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Rifampicin Inhibits α-Synuclein Fibrillation and Disaggregates Fibrils

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Summary

The aggregation of α-synuclein in dopaminergic neurons of the *substantia nigra* is a critical step in the pathogenesis of Parkinson's disease. We show that the antibiotic rifampicin inhibited α-synuclein fibrillation and disaggregated existing fibrils in a concentration-dependent manner. Size-exclusion chromatography data indicated that rifampicin stabilized α-synuclein as both a monomer and soluble oligomers comprised of partially folded α-synuclein. Experiments using aged samples of rifampicin indicated that the most active species in inhibiting fibrillation and disaggregating fibrils is an oxidation product of rifampicin, which was confirmed in experiments under anaerobic conditions. These results indicate that rifampicin-mediated inhibition of α-synuclein fibrillation and disaggregation of fibrils involves preferential stabilization of monomeric and soluble oligomeric forms, and that rifampicin potentially may have therapeutic application for Parkinson's disease.

Introduction

Human α-synuclein is a 140 residue, highly conserved presynaptic protein that is abundant in various regions of the brain [1, 2]. It is found as both a soluble cytoplasmic protein and associated with presynaptic vesicles, and it may play a role in synaptic release of neurotransmitters [1, 3]. Structurally, purified α-synuclein is a natively unfolded protein at neutral pH [4–9]. Pathologically, Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons from the *substantia nigra* region of the brain, with some surviving nigral dopaminergic neurons containing cytosolic filamentous inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs) [10]. α-synuclein is a major fibrillar component of LBs and LNs [11, 12]. The aggregation of α-synuclein is thought to be a critical step in the pathogenesis of PD and several other neurodegenerative disorders [13]. Recently, it has been suggested that the precursors of fibrils, oligomers of α-synuclein, might be more toxic than fibrils [14, 15].

The mechanisms of in vivo α-synuclein aggregation and toxicity are not well understood. However, it has been shown that many environmental factors, including heavy metals and pesticides, may facilitate the aggregation process [16, 5, 17–20]. Recently, a commercially available library of drug-like molecules was analyzed to establish potential inhibitors of α-synuclein fibrillation [21]. Fifteen of 169 compounds screened inhibited fibril formation of α-synuclein. All but one of the inhibitors were catecholamines, including dopamine and L-dopa, norepinephrine, and epinephrine. It was concluded that the inhibitory activity of dopamine depended on its oxidative ligation to α-synuclein [21].

Epidemiological studies have indicated that leprosy patients had significantly lower probability of senile dementia development if they had been under antileprosy treatment with dapsone or rifampicin (Figure 1) and closely related drugs for the preceding several years [22, 23]. For example, it has been shown that the overall prevalence of senile dementia was 2.9% in 1410 patients who were continuously on antileprosy treatment, compared with 6.25% in 1761 untreated patients [24]. Based on this observation, it has been suggested that some drugs being used for leprosy might prevent Aβ aggregation, thus resulting in the absence of amyloid deposition [25]. This hypothesis has been tested with two antileprosy drugs, depasone and rifampicin, and it has been found that rifampicin and its analogs, *p*-benzoquinone and hydroquinone, inhibited Aβ1-40 or Aβ1-42 aggregation and neurotoxicity in vitro [25–27]. Similar rifampicin effects have been recently reported for human islet amyloid polypeptide, amylin [26]. Rifampicin is a semisynthetic derivative of the rifamycins, a class of antibiotics that are fermentation products of *Nocardia mediterranei* [28]. The common structure of rifamycins is a naphthohydroquinone or naphthoquinone chromophore spanned by an aliphatic chain. In addition to treatment for leprosy, rifampicin is widely used in some countries for the treatment of tuberculosis [29].

Observations that catecholamines inhibited fibril formation of α-synuclein [21, 30] and that rifampicin and related drugs prevented aggregation and neurotoxicity of Aβ peptide and amylin [25–27] led us to investigate the effect of rifampicin on α-synuclein aggregation in vitro. Although the mechanism of rifampicin inhibition of fibrillation is unknown, it has been suggested that it may act as a free radical scavenger. In the present study, we show that rifampicin can inhibit α-synuclein fibrillation and efficiently disaggregates preformed fibrils in a concentration-dependent manner. Subsequent analysis suggested that these effects result from the preferential stabilization of monomeric and soluble oligomeric forms of the protein by rifampicin or its derivatives. Thus, rifampicin may potentially have therapeutic application for Parkinson's disease.

Results

Rifampicin Inhibits α-Synuclein Fibrillation in a Dose-Dependent Manner

Thioflavin T (ThT) is a fluorescent dye, which interacts with fibrils relatively specifically, and is widely used for the detection of amyloid fibrils [31, 32]. We first examined the effect of rifampicin on the kinetics of fibrillation

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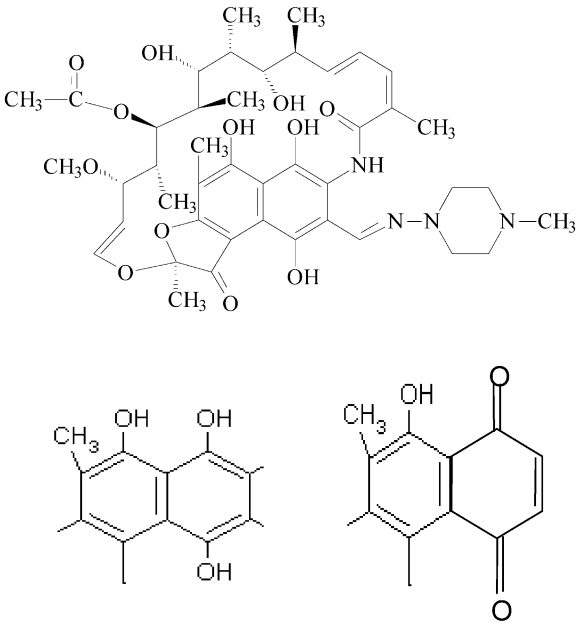

Figure 1. The Structure of Rifampicin, Its Naphthyl Core, and Quinone Form

The lower structures show the naphthyl core of rifampicin (left) and the corresponding quinone form (right).

of α-synuclein. As shown in Figure 2A, the fibrillation of α-synuclein is completely inhibited by 100 μM rifampicin as monitored by ThT. Confirmation that fibrillation was inhibited by rifampicin was obtained by static light scattering experiments (data not shown) and by measuring the amount of insoluble material as a function of time using the Lowry assay to determine the concentration of protein in both supernatant and pellet (Figure 2B) (aliquots were removed at various times during the incubation and centrifuged, prior to the assays). In addition, pellets and supernatants were analyzed using SDS PAGE: the data (data not shown) indicate that most of the protein (≥90%) was in the insoluble fraction in the control experiment, whereas in the presence of rifampicin most of the protein was in the soluble fraction, confirming that rifampicin inhibited aggregation. Figures 2B and 2C show that rifampicin inhibits the aggregation of α-synuclein in a concentration-dependent manner, based on both ThT fluorescence and protein deposited, as measured by the Lowry assay. Near-maximal inhibition is obtained with 50 μM rifampicin.

