tal cancers. The confinement of the gene to a small amplicon in a subset of these tumors provides important, complementary evidence for the role of this gene in metastasis; the major genes previously shown to be amplified in naturally occurring cancers are all oncogenes (*18*, *23*, *24*). Extra copies of chromosome 8q DNA sequences have been observed in the advanced stages of many different tumor types, including advanced colon cancers (*25–28*). It has been suggested that the c-*MYC* gene on chromosome 8q24.12 is the target of such 8q overrepresentation. In the three metastatic lesions we examined, c-*MYC* was not amplified and was in fact located ~14 Mb from the boundaries of the *PRL-3* amplicon. It will, therefore, be of interest to evaluate the expression and genomic representation of *PRL-3* in the metastases of other cancer types.

Further experiments will be required to determine the biochemical mechanisms through which *PRL-3* influences neoplastic growth and to establish its causative role in the metastatic process. However, one of the most important ramifications of the work described here concerns its potential therapeutic implications. Most of the previously described genetic alterations in colorectal cancers involve inactivation of tumor suppressor genes. The proteins produced from these genes are difficult to target with drugs, because they are inactive or absent in the cancer cells (*29*). In contrast, enzymes whose expression is elevated in cancer cells, like that encoded by *PRL-3*, provide excellent targets for drug discovery purposes.

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7. Examples of the nonepithelial transcripts identified in the initial libraries are vitronectin and lysozyme. These genes are transcribed in stromal cells and accounted for 0.4 and 0.1%, respectively, of the tags in SAGE libraries prepared from unpurified metastatic lesions but were not found in the SAGE library derived from purified metastatic epithelial cells. Similarly, transcripts made in hepatocytes, like apolipoprotein C–III, accounted for 0.3% of the tags from unpurified metastasis libraries but were not found in the libraries from purified cells. These differences in vitronectin, lysozyme, and apoliprotein C–III expression among SAGE libraries derived from purified and unpurified epithelial cells were statistically significant ($P < 0.0001$, χ^2 test).
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Kinetic Stabilization of the α-Synuclein Protofibril by a Dopamine–α-Synuclein Adduct

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The substantia nigra in Parkinson's disease (PD) is depleted of dopaminergic neurons and contains fibrillar Lewy bodies comprising primarily α-synuclein. We screened a library to identify drug-like molecules to probe the relation between neurodegeneration and α-synuclein fibrilization. All but one of 15 fibril inhibitors were catecholamines related to dopamine. The inhibitory activity of dopamine depended on its oxidative ligation to α-synuclein and was selective for the protofibril-to-fibril conversion, causing accumulation of the α-synuclein protofibril. Adduct formation provides an explanation for the dopaminergic selectivity of α-synuclein–associated neurotoxicity in PD and has implications for current and future PD therapeutic and diagnostic strategies.

A central role for α-synuclein fibrilization in PD (*1*, *2*) is supported by the fact that fibrillar α-synuclein is a major component of Lewy bodies (*3*, *4*) and that the α-synuclein gene has been linked to autosomal dominant PD (FPD) (*5*, *6*). Although both FPD α-synuclein mutations [$Ala^{53} \rightarrow$ Thr (A53T) and $Ala^{30} \rightarrow$ Pro (A30P)] accelerate the formation of nonfibrillar, oligomeric protofibrils in vitro, A30P inhibits the conversion of protofibrils to fibrils (*7*). This finding suggests that protofibrils may be pathogenic and fibrils inert, or even protective (*2*). Transgenic mice that express wild-type human α-synuclein (*8*) exhibit PD-like dopaminergic deficits and motor dysfunction, and they develop neuronal inclusions that, in contrast to those

