing 0.1% sodium azide. The samples were then centrifuged for 20 min at 113000*g* and the supernatants run on a Sephadex G-25 column, to separate oligomers from unbound inhibitor (*52*). The eluates were then fractionated on a Superdex 200 gel filtration column, as described above. Protein concentrations were determined using HPLC and the BCA protein assay kit (Pierce).

Cytotoxicity Assay. A solution (100 μL) containing 10 000 SH-SY5Y cells in DMEM-F12 with 10% fetal calf serum was added to each well of a 96-well microtiter plate and left for 24 h in a humidified incubator at 37 °C and 5% CO_2. The medium was then replaced with 100 μL of serum-free medium, followed by the addition of the monomeric, soluble oligomeric, protofibrillar, and filamentous α-synuclein. Following a 6 h incubation, the cytotoxic effect of α-synuclein was assessed by measuring cellular redox activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), following the manufacturer's (Sigma) instructions.

RESULTS

Inhibition of α-Synuclein Filament Assembly. Seventy-nine compounds belonging to 12 different chemical classes (listed in Table 1; see the Supporting Information for chemical structures) were tested for their ability to inhibit the assembly of full-length human α-synuclein into filaments, which was assessed by electron microscopy (Figure 1), thioflavin S fluorescence, and sarkosyl insolubility (Figure 2). By SDS−PAGE, a large reduction in the level of sarkosyl-insoluble α-synuclein and a corresponding increase in the level of sarkosyl-soluble protein were observed in the presence of several polyphenols (A17, A21, A26, and A31), phenothiazine E5, polyene macrolide F2, porphyrin G2, and Congo red derivative J2 (Figure 3). In the presence of inhibitors, dimeric and oligomeric, SDS-stable α-synuclein appeared in the sarkosyl-soluble fraction (Figure 3). Anthracyclines, benzothiazoles, lignans, steroids, 4,5-dianilinophthalimide (DAPH), and methyl yellow failed to inhibit α-synuclein filament formation, and SDS-stable oligomers were not observed. A representative example of each class of compound is shown in Figure 3.

Filament formation was quantified by measuring the levels of sarkosyl-insoluble α-synuclein and ThS fluorescence in the presence of each compound. In general, a good correlation was observed between the amounts of ThS fluorescence and sarkosyl-insoluble α-synuclein. However, for some benzothiazoles (C2 and C4), phenothiazines (E2, E3, and E6), porphyrin G3, and Congo red derivative J5, a large reduction in ThS fluorescence was observed, with only a slight change in the levels of sarkosyl-insoluble α-synuclein (Figure 2). The results observed by electron microscopy were in complete agreement with those from the sarkosyl experiments (Figure 1).

The IC_{50} values of all 79 compounds for inhibiting α-synuclein filament assembly were determined by quantifying the levels of sarkosyl-insoluble α-synuclein (Table 1). The effects of the same compounds on the assembly of Aβ and tau were assessed in parallel. For all three proteins, the inhibition of filament formation was concentration-dependent (data not shown). Strong inhibition of α-synuclein filament assembly (IC_{50} values of <10 μM) was observed with the polyphenol compounds baicalein (A2), delphinidin (A9), dopamine chloride (A12), epigallocatechin gallate (A16), exifone (A17), (−)-gallocatechin (A18), (−)-gallocatechin gallate (A19), gossypetin (A21), hinokiflavone (A22), hypericin (A23), procyanidin B1 (A29), procyanidin B2 (A30), rosmarinic acid (A33) and theaflavine (A37), the porphyrin ferric dehydroporphyrin IX (G1), Congo red (J1), and its derivative 1-bromo-2,5-bis(3-carboxystyryl)benzene (BSB) (J3). Vitamin E (α-tocopherol, A38) also exhibited a strong inhibitory effect, with an IC_{50} value of 10.9 μM. Other polyphenols (A4, A7, A13−A15, A25, A26, A28, A31, A32, A38, and A39), phenothiazines (E5 and E7), a polyene macrolide (F1), porphyrins (G2 and G3), and Congo red derivatives (J2 and J4) inhibited the assembly of α-synuclein