In order to elucidate the molecular mechanism of rifampicin inhibition of α-synuclein aggregation, the time course of changes in the far-UV CD spectra of the soluble fraction of the protein in the absence or in the presence of 100 μM rifampicin were studied. To this end, aliquots of the incubation mixture were withdrawn at desired time points and spun at 14,000 × g for 15 min to remove insoluble material, and the supernatants were subjected to far-UV CD analysis. Figure 3A shows that within the time interval from 0 and 24 hr in the absence of rifampicin the far-UV CD spectrum of the soluble α-synuclein fraction was indicative of a substantially unfolded protein. This was manifested by a minimum in

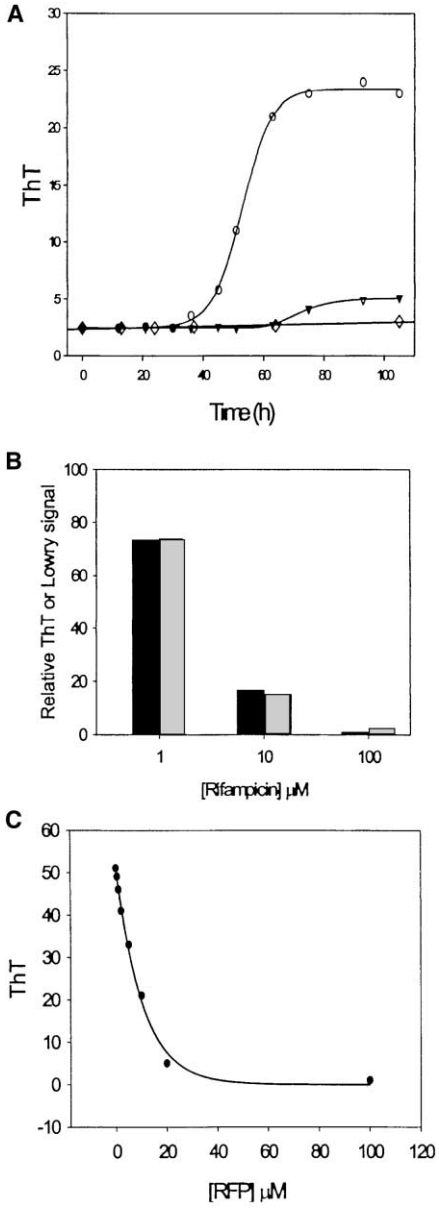

Figure 2. Rifampicin Inhibits α-Synuclein Fibrillation

(A) Kinetics of fibril formation monitored by ThT fluorescence. Control (circles), with 50 μM (triangles), and 100 μM rifampicin (diamonds). Increasing concentrations of rifampicin lead to slower kinetics of fibrillation and fewer fibrils.

(B and C) Dose-dependent inhibition of α-synuclein fibrillation by rifampicin. (B) shows that the amount of insoluble α-synuclein decreases with increasing rifampicin, as shown by the decreased ThT signal (black) and the amount of protein in the pellet (gray, measured by the Lowry assay) at completion of the incubation compared to the value in the absence of rifampicin (100%). In (C), the final ThT signal (a measure of the amount of fibrils) is shown as a function of rifampicin concentration. The incubation conditions were 70 μM α-synuclein (pH 7.4), 100 mM NaCl, 37°C.

the vicinity of 198 nm and the absence of bands in the 210–230 nm region. However, the far-UV CD spectrum measured for the soluble fraction of α-synuclein after 40 hr of incubation possessed features characteristic of a partially folded conformation. Figure 3A shows that

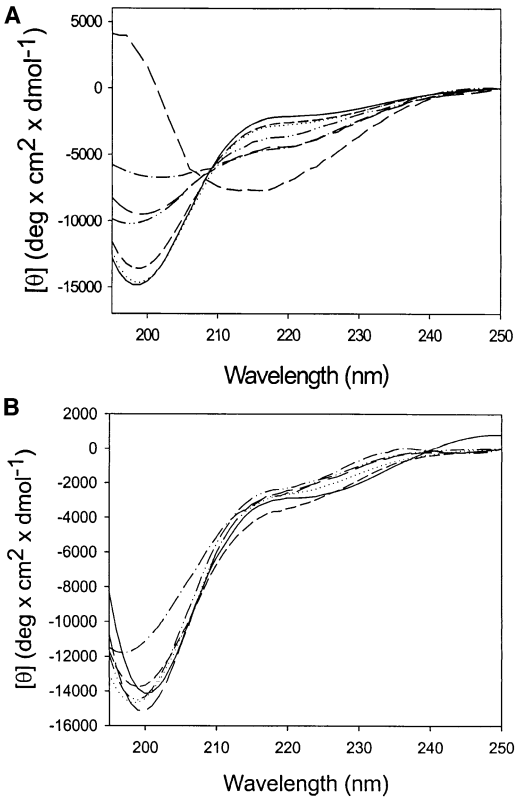

Figure 3. Rifampicin Prevents Significant Conformational Change in α-Synuclein

Far-UV circular dichroism spectra as a function of time. (A) Spectra of the supernatant in the absence of rifampicin, showing increasing amounts of β-structure with time; (B) in the presence of 100 μM rifampicin. Spectra were taken at 0 hr (solid line), 16 hr (dotted line), 24 hr (short dash), 40 hr (dash-dot-dot), 46 hr (long dash), 64 hr (dash-dot), and 72 hr (medium dash). Conditions are described under Experimental Procedures, and the spectra are corrected for protein concentration and any contribution of rifampicin.

the minimum in the vicinity of 200 nm decreased in intensity, whereas the negative intensity of the spectrum around 220 nm increased, reflecting incubation-induced formation of secondary structure. Previously, we have shown that such structural changes may be due to the formation of soluble oligomeric species comprised of partially folded α-synuclein [5, 20]. At 72 hr, after fibrillation was essentially complete, the soluble fraction of α-synuclein showed a far-UV CD spectrum typical of a β sheet protein, which we attribute to the presence of soluble oligomeric intermediates of α-synuclein. It is important to note that because the CD spectra are only of the soluble fractions of the reaction mixture, the actual intensities of the far-UV CD spectra were decreased starting from 46 hr, when significant fibrillation began. In contrast, incubation of α-synuclein with rifampicin (100 μM) for 46 hr was not accompanied by any significant changes in the CD spectra (Figure 3B), indicating the lack of change in the protein secondary structure. Only after incubation for 64 hr were minor changes in the far-UV CD spectrum observed, and the intensities of the spectra did not decrease after incubation for 64 hr in the presence of the rifampicin.