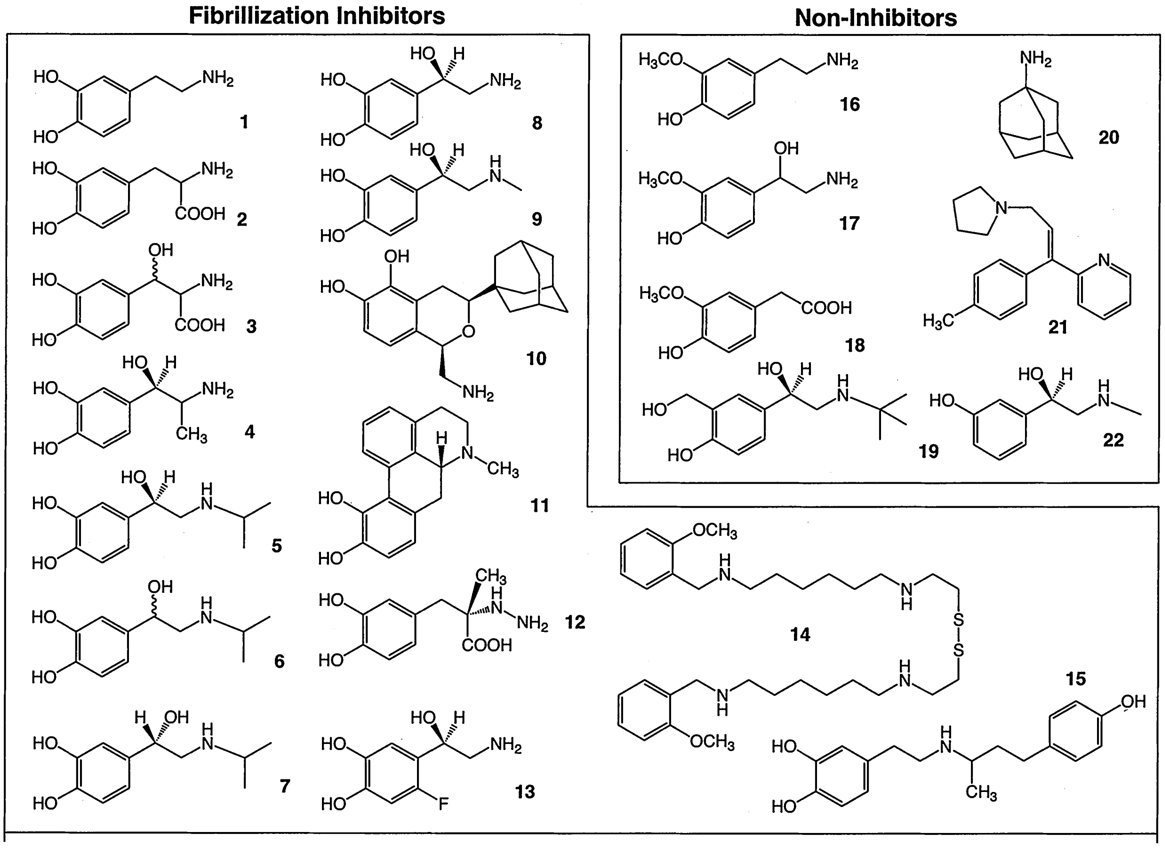

Fig. 1. Catechol-containing compounds inhibit α-synuclein fibrilization. Structures of 22 out of 169 compounds (assayed at 100 to 300 μM) from our initial screening library divided according to their activity in an in vitro thioflavin T (Thio T) assay (*7*). A53T α-synuclein (175 to 300 μM) was used in initial screens, but inhibition was confirmed with the wild type. Incubations were done with shaking (reading at 1 day) or without (reading at 30 days) at 37°C. Inhibitors decreased the Thio T signal by ≥50% in all four trials. This screen was not designed to precisely measure inhibitory activity. One out of 155 non-catechol compounds screened, **14**, met the above criteria. The requirement for a catechol ring was reinforced by the inactivity of compounds **16**, **17**, **18**, **19**, and **22**.

of the Parkinsonian humans and *Drosophila* (*9*), are entirely nonfibrillar. Crossing these mice with a second transgenic line expressing β-synuclein produces mice in which the number of nonfibrillar inclusions and the Parkinsonian phenotype are reduced, which suggests that protofibrils may cause disease (*10*).