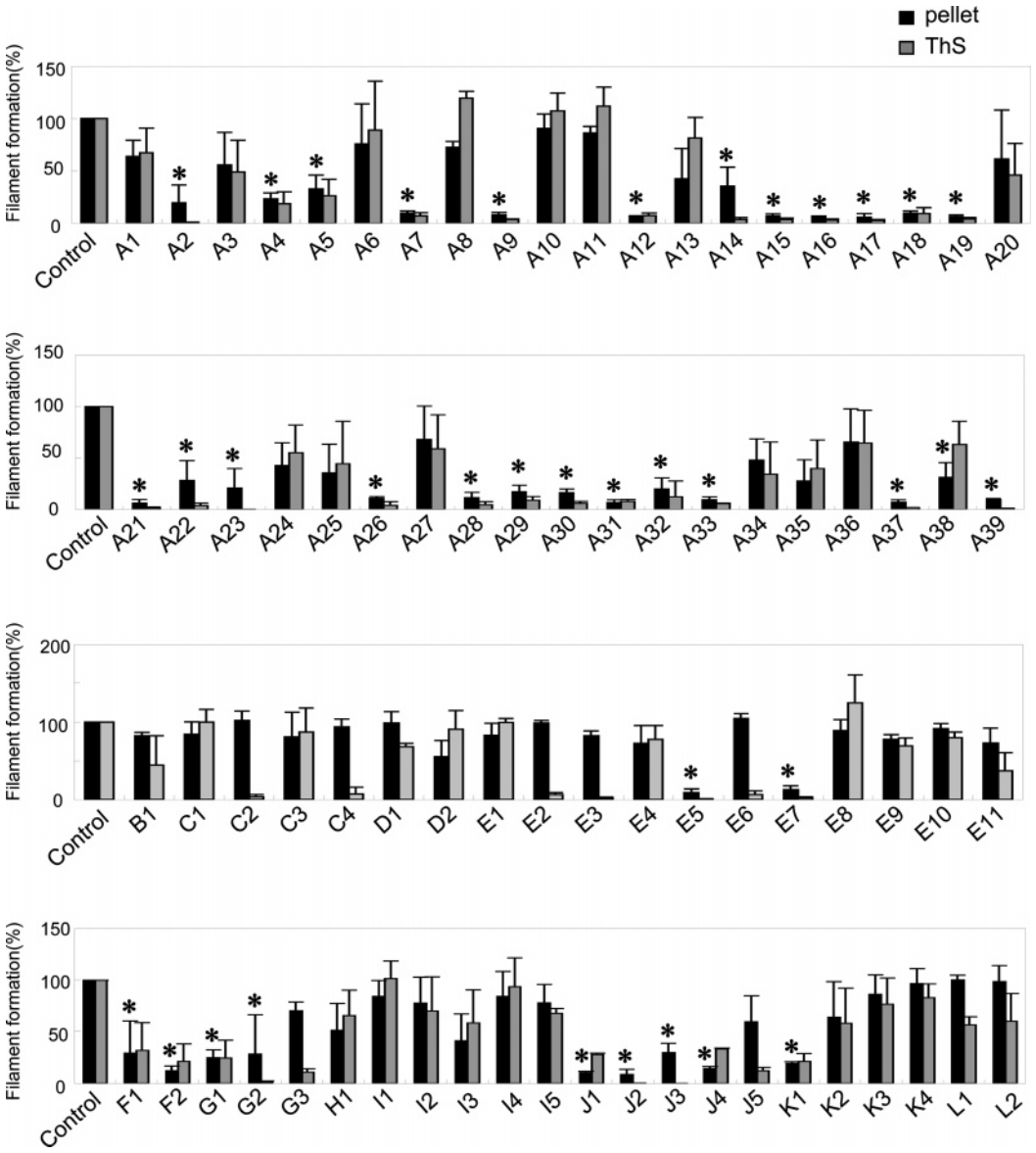

FIGURE 2: Quantitation of α-synuclein filament formation in the absence (Control) or presence of compounds using ThS fluorescence and sarkosyl insolubility. A significant reduction in the amount of sarkosyl-insoluble α-synuclein (black bars) and ThS fluorescence (gray bars) was observed in the presence of polyphenols (A2, A4, A5, A7, A9, A12, A14−A19, A21−A23, A26, A28−A33, A37, and A39), phenothiazines (E5 and E7), polyene macrolides (F1 and F2), porphyrins (G1 and G2), Congo red and its derivatives (J1−J4), and terpenoid K1. The results are expressed as means ± the standard error ($n = 3$; asterisks denote $p < 0.001$). With some compounds (C2, C4, E2, E3, E6, G3, and J5), ThS fluorescence was reduced, but the amount of sarkosyl-insoluble α-synuclein was not changed relative to controls.

less strongly (IC_{50} values of 10−40 μM). Rifampicin (H1) was weakly inhibitory (IC_{50} value of 46 μM).