Figure 4A compares size-exclusion chromatography (SEC) profiles measured for the soluble fractions of α-synuclein incubated in the absence or presence of 100 μM rifampicin. The position of the monomer peak (V_{el} = 18.5 ml, which corresponded to R_S = 35 ± 2 Å, a value appropriate for natively unfolded α-synuclein [33]) was identical in the presence or absence of rifampicin. After 42 hr of incubation in the absence of rifampicin, there was only a trace of monomer present in the supernatant, whereas in the presence of 100 μM rifampicin a large amount of monomer and some oligomer were observed in the solution. Thus, either a mixture of monomer and oligomer are formed in the presence of rifampicin, or the soluble oligomer(s) is not very stable and mostly dissociated during the chromatographic process. Regardless, the fact that the oligomers are present in the HPLC eluant after 15 min on the column indicates that the dissociation rate of the oligomers is quite slow. In either case, the results indicate that rifampicin prevents α-synuclein from fibrillation and deposition.

The HPLC SEC data provide a number of additional insights into the interaction of rifampicin with α-synuclein. As shown in Figure 4B, monitoring the absorbance at 480 nm, where only rifampicin absorbs, shows that the soluble material after 24 or 48 hr of incubation contains both monomeric and oligomeric α-synuclein and that, in both cases, rifampicin is tightly or covalently bound to the protein.

Further confirmation that rifampicin inhibits α-synuclein fibrillation comes from electron microscope (EM) images of amyloid fibrils formed from α-synuclein in the absence and presence of 100 μM rifampicin. In the absence of rifampicin (Figure 5A), long needle-like fibrils were observed with a diameter of 6–12 nm (the thicker fibrils appeared to arise from interaction of thinner ones). In the presence of rifampicin (Figure 5B), the amount of fibrils formed was minimal, and it was difficult to find fibrils on the grid. Fibrils that were observed to grow in the presence of rifampicin appear essentially thinner and considerably shorter that those grown in the absence of the antibiotic.

Effect of Rifampicin Oxidation

Aqueous solutions of rifampicin are unstable and break down to 3-formyl rifampicin and rifampicin quinone (favored by acidic and basic conditions, respectively). This degradation can be readily monitored spectrophotometrically around 480 nm [34]. Under the conditions used for α-synuclein fibrillation (pH 7.5, 37°C), we found the rate of degradation of 100 μM rifampicin to be biphasic, i.e., biexponential, with observed rate constants of 0.84 ± 0.06 and 0.067 ± 0.007 hr^{-1}, based on the decrease in absorbance at 483 nm. Thus, significant amounts of degradation products will be present during the incubation. This was confirmed by ESI mass spectrometry; the major component having a molecular mass of 221.7 Da, corresponding to the naphthyl core of rifampicin. Other major products had molecular masses of 353, 383 (quinone), 441, 455, and 528 Da. In order to determine whether rifampicin itself or its degradation products were responsible for the inhibition of α-synuclein fibrillation, we preincubated 100 μM rifampicin for

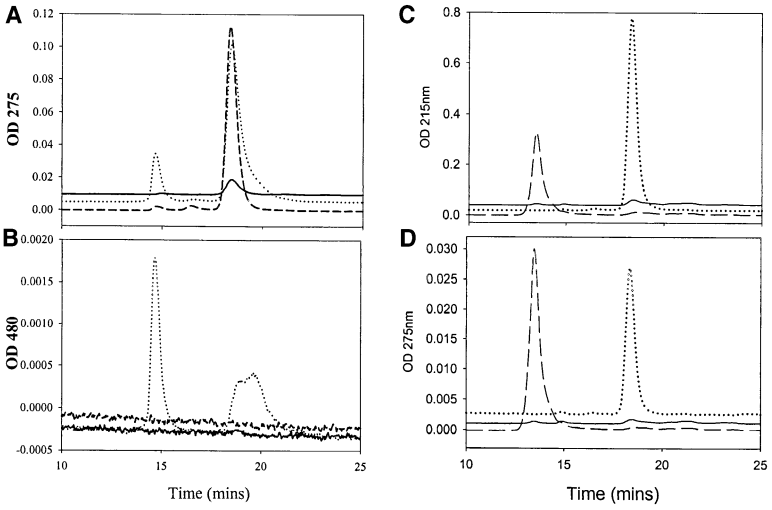

Figure 4. The Presence of Rifampicin Leads to Stable α-Synuclein Oligomers Containing Rifampicin

Size-exclusion chromatography (SEC) profiles for the soluble fractions of α-synuclein (70 μM) incubated in the absence or presence of 100 μM rifampicin. The monomer elutes at 18.5 min and the oligomer at 14.5 min.

(A and B) Inhibition of fibrillation by rifampicin. After 42 hr incubation in the absence of rifampicin (A) (solid line), there is just a trace of monomer in the supernatant. However, in the presence of 100 μM rifampicin (dotted line), both monomer and oligomer are observed. (B) Monitoring at 480 nm, where only rifampicin absorbs, shows that rifampicin is covalently associated with the monomer and oligomer (dotted line). A control of monomeric α-synuclein (dashed line) shows no signal at 480 nm.

(C and D) Disaggregation of α-synuclein fibrils (100 μM) by rifampicin (100 μM) leads to oligomer formation (dashed lines); the increased intensity of the oligomers at 275 nm (relative to 215 nm) reflect contributions from covalently bound rifampicin. The dotted lines are for α-synuclein monomers, and the solid black line is for the supernatant from incubation in the absence of rifampicin. Incubation conditions were pH 7.4, 100 mM NaCl, 37°C.

24 or 48 hr prior to initiating fibrillation. As shown in Figure 6A, under anaerobic conditions, in which the oxidative breakdown of rifampicin is inhibited, the inhibitory effect of rifampicin was much reduced, indicating that it is one of the oxidative products that is responsible for most of the inhibition. Given the relatively fast degradation of rifampicin in the presence of oxygen (under nonanaerobic conditions), there will always be some oxidative products present, and these will increase at longer incubation times. Interestingly, in the presence of antioxidants such as glutathione and ascorbic acid, which would be anticipated to minimize the oxidation