Drug-like molecules could be used in animal models to probe the relation between the conversion of α-synuclein to protofibrils (and then to fibrils) and the progression of neuronal loss and motor dysfunction. If the protofibril is pathogenic, then delaying the appearance of fibrillar inclusions by stabilizing protofibrillar intermediates may accelerate the onset and progression of motor abnormalities (*2*). In contrast, inhibiting the formation of both protofibrils and fibrils could slow disease progression (*10*). To identify compounds that inhibit the formation of α-synuclein amyloid fibrils (*7*), we screened a commercially available compound library (*11*). Fifteen of 169 compounds initially screened inhibited α-synuclein fibrilization

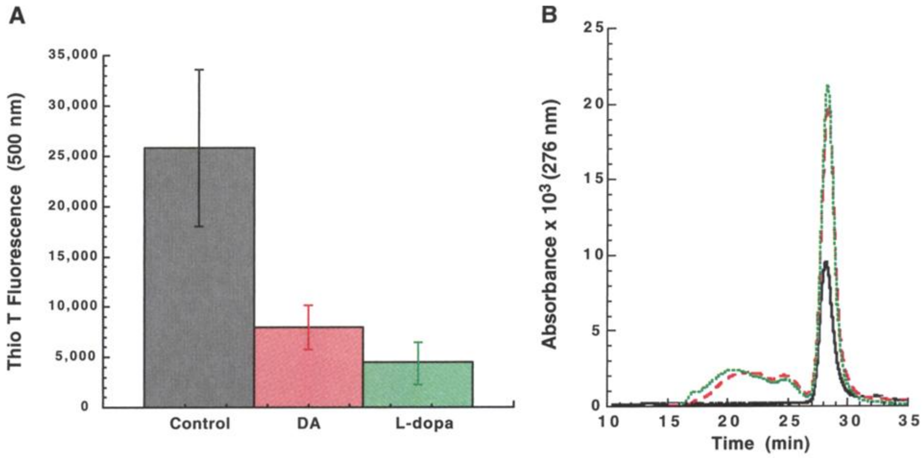

Fig. 2. Catecholamines inhibit α-synuclein fibril formation, modify monomer, and stabilize UV-active oligomers; effects are eliminated by antioxidants. Samples of 180 μM A53T α-synuclein were incubated alone or with equimolar DA or L-dopa at 37°C with agitation. After 3 days, aliquots from each incubation were analyzed by Thio T to measure fibrils (**A**) and were subjected to gel filtration chromatography to measure nonfibrillar species (**B**). In (A), DA and L-dopa inhibit α-synuclein fibril formation; the effect is reversed by the addition of 1% w/v $Na_2S_2O_5$ (53 mM) or by 3 molar equivalents each of *N*-acetyl cysteine and desferroximine (*36*). In (B), DA (dashed red line) and L-dopa (dotted green line) stabilize the formation of oligomeric α-synuclein (broad peak eluting between 17 and 27 min) and increase the UV activity of the monomer peak (28 min) as compared to time 0 (*36*). No protofibrils were detected in the control incubation (solid black line). Addition of 1% w/v $Na_2S_2O_5$ eliminates the catechol-induced effects (*36*).

(Fig. 1). All but one of the inhibitors were catecholamines, including dopamine (DA) (**1**) and L-dopa (**2**), norepinephrine (**8**), and epinephrine (**9**). The inhibitory activity of DA and L-dopa (Fig. 2A) was reversed by the addition of antioxidants, which suggested that

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catechol oxidation was responsible (*11, 12*).