The role of the carboxy-terminal region of α-synuclein was investigated by incubating α-synuclein(1−120) with the following at 200 μM: polyphenols dopamine chloride (A12) and exifone (A17), the phenothiazine lacmoid (E5), the porphyrin hematin (G2), and the Congo red derivative chlorazol black E (J2). All five compounds strongly inhibited the assembly of full-length α-synuclein; however, unlike exifone and lacmoid, dopamine chloride, hematin, and chlorazol black E did not inhibit filament formation of α-synuclein(1−120) (see the Supporting Information).

Inhibition of Aβ Fibril Formation. We investigated whether inhibition of Aβ fibril formation is accompanied by the formation of SDS-stable dimers and oligomers. As shown in Figure 4, both in the absence of compounds and in the presence of noninhibitory compounds, soluble dimer and oligomer levels were very low. In the presence of compounds that inhibit Aβ and α-synuclein assembly, such as some polyphenols (A17, A21, A26, and A33), the phenothiazine lacmoid (E5), and the porphyrin hematin (G2), a strong dimer band and more variable oligomer bands were observed in the sarkosyl-soluble fraction (Figure 4).

Cytotoxicity Studies. Protofibrils and soluble oligomeric species of α-synuclein were prepared by gel filtration chromatography (Figures 5 and 6). Protofibrils were defined as the material eluting in the void volume (fraction 1) of the column, as proposed previously (*59*). SH-SY5Y cells were exposed for 6 h to monomeric, soluble oligomeric, protofibrillar, or filamentous human α-synuclein. Cell viability was evaluated using reduction of MTT to MTT formazan and compared with that of vehicle-treated cells. In cells exposed to monomeric α-synuclein, there was no significant reduction in cell viability. This stood in marked contrast to

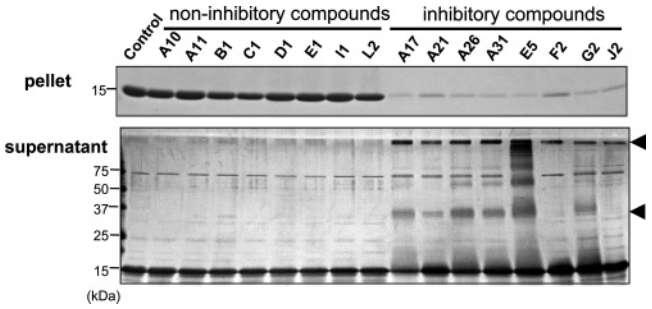

FIGURE 3: Formation of soluble α-synuclein oligomers in the presence of inhibitory compounds. Sarkosyl-insoluble (pellet, top panel) and sarkosyl-soluble (supernatant, bottom panel) α-synuclein was prepared following incubation of α-synuclein in the absence (Control) or presence of compounds (A10, A11, A17, A21, A26, A31, B1, C1, D1, E1, E5, F2, G2, I1, J2, and L2). Via SDS−PAGE, SDS-stable, high-molecular weight α-synuclein (arrowheads) was detected in the supernatants of samples incubated with inhibitory compounds, but not in the supernatants of controls or samples incubated with noninhibitory compounds. The presence of high-molecular weight α-synuclein in the supernatant was accompanied by a reduction in the levels of monomeric α-synuclein in the pellet. A typical experiment is shown. Similar results were obtained in three separate experiments.