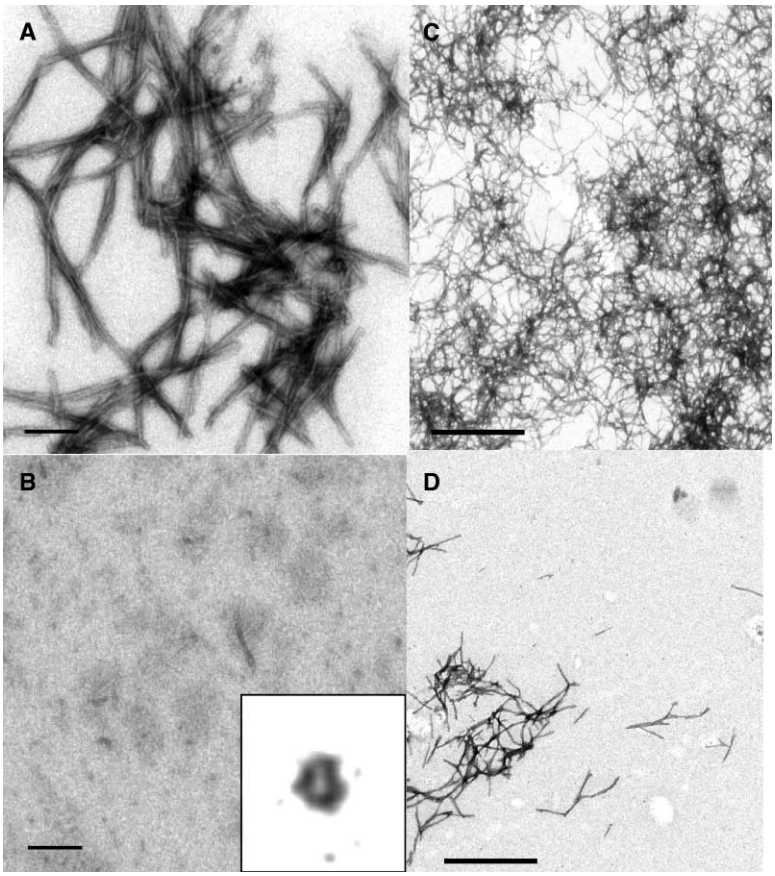

Figure 5. Rifampicin Inhibits α-Synuclein Fibril Formation and Disaggregates Existing Fibrils

TEM images of α-synuclein fibrils (A) after incubation of 35 μM α-synuclein (pH 7.4), 37°C, for 72 hr. In the presence of 100 μM rifampicin (B), very few fibrils are observed. The inset shows a close-up of an annular oligomeric intermediate (outer diameter 40–50 nm): both spherical and annular intermediates were observed, as well as very short fibrils. Similarly, incubation of α-synuclein fibrils (56 μM, grown over 96 hr) for 68 hr in the absence (C) and presence of 100 μM rifampicin (D) demonstrates rifampicin-induced dissociation of fibrils. The scale bar is 160 nm for (A) and (B) and 2 μm in (C) and (D).

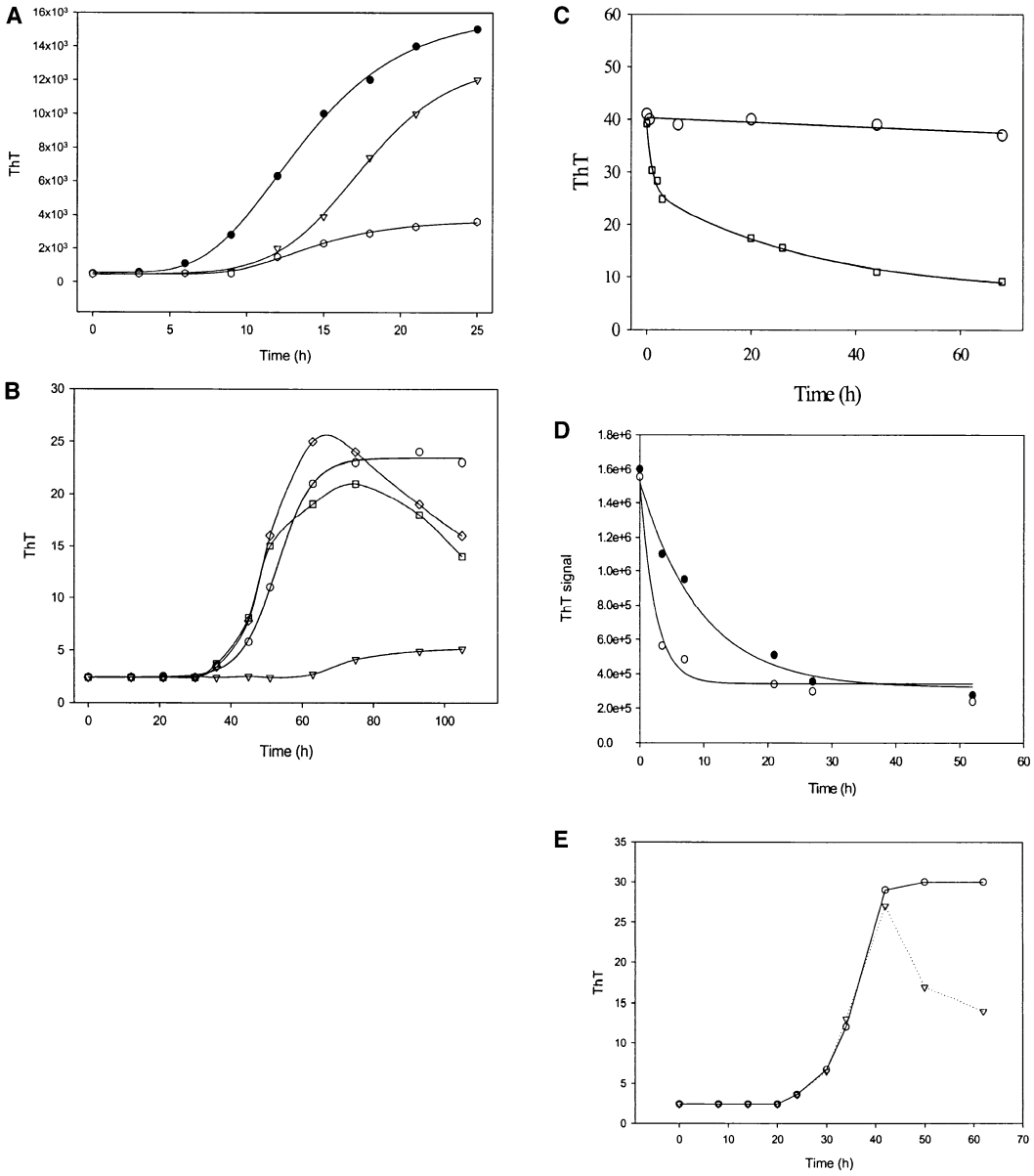

Figure 6. Oxidized Rifampicin Is More Effective Than Rifampicin in Fibril Inhibition and Disaggregation

(A and B) Fibrillation of α-synuclein monitored by ThT. (A) α-synuclein incubated under anaerobic conditions: control (filled circles), 100 μM freshly prepared rifampicin (triangles), 100 μM 24 hr aged rifampicin (open circles). (B) Glutathione and ascorbic acid (AA) prevent oxidation of rifampicin and thereby prevent rifampicin inhibition of α-synuclein fibrillation. Control (circles), 50 μM rifampicin plus 400 mM AA (triangles), or GSH (diamonds), 50 μM rifampicin (inverted triangles). The decreased ThT signal at long times in the presence of GSH or AA is attributed to rifampicin-induced disaggregation of fibrils (see main text). Conditions were 30 μM α-synuclein, pH 7.4, 100 mM NaCl, 37°C.