Under inhibitory conditions, protofibrils accumulated (*13*) and the ultraviolet (UV) absorption of the residual monomeric α-synuclein increased (Fig. 2B). Both phenomena were eliminated by sodium metabisulfite, suggesting that fibril inhibition, protofibril accumulation, and monomer modification may be consequences of covalent modification by the dopamine-derived orthoquinone [DAQ; see (*11*)]. Although α-synuclein does not contain cysteine, which reacts with DAQ in vitro and in vivo (*14*), its natively unfolded structure (*15*) may expose other reactive side chains such as Tyr, Lys, or Met. The DA-modified α-synuclein monomer produced a long-wavelength (468 nm) fluorescence emission, consistent with a Tyr-derived radical coupling adduct (Fig. 3A) (*16*). Because analysis by mass spectrometry suggested that adduct formation occurred at low levels (less than 10% of parent ion intensity was potentially due to adduct), the possible incorporation of radiolabeled DA was monitored. [^{3}H]DA incorporation (DA concentration = 2 mM) into monomeric (Fig. 3B; Fig. 3D, left panel) and then protofibrillar (Fig. 3C; Fig. 3D, right panel) α-synuclein occurred at a rate comparable to DA polymerization, a process that is known to occur in vivo (*17, 18*). After purification of the monomeric adduct, the average level of DA incorporation (moles of [^{3}H]DA per mole of protein) was estimated to be 5 to 10% (Fig. 3E, blue bars). The monomeric adduct was stable to boiling (Fig. 3E, red bars) and stained [SDS–polyacrylamide gel electrophoresis (PAGE)] with a redox-active system that identifies catechol-containing proteins (*19*). Together, these data are consistent with the formation of low levels of one or more DA–α-synuclein covalent adducts, possibly derived from radical coupling (DAQ to Tyr) and/or nucleophilic attack (e.g., Lys forming a Schiff base with DAQ) (*20, 21*).

The partially modified α-synuclein monomer was separated from protofibrillar material and from free DA, and its fibrilization in the absence of DA was compared to that of unmodified α-synuclein monomer (Fig. 4, yellow versus black bars). In addition, parallel incubations in which the adduct was diluted with unmodified α-synuclein (total α-synuclein concentration was constant) were also followed (Fig. 4, red and green bars). Fibril formation (Fig. 4A) was fastest in the unmodified incubation, and the degree of inhibition was correlated to the concentration of adduct(s). Protofibrils were observed as transient intermediates in every incubation and were most long-lived in the incubations containing trace amounts of DA–α-synuclein adduct (Fig. 4B, green bars). This effect was observed even in the case where the DA adduct was diluted 1:4 and represented about 1 to 3% of the total α-synuclein (Fig. 4B, red bars). Nucleation-dependent protein oligomerization processes can be inhibited by