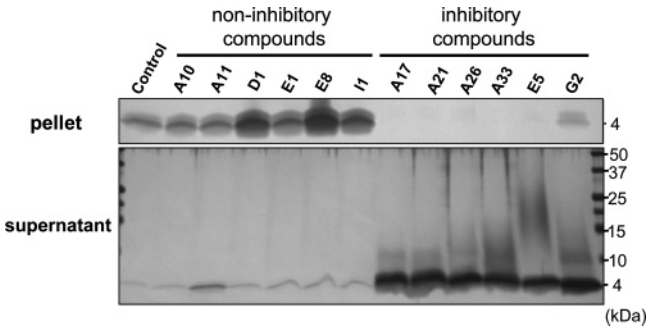

FIGURE 4: Formation of soluble Aβ oligomers in the presence of inhibitory compounds. Sarkosyl-insoluble (pellet, top panel) and sarkosyl-soluble (supernatant, bottom panel) Aβ was prepared following incubation of Aβ in the absence (Control) or presence of compounds (A10, A11, A17, A21, A26, A33, D1, E1, E5, E8, G2, and I1). Via SDS−PAGE, SDS-stable, soluble oligomeric Aβ was detected in the presence of inhibitory compounds, but not in the presence of noninhibitory compounds. A typical experiment is shown. Similar results were obtained in three separate experiments.

α-synuclein filaments, which were cytotoxic. When they were used at 500 nM, approximately 60% of cells were killed, with ~50% cell death at 50 nM filaments (Figure 5). Protofibrils were also cytotoxic, with 30% cell death at 500 nM and 10% cell loss at 50 nM. The toxicities of filaments and protofibrils were concentration-dependent (Figure 5). Next, we tested the cytotoxicity of soluble oligomeric species obtained following incubation of α-synuclein in the presence of inhibitory compounds dopamine chloride (A12), exifone (A17), lacmoid (E5), and hematin (G2). In presence of each inhibitory compound, five protein peaks were resolved by gel filtration (Figure 6) and tested individually in the cytotoxicity assay. In contrast to filaments and protofibrils, none of the soluble oligomeric fractions of α-synuclein that were tested exhibited cytotoxicity in this assay (Figure 6).

DISCUSSION

Thioflavin S fluorescence, levels of sarkosyl-insoluble α-synuclein, and electron microscopic examination were used

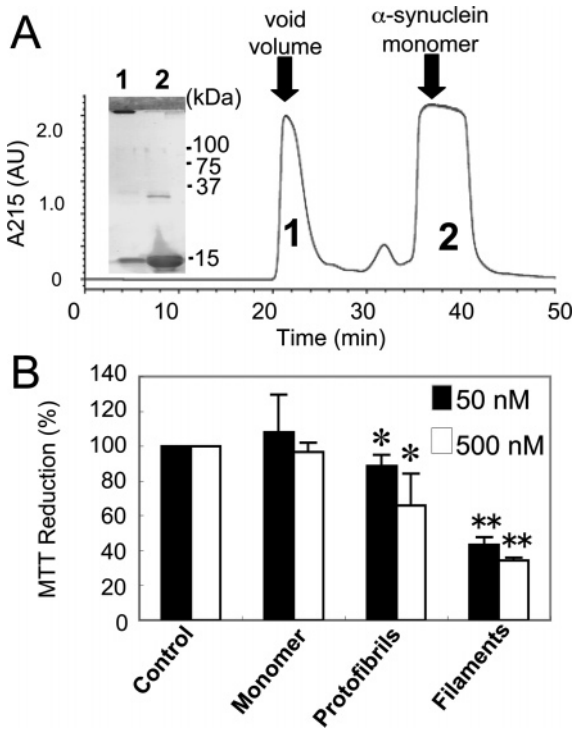

FIGURE 5: Cytotoxicity of α-synuclein monomer, protofibrils, and filaments. α-Synuclein protofibrils and soluble monomer were fractionated by gel filtration chromatography and analyzed by Western blotting (WB) using antibody syn102. The cytotoxicity of α-synuclein monomers, protofibrils, and filaments at 50 nM (black bars) or 500 nM (white bars) was assessed using the MTT assay. Protofibrils and filaments exhibited dose-dependent cytotoxicity. The results are presented as percent MTT reduction, with the values obtained in the absence of added α-synuclein taken as 100%. They are expressed as means ± the standard error ($n = 6$; one asterisk denotes $p < 0.01$, and two asterisks denote $p < 0.001$).

to investigate the effects of 79 compounds belonging to 12 chemical classes on the assembly of α-synuclein into filaments. Compounds belonging to seven classes (polyphenols, phenothiazines, polyene macrolides, porphyrins, rifamycins, Congo red and its derivatives, and terpenoids) inhibited filament assembly. The three methods used for monitoring filament assembly gave similar results in the presence of inhibitors, except for thioflavin S (a benzothiazole), which could not be used for testing inhibition by some benzothiazoles and phenothiazines. It could also not be used in the presence of Congo red and its derivatives.