(C–E) Rifampicin disaggregates α-synuclein fibrils. (C) Fibrils of α-synuclein (0.8 mg/ml) were incubated at pH 7.4, 37°C in the absence (circles) and presence (squares) of 100 μM rifampicin. (D) Oxidized rifampicin (preincubated for 24 hr) (open circles) is more effective at disaggregating α-synuclein fibrils compared to fresh rifampicin (filled circles). (E) The addition of rifampicin (100 μM, triangles) after 24 hr of α-synuclein incubation leads to disaggregation of fibrils. In each case, the reaction was monitored by ThT fluorescence, and the conditions were pH 7.4, 37°C. The ThT signal magnitudes in different panels vary because samples were measured with two different systems (see Experimental Procedures).

of rifampicin, the addition of rifampicin to α-synuclein incubations led to minimal inhibition (Figure 6B). We interpret this to mean that the antioxidants resulted in maintenance of a high concentration of nonoxidized rifampicin, and this had little inhibitory effect. At longer time periods of incubation with both rifampicin and glutathione or ascorbic acid, the ThT signal began to decrease. This is most readily explained by assuming that some oxidation of rifampicin occurred, but at a slow rate, and the resulting oxidized rifampicin inhibited further fibrillation and disaggregated existing fibrils (see below).

ESI mass spectrometry was used to examine α-synuclein after incubation in the presence and absence of rifampicin: no detectable differences were observed,

suggesting that the soluble α-synuclein (monomer and oligomers) had not been covalently modified by rifampicin or its degradation products, at least under the experimental conditions used. However, if a covalent imine adduct had formed between oxidized rifampicin and the lysine side chains of α-synuclein, it is possible that it would have been hydrolyzed under the acidic conditions used for preparing the MS sample.

Rifampicin Dissolves α-Synuclein Fibrils

We next addressed the question of whether rifampicin could disaggregate existing fibrils of α-synuclein. Figure 6C shows that coincubation of α-synuclein fibrils with 100 μM rifampicin is accompanied by a large decrease in ThT signal as well as a substantial decrease in the amount of insoluble material (based on Lowry assays; data not shown). Thus, rifampicin can dissolve preformed α-synuclein fibrils. The kinetics of rifampicin-induced disaggregation of α-synuclein fibrils are biphasic, with the half-life for the faster, main process being ~1 hr under the experimental conditions used. If the rifampicin was allowed to undergo oxidative degradation prior to addition to the fibrils, the kinetics of disaggregation were significantly faster, indicating that both breakdown products and intact rifampicin are active in disaggregation (Figure 6D). The addition of 50 μM rifampicin to α-synuclein that had been incubated for 24 hr led to a decrease in the ThT signal, consistent with the disaggregation of fibrils (Figure 6E).

Further confirmation that rifampicin dissolves α-synuclein fibrils and leads to the formation of soluble oligomers was obtained from SEC HPLC experiments. When α-synuclein fibrils were incubated at 37°C in buffer (pH 7.4) and then centrifuged, the supernatant contained virtually no soluble protein (Figures 4C and 4D). However, if the fibrils were incubated with 100 μM rifampicin, the HPLC trace showed only soluble oligomers. Furthermore, the intensity of the peak at 275 nm compared to 215 nm indicated that rifampicin was bound to the oligomers.

The results of EM analysis also demonstrated the rifampicin-induced dissociation of preformed α-synuclein fibrils (Figure 5) as the amount of fibrillar material adsorbed on the grid decreased drastically after 68 hr incubation of α-synuclein fibrils in the presence of 100 μM rifampicin (compare Figures 5C and 5D).

We used circular dichroism to investigate the secondary structure of α-synuclein in the soluble oligomers formed after disaggregation in the presence of rifampicin. Figure 7 compares the far-UV CD spectra of natively unfolded α-synuclein and the soluble fractions extracted from incubation of α-synuclein fibrils in the absence or in the presence of rifampicin. Both spectra corresponding to the soluble fractions had shapes typical of β-structural proteins. The CD analysis clearly demonstrates that the conformation of α-synuclein in the soluble species resulting from rifampicin-induced dissociation of fibrils was predominantly β-structure. Consequently, the SEC HPLC and far-UV CD data indicate that the rifampicin-disaggregated fibrils were stabilized in the form of large soluble oligomers enriched in β-structure.

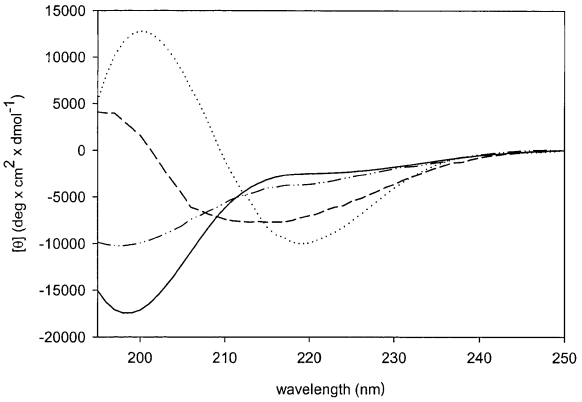

Figure 7. Rifampicin-Induced Disaggregation of α-Synuclein Fibrils Leads to Conformational Changes

Far-UV circular dichroism spectra of α-synuclein: control, monomeric α-synuclein (solid line); supernatant after incubation of α-synuclein fibrils with 100 μM rifampicin (dotted); supernatant after incubation of α-synuclein with rifampicin (dash-dot-dot); and for comparison, the supernatant after incubation of α-synuclein in the absence of rifampicin (dashed). Conditions are described under Experimental Procedures.

Discussion

The aggregation of α-synuclein is a key factor in the development of Parkinson's disease and related α-synucleinopathies [13, 35]. Consequently, molecules that inhibit fibrillation or disaggregate fibrils of α-synuclein may lead to therapies to prevent or control Parkinson's disease as well as to a better understanding of the process. Substantial evidence suggests that factors that destabilize the proteins involved in deposition diseases lead to the population of partially folded intermediates, which are critical species in the aggregation process [36]. In contrast, for natively unfolded proteins, such as α-synuclein, it is factors that lead to partial folding that are critical in aggregation [9]. The underlying kinetic basis of fibrillation is consistent with a nucleated polymerization and can be considered to consist of two components, a lag phase leading to formation of the nucleus, followed by rapid formation of fibrils, which grow through exponential elongation and possible subsequent lateral interactions. Several recent observations suggest that oligomeric intermediates (sometimes called protofibrils) present during aggregation may be more cytotoxic then fibrils themselves [37–43]. The minimum kinetic scheme for α-synuclein aggregation/fibrillation involves the initial formation of a critical partially folded intermediate that undergoes partitioning either to fibrils, soluble oligomers, or amorphous aggregates.