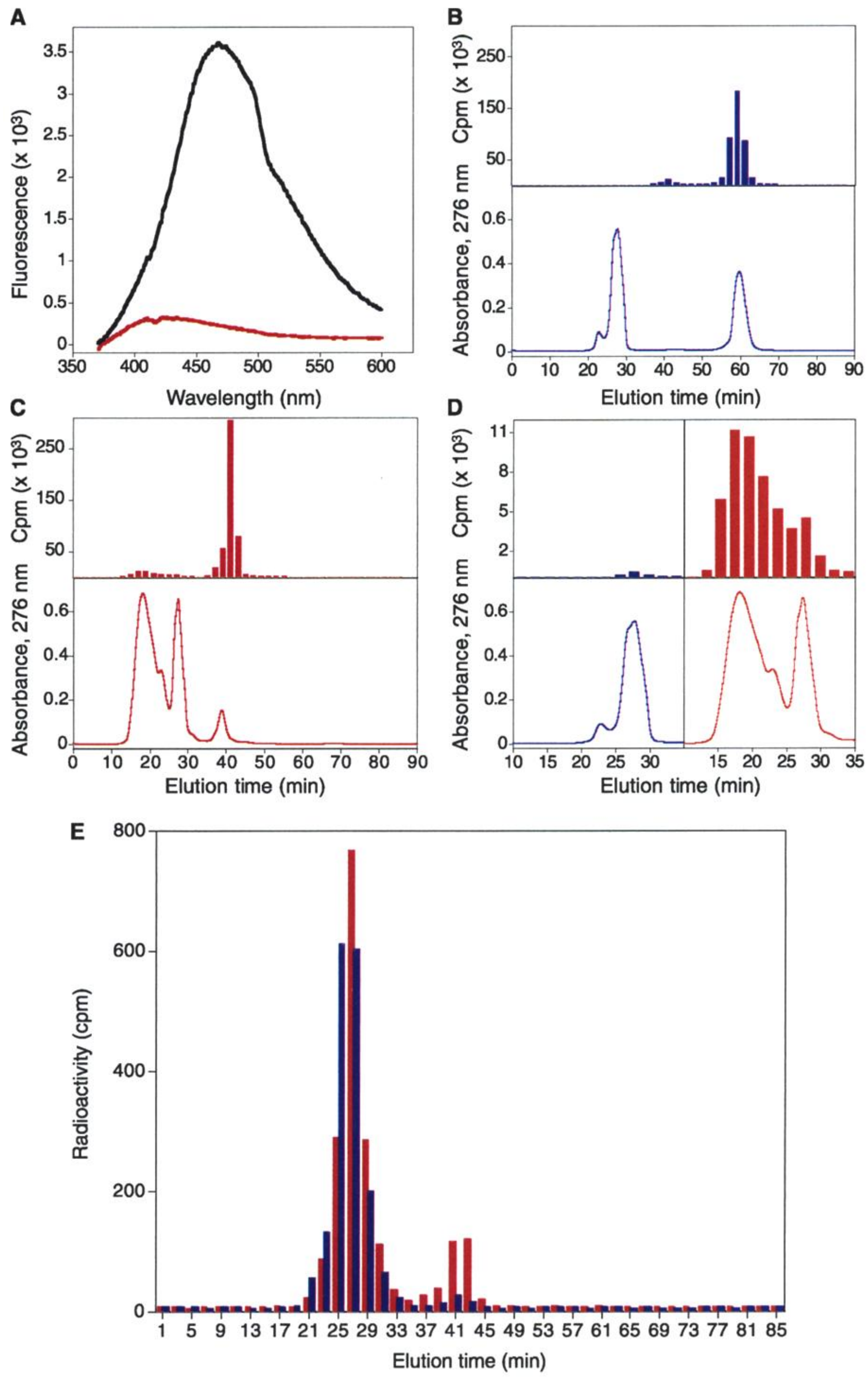

Fig. 3. DAQ reacts with α-synuclein to form fluorescent covalent adducts. (**A**) Fluorescence emission spectra of monomeric DA-treated A53T (8.9 μM, black line) and untreated A53T (18 μM, red line). Excitation wavelength, 360 nm; wavelength of maximal emission (λ_{max}): DA-treated A53T, 468 nm; untreated A53T, 426 nm. (**B** to **D**) Gel filtration of a solution of A53T (1 mM) treated with [2,5,6-^{3}H]DA (2 mM, 5 mCi/mmol), monitored by scintillation counting (top) and absorbance at 276 nm (bottom). The solution was analyzed immediately (B) or after incubation for 11 days at 37°C without rotation (C). The left and right panels of (D) correspond to data from (B) and (C), respectively, showing the elution of oligomeric and monomeric α-synuclein between 10 and 35 min (DA elutes at 60 min, and its oxidative polymerization product, which is also formed in the absence of α-synuclein, elutes at 39 min). (**E**) Gel-purified monomer [from (C)] was analyzed before (blue bars) and after (red bars) boiling for 10 min.

trace impurities, which act like crystal poisons (*22*). This effect, which may explain the results above, has been demonstrated in the case of α-synuclein fibrilization, where a small relative amount of human α-synuclein inhibits fibrilization of its mouse homolog (*13*).

Cell death in PD largely affects the substantia nigra. The selective loss of dopaminergic