A number of polyphenols [baicalein, delphinidin, dopamine chloride, epigallocatechin gallate, exifone, (−)-gallocatechins, gossypetin, hinokiflavone, hypericin, procyanidins, rosmarinic acid, and theaflavine], the porphyrin compound ferric dehydroporphyrin IX, and Congo red and its derivative BSB inhibited α-synuclein filament assembly with IC_{50} values of <10 μM. Other polyphenols [cyanidin, (−)-epicatechin 3-gallate, epigallocatechin, myricetin, purpurogallin, (+)-α-tocopherol, and 2,3,4-trihydroxybenzophenone], as well as the phenothiazine lacmoid, the porphyrin hematin, and the Congo red derivatives chlorazol black E and 1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxystyryl)benzene (FSB), inhibited filament formation with IC_{50} values of 10−20 μM. The phenothiazine perphenazine, the polyene macrolides amphotericin B and filipin III, the terpenoid asiatic acid, and rifampicin were less potent. Anthracyclines, benzothiazoles, lignans, steroids, DAPH, and methyl yellow were without

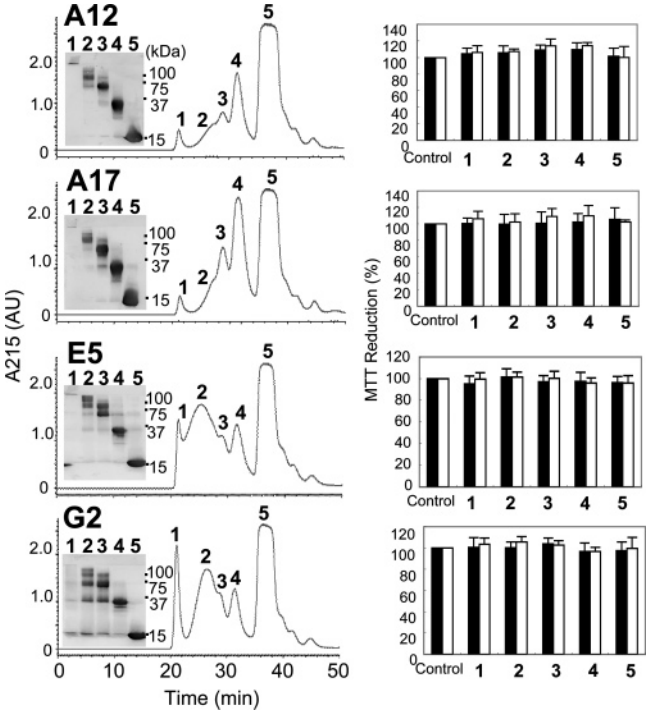

FIGURE 6: Soluble α-synuclein oligomers formed in the presence of inhibitory compounds do not reduce the viability of SH-SY5Y cells. α-Synuclein soluble oligomers were fractionated by gel filtration chromatography and analyzed by WB using antibody syn102. In the presence of inhibitory compounds A12, A17, E5, and G2, SDS-resistant dimers (fraction 4) and oligomers (fractions 1−3) formed. Fraction 5 corresponds to the α-synuclein monomer. In the absence of inhibitory compounds, oligomers did not form (not shown). The cytotoxicity of soluble oligomers was assessed using the MTT assay. In contrast to protofibrils and filaments, monomeric α-synuclein and fractions 1−5 of oligomeric α-synuclein at 50 nM (black bars) or 500 nM (white bars) were not cytotoxic. The results are presented as the percent MTT reduction, with the values obtained in the absence of added α-synuclein taken as 100%. They are expressed as means ± the standard error (n = 6).