The data presented here indicate that rifampicin, in the 1–10 μM concentration range, leads to inhibition of α-synuclein fibril formation. Furthermore, the results demonstrate that existing fibrils are disaggregated by similar concentrations of rifampicin. In both cases, inhibition and disaggregation, the end product is a mixture of a large soluble oligomer of α-synuclein, with substantial β sheet structure, and monomer. In addition, our observations reveal that an oxidized form (or forms) of rifampicin is more potent than the parent compound

itself, and that rifampicin or a fragment is present in the monomers and oligomers resulting from inhibition or disaggregation, suggesting covalent incorporation. In the context of the minimum kinetic scheme for α-synuclein aggregation, rifampicin and its oxidation products favor the pathway leading to soluble oligomers and stabilization of these species. The fact that less than stoichiometric amounts of rifampicin are required for inhibition is attributed to the formation of heterologous oligomers of rifampicin-modified and unmodified α-synuclein.

An understanding of the underlying molecular mechanism of rifampicin-induced inhibition and disaggregation is complicated by the instability of rifampicin. Since our data show that samples of rifampicin that had been preincubated for 24 or 48 hr were more effective inhibitors, and that much less inhibition was observed by rifampicin under anaerobic conditions or in the presence of antioxidants, in which oxidation is prevented, it is likely that an oxidation product is the major species responsible for inhibition of fibrillation. Thus, we believe that it is a quinone form of rifampicin that is the major active species in the inhibition. The inhibition of α-synuclein fibrillation by dopamine and related catecholamines has been reported [21, 30]. Conway et al. [21] proposed that the mechanism of inhibition involved the orthoquinone derivative of dopamine forming a covalent adduct with 5%–10% of the α-synuclein. The small amounts of covalently modified α-synuclein inhibited fibrillation, leading to the accumulation of protofibrils.

Quinones are susceptible to nucleophilic attack via Michael addition or imine (Schiff-base) formation by the lysine side chains of α-synuclein, leading to covalent modification. The spectrum of α-synuclein in the oligomer stabilized by rifampicin shows evidence for such covalent modification, in that the absorbance spectrum of the oligomer from α-synuclein plus rifampicin shows the spectral signal of rifampicin or a derivative, although, as noted in the Results, covalent modification of the protein was not confirmed by mass spectrometry. The SEC HPLC data show that rifampicin or its oxidation products result in the formation of a slowly dissociating oligomer of α-synuclein. It is the presence of this stable oligomer that is most likely responsible for the lack of fibrillation. The oligomers were also observed by EM and light scattering.

Interestingly, although oxidized rifampicin is more effective, rifampicin itself is still quite efficient in disaggregating fibrils of α-synuclein. Consequently, the issue of whether rifampicin will be oxidized or not in vivo is not critical to its potential utility in treating PD. The results with rifampicin are very similar to those we have recently observed with baicalein, a flavonoid, which also inhibits α-synuclein fibrillation and disaggregates existing fibrils by stabilizing a soluble oligomer [44]. As with rifampicin, an oxidized quinone form is more effective in inhibition then the parent compound, but there is little difference in disaggregation.

These observations have important implications for our understanding of protein deposition diseases. If fibrillar α-synuclein contributes to degeneration of nigral neurons, then disassembly of fibrils by rifampicin may reverse or slow down the progression of the disease. However, if the products of fibril inhibition or disaggregation are neurotoxic, then the accumulation of stable fibrillar deposits, such as Lewy bodies in Parkinson's disease, could be a marker of neurons that are relatively resistant to the neurodegenerative process. In addition, if the end products of rifampicin inhibition/dissociation of α-synuclein fibrils, the soluble oligomers, are neurotoxic, then therapeutic treatments based on rifampicin will not be feasible.

Whereas there is strong support that the incidence of dementia is decreased in leprosy patients taking rifampicin, there are conflicting accounts of whether rifampicin is effective in inhibiting formation of fibrils from Aβ [25, 26, 45–47]. Interestingly, a leprosy genetic susceptibility locus has been mapped to a region shared by the Parkinson's disease gene PARK2 [48].

Significance

We show that the antibiotic rifampicin, in substoichiometric low micromolar concentrations, inhibits α-synuclein fibrillation and disaggregates existing fibrils in a concentration-dependent manner. In both inhibition and disaggregation, the end product is a mixture of monomer and large soluble oligomers of α-synuclein with substantial β sheet structure. The results indicate that an oxidized form (or forms) of rifampicin is more potent than the parent compound itself, and that rifampicin or a structural fragment is present in the monomers and oligomers resulting from inhibition or disaggregation, suggesting covalent incorporation, most likely by the quinone form. The data indicate that in the presence of rifampicin the aggregation of α-synuclein involves kinetic partitioning to a pathway leading to relatively stable soluble oligomers, rather than fibrils. These results indicate that rifampicin potentially may have therapeutic application for Parkinson's disease.

Experimental Procedures

Materials

Rifampicin and Thioflavin T (ThT) were obtained from Sigma and Fluka, respectively. All other chemicals were of analytical grade from Fisher.

Synuclein Purification

Human wild-type α-synuclein was expressed in the *E. coli* BL21(DE3) cell line transfected with pRK172/α-synuclein WT plasmid (the kind gift of M. Goedert, MRC Cambridge) and purified as previously described [49]. Lyophilized protein was dissolved immediately before use in deionized, purified H_2O, and the pH was adjusted to 10 ± 0.5 with 0.1 N NaOH (about 3 mM final) to solubilize any aggregated protein. After 10 min incubation at room temperature, the pH was readjusted to 7–8 with 0.1 N HCl. The protein concentration was determined by measuring the absorbance at 275 nm and using an extinction coefficient of 0.40 $mg^{-1}cm^2$. For fibrillation assays, the solution was centrifuged at 14,000 rpm for 10 min prior to starting the incubation.

Fibril Formation

α-synuclein was incubated for various time periods under the following conditions. Protein concentration was kept at 1.0 mg/ml (70 μM) in 25 mM Tris-HCl and 0.1 M NaCl at pH 7.6 (fibrillation buffer). Samples (0.5 ml) of protein solution in glass vials with or without rifampin were stirred with micro-stir bars at 37°C. Aliquots of 5 μl of the reaction mixture were removed from the incubated sample and added to 1.0 ml of 10 μM ThT in fibrillation buffer, from which

fibril formation was monitored by characteristic changes in ThT fluorescence. Incubations were run at least in duplicate, and all experiments were repeated several times. The estimated errors in the averaged kinetics for nucleation and elongation are ± 15%. Although the fluorescence of ThT is quenched by large concentrations of rifampicin, this was not a problem under the experimental conditions used, due to the very low concentrations present in the assay.