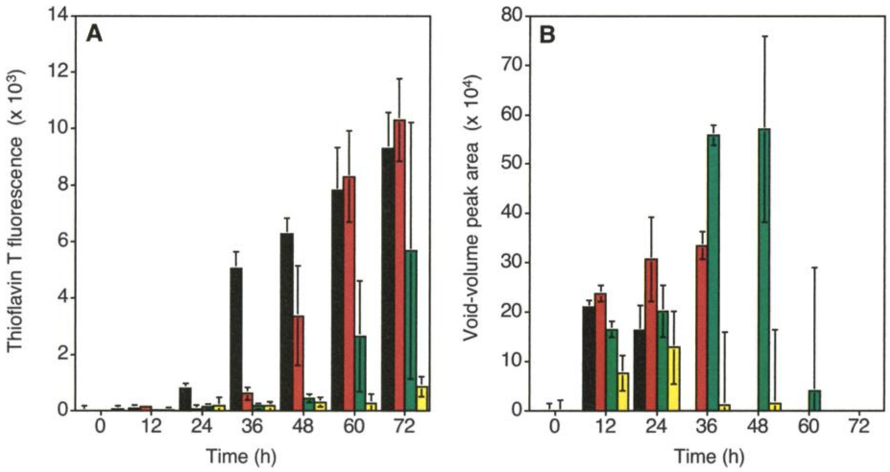

Fig. 4. Monomeric DA–α-synuclein adduct inhibits α-synuclein fibrilization but prolongs the lifetime of the protofibrillar intermediates. Incubations containing unmodified wild type (600 μM, black bars), DA-modified wild type (600 μM, yellow bars), or mixtures of the unmodified and modified proteins [450 μM unmodified + 150 μM modified (red bars); 300 μM unmodified + 300 μM modified (green bars)] were rotated at 37°C and analyzed at various times. (**A**) The Thio T assay shows that fibril formation is fastest in unmodified incubation and slowest in the incubation containing the highest level of DA adduct. (**B**) Void-volume (protofibril) peak area determined by gel filtration and UV (absorbance at 215 nm). At time points where no protofibril was detected (e.g., 72 hours), the bar was omitted. Absorbance attributed to a small amount of oligomeric material present in the unmodified sample at time 0, as a result of freeze-thawing, was subtracted from subsequent measurements. Protofibrils were most persistent at trace levels of adduct (green bars), but their formation is inhibited at a higher level of modification (yellow bars), indicating that high levels of adduct also inhibit the monomer-to-protofibril transition.

neurons is mimicked whether Parkinsonism is induced in *Drosophila* by brain-wide expression of α-synuclein (*9*) or in rats by chronic systemic exposure to rotenone, a complex I inhibitor (*23*). Thus, three established risk factors—oxidative stress, DA, and α-synuclein—may, in combination, stabilize protofibrillar α-synuclein and promote the pathogenesis of PD (*1, 11*). As a prerequisite, α-synuclein levels must exceed the critical concentration for oligomerization. In rare familial forms of PD, that threshold may be lowered by α-synuclein point mutations (*7*). In other forms of PD, elevated levels of wild-type α-synuclein could result from its inefficient degradation due to PD-associated parkin mutations (*24, 25*) or its overexpression due to PD-associated promoter polymorphisms (*26, 27*). The α-synuclein message is up-regulated in PD substantia nigra, but not in cerebellum or cortex (*28*). Once the critical concentration of α-synuclein has been exceeded, the cytoplasmic concentration of DAQ may be one of several factors that determine the amount and lifetime of potentially pathogenic α-synuclein protofibrils. DAQ results from a combination of oxidative stress and elevated cytoplasmic DA concentration, both of which are independently associated with PD (*14*). The importance of cytoplasmic DA in PD cell death is supported by two facts: (i) The dopaminergic neurons of the ventral tegmental area—which are resistant relative to the substantia nigra—express high levels of VMAT2, which promotes vesicular sequestration of DA, and low levels of DAT, which pumps DA into the cytoplasm (*29*); and (ii) neurons containing neuromelanin, a polymerization product of DAQ, are relatively sensitive to PD-induced cell death (*18*). Differences in transport and vesicular packaging may also explain why the noradrenergic system is relatively spared in PD, even though epinephrine and norepinephrine are oxidatively labile and capable of adduct formation. DA and some of its analogs are neurotoxic in cell culture (*30*), and this effect has been linked to α-synuclein (*31*). A distinct but overlapping combination of factors may contribute to the pathogenesis of diffuse Lewy body disease (DLBD), a dementia that is characterized by α-synuclein inclusions and degeneration of the cortex. Thus, high cortical expression of α-synuclein [mRNA level is elevated in DLBD (*28*)] may combine with other trace α-synuclein modifications (*32*) to stabilize protofibrils.

Decreasing α-synuclein expression and preventing protofibril accumulation are two attractive therapeutic strategies against PD. The latter could be accomplished by decreasing the cytoplasmic concentration of DA, an endogenous protofibril stabilizer, while avoiding depletion of synaptic DA, which ameliorates PD symptoms. Although there is no consistent evidence regarding a potentially neurotoxic effect of L-dopa therapy that is suggested by this work (*33*), clinical trials to address this issue are in progress (*34*).

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