effect (IC_{50} values of >80 μM). These findings establish that polyphenols constitute a major class of compounds that can inhibit the assembly of α-synuclein. Many polyphenols are natural substances present in beverages obtained from plants, fruits, and vegetables. Besides inhibiting the aggregation of α-synuclein, Aβ, and tau, they are also known to have neuroprotective effects in a number of paradigms (*60*). Of the 39 compounds that were tested, 26 inhibited α-synuclein assembly, 29 inhibited Aβ assembly, and 19 inhibited tau filament formation. In particular, some catechins and vitamin E (α-tocopherol) were inhibitory. We confirmed and extended recent work showing inhibition of α-synuclein filament formation by baicalein, dopamine, and a number of catecholamines. Previous work suggested that two adjacent phenolic OH groups may be required for the inhibition of α-synuclein filament formation via covalent modification (*48*−*51*). Of the 27 inhibitory polyphenols identified here, 23 had two adjacent phenolic OH groups. Furthermore, all polyphenols with three OH groups in the ring (a total of 15 compounds) were inhibitory (Supporting Information), suggesting that adjacent phenolic OH groups may indeed play an important role. However, some compounds (hinokiflavone, hypericin, and α-tocopherol) lacking adjacent phenolic OH groups were also inhibitory. Additional studies are needed to better understand these structure−activity relationships.

Porphyrins have previously been shown to inhibit the assembly of Aβ and tau (*47*). Prior to the work presented here, phthalocyanine tetrasulfonate was the only porphyrin known to inhibit α-synuclein filament assembly (*52*). We found a weak inhibitory effect of phthalocyanine tetrasulfonate, with stronger inhibition by the porphyrins ferric dehydroporphyrin IX and hematin. In contrast to a previous report (*52*), we failed to detect disassembly of α-synuclein filaments in the presence of phthalocyanine tetrasulfonate. Consistent with previous work (*53*), rifampicin inhibited α-synuclein assembly, albeit not very potently. Prior to the study presented here, inhibition of α-synuclein filament formation by phenothiazines had not been reported. Of the 11 compounds that were tested, lacmoid and perphenazine were inhibitory. As shown previously (*47*), compounds lacking a side chain at position 9 of the phenothiazine ring were inhibitory toward the assembly of Aβ and tau. However, this was not true of α-synuclein aggregation, suggesting a difference in conformation between oligomers and filaments of α-synuclein and those of Aβ and tau. Congo red was strongly inhibitory toward the assembly of α-synuclein and tau. The same was true of Aβ fibril formation, confirming previous results (*61*, *62*). Moreover, Congo red has also been shown to prevent the aggregation and infectivity of the prion protein (*63*, *64*) and the aggregation of huntingtin (*65*, *66*). Congo red derivatives BSB and FSB also inhibited the assembly of Aβ, α-synuclein, and tau. Unlike Congo red, they cross the blood−brain barrier and are currently being developed as reagents for visualizing Aβ deposits in vivo by positron emission tomography (PET) and magnetic resonance imaging (MRI) (*67*−*69*). BSB has also been shown to label some α-synuclein deposits in brain sections from cases of MSA and DLB and some tau deposits in cases of Alzheimer's disease, progressive supranuclear palsy, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (*70*). Overall, a number of compounds inhibited the assembly of Aβ, α-synuclein, and tau, in line with findings suggesting that soluble amyloid oligomers can have structural features in common (*71*). Almost all the compounds that inhibited α-synuclein assembly also inhibited Aβ assembly. Exceptions were (−)-epicatechin, procyanidin B2, α-tocopherol, perphenazine, and asiatic acid. Conversely, several compounds inhibited Aβ assembly without interfering with α-synuclein and tau filament formation. They included all the benzothiazoles that were tested, several polyphenols (curcumin, gingerol, kaempferol, naringenin, and rutin), the anthracycline daunorubicin hydrochloride, the Congo red derivative Ponceau S, the terpenoid ginkgolide B, and DAPH and methyl yellow.

Previous work has shown that the C-terminal region of α-synuclein is a negative regulator of filament assembly (*26*, *29*, *32*), probably through long-range interactions with part of the hydrophobic region (*33*, *34*). We therefore investigated the ability of dopamine chloride, exifone, lacmoid, hematin, and chlorazol black E to inhibit the assembly of α-synuclein (1−120). In contrast to that of full-length α-synuclein, the assembly of α-synuclein(1−120) was not inhibited by dopamine chloride, hematin, or chlorazol black E. Exifone and lacmoid inhibited but did not abolish filament formation of α-synuclein(1−120). These findings establish an important