Fluorescence measurements were performed in semimicro-quartz cuvettes (Hellma) with a 1 cm excitation light path using a FluoroMax-3 spectrofluorometer (Instruments S. A., Inc.) or using a fluorescent plate reader (Fluoroskan CF). ThT fluorescence was recorded immediately after addition of the aliquots to the ThT solution from 470 to 560 nm with excitation at 450 nm, an increment of 1 nm, an integration time of 1 s, and slits of 5 nm for both excitation and emission. For each sample, the signal was obtained as the ThT intensity at 482 nm, from which a blank measurement recorded prior to addition of α-synuclein to the ThT solution was subtracted. All data were processed using DataMax/GRAMS software.

Circular Dichroism Measurements

CD spectra were obtained with an Aviv 60DS spectrophotometer using an α-synuclein concentration of 1.0 mg/ml. Spectra were recorded in a 0.01 cm cell from 250 to 195 nm with a step size of 0.5 nm, a bandwidth of 1.5 nm, and an averaging time of 5 sec at room temperature. For all spectra, an average of five scans was obtained. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra. For incubated solutions, the sample was centrifuged if necessary, and the protein concentration of the sample was determined using the Lowry assay. Conditions were pH 7.4, 100 mM NaCl, 25°C or 37°C, unless otherwise indicated.

Size-Exclusion Chromatography

The hydrodynamic dimensions (Stokes radius, R_S) of α-synuclein were measured by size-exclusion chromatography on a BioSep-SEC-S 2000 column (from Phenomenex) using a Waters HPLC system. Size-exclusion analysis was performed using 50 mM Na phosphate/0.1 M Na_2SO_4 (pH 7.0) buffer as mobile phase. A set of globular proteins (Gel Filtration Chromatography Standards from Bio-Rad Laboratories) with known R_S values was used in order to create a calibration curve, $1000/V_{el}$ versus R_S [50–53].

Electron Microscopy

Transmission electron micrographs were collected using a JEOL JEM-100B microscope operating with an accelerating voltage of 80 kV. Typical nominal magnifications ranged from 20,000–75,000×. An aliquot of 5 μl of sample was loaded on a 300 mesh copper grid, which was Formvar coated and carbon stabilized. The grid was then gently washed with water and stained with 1% aqueous uranyl acetate.

Mass Spectrometry

α-synuclein was incubated with 100 μM rifampicin at pH 7.5, 37°C for 2–4 hr, and the resulting solution was analyzed using a MicroMass Quattro II electrospray instrument. Samples for MS analysis were prepared by diluting 2 μl of protein solution in 200 μl of 50% acetonitrile/50% (pH 2.0) HCl mixture and introduced via a Harvard Apparatus (Holliston, MA) syringe pump at a flow rate of 6 μl/min. The source temperature was set to 50°C, and the capillary voltage was 3.0 kV.

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References

1. Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H.A., Kittel, A., and Saitoh, T. (1995). The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. Neuron *14*, 467–475.
2. Irizarry, M.C., Kim, T.W., McNamara, M., Tanzi, R.E., George, J.M., Clayton, D.F., and Hyman, B.T. (1996). Characterization of the precursor protein of the non-A beta component of senile plaques (NACP) in the human central nervous system. J. Neuropathol. Exp. Neurol. *55*, 889–895.
3. Jakes, R., Spillantini, M.G., and Goedert, M. (1994). Identification of two distinct synucleins from human brain. FEBS Lett. *345*, 27–32.
4. Weinreb, P.H., Zhen, W.G., Poon, A.W., Conway, K.A., and Lansbury, P.T., Jr. (1996). NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. Biochemistry *35*, 13709–13715.
5. Uversky, V.N., Lee, H.J., Li, J., Fink, A.L., and Lee, S.J. (2001). Stabilization of partially folded conformation during alpha-synuclein oligomerization in both purified and cytosolic preparations J. Biol. Chem. *276*, 43495–43498.
6. Eliezer, D., Kutluay, E., Bussell, R., Jr., and Browne, G. (2001). Conformational properties of alpha-synuclein in its free and lipid-associated states J. Mol. Biol. *307*, 1061–1073.
7. Dunker, A.K., Lawson, J.D., Brown, C.J., Williams, R.M., Romero, P., Oh, J.S., Oldfield, C.J., Campen, A.M., Ratliff, C.M., Hipps, K.W., et al. (2001). Intrinsically disordered protein. J. Mol.Graph. Model. *19*, 26–59.
8. Wright, P.E., and Dyson, H.J. (1999). Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm J. Mol. Biol *293*, 321–331.
9. Uversky, V.N., Li, J., and Fink, A.L. (2001). Evidence for a partially folded intermediate in alpha-synuclein fibril formation J. Biol. Chem. *276*, 10737–10744.
10. Forno, L.S. (1996). Neuropathology of Parkinson's disease. J. Neuropathol. Exp. Neurol. *55*, 259–272.
11. Spillantini, M.G., Schmidt, M.L., Lee, V.M.Y., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in lewy bodies. Nature *388*, 839–840.
12. Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and Goedert, M. (1998). *a*-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc. Natl. Acad. Sci. USA *95*, 6469–6473.
13. Trojanowski, J.Q., and Lee, V.M. (2003). Parkinson's disease and related alpha-synucleinopathies are brain amyloidoses. Ann. N.Y. Acad. Sci. *991*, 107–110.
14. Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., and Lansbury, P.T., Jr. (2002). Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. J. Mol. Biol *322*, 1089–1102.
15. Volles, M.J., and Lansbury, P.T., Jr. (2003). Zeroing in on the pathogenic form of alpha-synuclein and its mechanism of neurotoxicity in Parkinson's disease biochemistry *42*, 7871–7878.
16. Uversky, V.N., Li, J., and Fink, A.L. (2001). Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease FEBS Lett. *500*, 105–108.
17. Uversky, V.N., Li, J., and Fink, A.L. (2001). Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular link between Parkinson's disease and heavy metal exposure. J. Biol. Chem. *276*, 44284–44296.
18. Manning-Bog, A.B., McCormack, A.L., Li, J., Uversky, V.N., Fink, A.L., and Di Monte, D.A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice. J. Biol. Chem. *277*, 1641–1644.
19. Uversky, V.N., Li, J., Zhu, M, Bower, K, and Fink, A.L. (2002). Synergistic effects of pesticides and metals on the fibrillation of alpha-synuclein: Implications for Parkinson's disease. Neurotoxicology *23*, 527–536.
20. Li, J., Uversky, V.N., and Fink, A.L. (2002). Conformational behavior of human alpha-synuclein is modulated by familial Par-