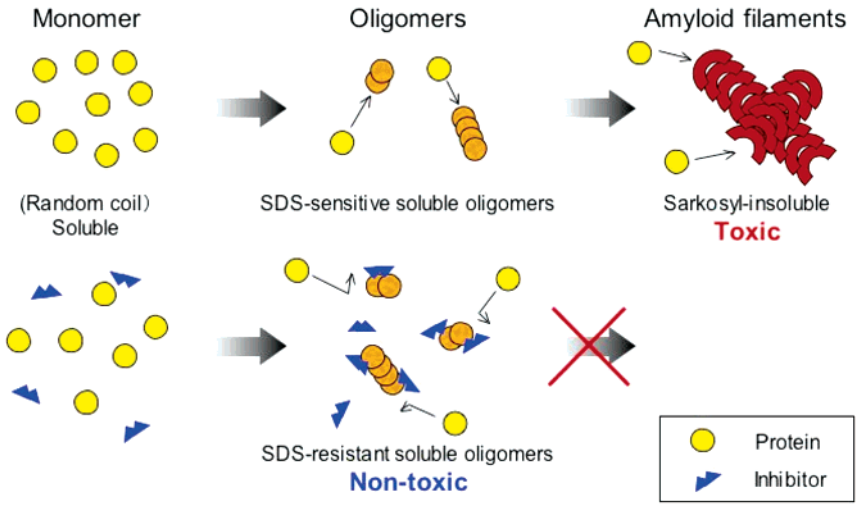

FIGURE 7: Model for the inhibition of amyloid filament formation by small compounds.

role for the C-terminal region of α-synuclein in the inhibition of filament assembly and suggest that the compounds used may bind to this region. They are in line with a recent study reporting that dopaminochrome inhibited assembly by binding to residues 125−129 of α-synuclein (*49*). Consistent with this report, we also found that covalent modification of α-synuclein was not required to prevent filament formation (not shown). Furthermore, binding of single-chain antibodies to the C-terminal region of α-synuclein has been shown to inhibit filament assembly (*72*). It remains to be determined whether the binding of inhibitory compounds and antibodies influences the long-range interactions between the C-terminus of α-synuclein and the hydrophobic region.

Analysis of the sarkosyl-soluble fraction of α-synuclein assemblies by SDS−PAGE revealed the presence of dimeric and oligomeric protein in the presence of inhibitory compounds. This was in marked contrast to the sarkosyl-insoluble, SDS-sensitive α-synuclein which formed in the absence of inhibitors. Dimers and oligomers were also observed upon incubation of Aβ with inhibitory compounds. These findings are reminiscent of what was observed when heparin-induced filament formation of tau protein was inhibited by some of the same compounds (*47*). It suggests that the compounds investigated here may inhibit filament formation of α-synuclein by stabilizing soluble, prefibrillar intermediates (Figure 7). The latter, whose precise mechanism of formation remains to be determined, may be intermediates in the pathway leading from monomeric to filamentous α-synuclein. A recent study (*73*) has shown that heat shock protein Hsp70 inhibits α-synuclein filament formation by binding to soluble prefibrillar intermediates, suggesting that small organic molecules and chaperone proteins may inhibit assembly of α-synuclein through similar mechanisms. Since soluble amyloid oligomers of Aβ, α-synuclein, and tau appear to have structural features in common (*71*), a number of compounds that inhibited the assembly of Aβ, α-synuclein, and tau may recognize and interact with this common structure. Other compounds only inhibited Aβ aggregation, indicating that they may recognize a conformation specific to Aβ.

The cytotoxicity of soluble oligomers was investigated in SH-SY5Y cells and compared with that of monomeric α-synuclein, protofibrils, and filaments. Monomers and soluble oligomers did not reduce cell viability. By contrast, protofibrils and filaments were markedly cytotoxic. These results indicate that the compounds investigated here inhibited not only filament formation but also the toxicity of α-synuclein. They demonstrate a link between filament formation and toxicity in this system, in agreement with earlier findings (*74*). Future experiments will investigate whether the same inhibitors have similar effects in model systems where α-synuclein filaments are present intracellularly rather than being added from the outside. Currently, it is not clear which α-synuclein species are the most detrimental to nerve cells and glial cells in Lewy body diseases and MSA, and in animal models thereof (*75*−*77*). This notwithstanding, these findings indicate that the soluble oligomeric species formed in the presence of inhibitory compounds may not be toxic to nerve cells and may hence have therapeutic potential.

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SUPPORTING INFORMATION AVAILABLE

List of compounds tested and quantitation of α-synuclein (1−120) filament formation in the absence or presence of inhibitory compounds using sarkosyl insolubility. This material is available free of charge via the Internet at http://pubs.acs.org.

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