kinson's disease point mutations A30P and A53T. Neurotoxicology *23*, 553–567.
21. Conway, K.A., Rochet, J.C., Bieganski, R.M., and Lansbury, P.T., Jr. (2001). Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science *294*, 1346–1349.
22. Namba, Y., Kawatsu, K., Izumi, S., Ueki, A., and Ikeda, K. (1992). Neurofibrillary tangles and senile plaques in brain of elderly leprosy patients. Lancet *340*, 978.
23. Chui, D.H., Tabira, T., Izumi, S., Koya, G., and Ogata, J. 1994 Decreased beta-amyloid and increased abnormal Tau deposition in the brain of aged patients with leprosy. Am. J. Pathol. *145*, 771–775.
24. McGeer, P.L., Harada, N., Kimura, H., McGreer, E.G., and Schulzer, M. (1992). Dementia *3*, 146–149.
25. Tomiyama, T., Shoji, A., Kataoka, K., Suwa, Y., Asano, S., Kaneko, H., and Endo, N. (1996). Inhibition of amyloid beta protein aggregation and neurotoxicity by rifampicin. Its possible function as a hydroxyl radical scavenger. J. Biol. Chem. *271*, 6839–6844.
26. Tomiyama, T., Kaneko, H., Kataoka, K., Asano, S., and Endo, N. (1997). Rifampicin inhibits the toxicity of pre-aggregated amyloid peptides by binding to peptide fibrils and preventing amyloid-cell interaction. Biochem. J. *322*, 859–865.
27. Tomiyama, T., Asano, S., Suwa, Y., Morita, T., Kataoka, K., Mori, H., and Endo, N. (1994). Rifampicin prevents the aggregation and neurotoxicity of amyloid beta protein in vitro. Biochem. Biophys. Res. Commun. *204*, 76–83.
28. Furesz, S. (1970). Chemical and biological properties of rifampicin. Antibiot. Chemother. *16*, 316–351.
29. Hartmann, G., Honikel, K.O., Knusel, F., and Nuesch, J. 1967 The specific inhibition of the DNA-directed RNA synthesis by rifamycin. Biochim. Biophys. Acta *145*, 843–844.
30. Li, J., Zhu, M., Manning-Bog, A., Di Monte, D., and Fink, A.L. (2004). Dopamine and L-dopa disaggregate amyloid fibrils: Implications for Parkinson's and Alzheimer's disease. FASEB J. *18*, 962–964.
31. Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989). Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, thioflavin T. Anal. Biochem. *177*, 244–249.
32. Levine, H. (1993). Thioflavine-T interaction with synthetic Alzheimer's disease β-amyloid peptides: detection of amyloid aggregation in solution. Protein Sci. *2*, 404–410.
33. Uversky, V.N., Li, J., Souillac, P.O., Millett, I.S., Doniach, S., Jakes, R., Goedert, M., and Fink, A.L. (2002). Biophysical properties of the synucleins and their propensities to fibrillate: inhibition of alpha-synuclein assembly by beta- and gamma-synucleins J. Biol. Chem. *277*, 11970–11978.
34. Walash, M.I., Belal, F., Metwally, M.E., and Hefnawy, M.M. (1993). Fluorimetric determination of tauromustine (a novel antitumour agent) in formulations and biological fluids. J. Pharm. Biomed. Anal. *11*, 777–780.
35. Goedert, M. (2001). Parkinson's disease and other alpha-synucleinopathies. Clin. Chem. Lab. Med. *39*, 308–312.
36. Fink, A.L. (1998). Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold. Des. *3*, R9–R23.
37. Michikawa, M., Gong, J. S., Fan, Q.W., Sawamura, N., and Yanagisawa, K. (2001). A novel action of Alzheimer's amyloid beta protein Abeta: oligomeric a beta promotes lipid release. J. Neurosci. *21*, 7226–7235.
38. Rochet, J.C., Conway, K.A., and Lansbury, P.T., Jr. (2000). Inhibition of fibrillization and accumulation of prefibrillar oligomers in mixtures of human and mouse alpha-synuclein. Biochemistry *39*, 10619–10626.
39. Ding, T.T., Lee, S.J., Rochet, J.C., and Lansbury, P.T., Jr. (2002). Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. Biochemistry *41*, 10209–10217.
40. El Agnaf, O.M., Nagala, S., Patel, B.P., and Austen, B.M. (2001). Non-fibrillar oligomeric species of the amyloid ABri peptide, implicated in familial British dementia, are more potent at inducing apoptotic cell death than protofibrils or mature fibrils. J. Mol. Biol. *310*, 157–168.
41. Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., and Lansbury, P.T., Jr. (2002). Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. J. Mol. Biol. *322*, 1089–1102.
42. Volles, M.J., and Lansbury, P.T., Jr. (2003). Zeroing in on the pathogenic form of alpha-synuclein and its mechanism of neurotoxicity in Parkinson's disease. Biochemistry *42*, 7871–7878.
43. Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science *300*, 486–489.
44. Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F., and Fink, A.L. (2004). The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils. J. Biol. Chem. *279*, 26846–26857.
45. Ono, K., Hasegawa, K., Yoshiike, Y., Takashima, A., Yamada, M., and Naiki, H. (2002). Nordihydroguaiaretic acid potently breaks down pre-formed Alzheimer's beta-amyloid fibrils in vitro. J. Neurochem. *81*, 434–440.
46. van Nostrand, W.E., and Melchor, J.P. (2001). Disruption of pathologic amyloid beta-protein fibril assembly on the surface of cultured human cerebrovascukar smooth muscle cells. Amyloid *8*, 20–27.
47. Endoh, M., Kunishita, T., and Tabira, T. (1999). No effect of antileprosy drugs in the prevention of Alzheimer's disease and beta-amyloid neurotoxicity. J. Neurol. Sci. *165*, 28–30.
48. Mira, M.T., Alcais, A., Nguyen, V.T., Moraes, M.O., Di Flumeri, C., Vu, H.T., Mai, C.P., Nguyen, T.H., Nguyen, N.B., Pham, X.K., et al. (2004). Susceptibility to leprosy is associated with PARK2 and PACRG. Nature *427*, 636–640.
49. Yamin, G., Uversky, V.N., and Fink, A.L. (2003). Nitration inhibits fibrillation of human alpha-synuclein in vitro by formation of soluble oligomers. FEBS Lett. *542*, 147–152.
50. Ackers, G.K. (1970). Analytical gel chromatography of proteins. Adv. Protein Chem. *24*, 343–446.
51. Corbett, R.J., and Roche, R.S. (1984). Use of high-speed size-exclusion chromatography for the study of proteins. Biochemistry *23*, 1888–1894.
52. Uversky, V.N. (1993). Use of fast protein size-exclusion liquid chromatography to study the unfolding of proteins which denature through the molten globule. Biochemistry *32*, 13288–13298.
53. Uversky, V.N. (1994). Gel-permeation chromatography as a unique instrument for quantitative and qualitative analysis of protein denaturation and unfolding. Int. J. Biochem. *1*, 103–114.