# Identification of Potent Small Molecule Inhibitors of Alpha-Synuclein Aggregation in Cell Culture and by *In Vitro* Screening

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## Abstract

A rotenone-induced cell culture cell model of Parkinson's disease (PD) was primarily utilized to test the ability of ProteoTech's small molecule library of compounds (representing new chemical entities) to inhibit aggregation of α-synuclein, a major component of PD Lewy bodies. In this cell culture model, A53T mutant α-synuclein was overexpressed in human BEM-17 neuroblastoma cells. Cells exposed to 1 or 5μM rotenone accumulated α-synuclein aggregates containing a large amount of beta-sheet secondary structure as detected by positive Thioflavin S fluorescence and quantitative image analysis. Treatment of rotenone-treated cells with different novel small molecule compounds identified 4 compounds (referred to as PD-61, 86, 31 and 13) that profoundly reduce the accumulation of Thioflavin S positive α-synuclein aggregates by 87-91%, 73-91%, 40-84% and 57-70%, respectively. A marked reduction of α-synuclein aggregation by these compounds was confirmed using Thioflavin T fluorometry, Congo red binding assays and circular dichroism spectroscopy. In addition, to their potent α-synuclein anti-aggregation properties, these compounds also provide good protection against rotenone-induced neurotoxicity in cell culture and in a C. elegans survival assay. These studies indicate that we have identified unique small molecule compounds that have potent α-synuclein anti-aggregation and neuroprotective properties and thus may serve as promising new therapeutics for PD and related disorders.

# Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that is pathologically characterized by the presence of intracytoplasmic Lewy bodies, the components that are filaments of an 140-amino acid protein known as α-synuclein. Mutations in the α-synuclein protein have been directly lined to PD. α-synuclein fibrillogenesis and Lewy body formation are considered important therapeutic targets for the treatment of PD and related disorders.

During the last 7 years, ProteoTech scientists, led by Dr. Alan Snow, in collaboration with some of the world's leading scientists in amyloid disease research, have been working on the development of disease-modifying small molecule therapeutic for the treatment of Alzheimer's Disease (AD). One compound derived from a unique small molecule synthetic analog library established at ProteoTech, known as Exebryl-1™ has been shown in AD transgenic mice to markedly reduce brain beta-amyloid protein deposition in older animals (by 50-80%) and cause marked improvements/reversal in memory impairments (by 70%) following only 3 months of administration. Exebryl-1™ is currently in late pre-clinical development and phase I human trials are planned for mid-2008.

From the design of >250 analogs from this original class of compounds, ProteoTech scientists have discovered that select small molecule analogs from this library markedly and specifically inhibit/disrupt α-synuclein fibrillogenesis in vitro and are postulated to inhibit synuclein filament and Lewy body formation in vivo. In a 3-year LEAPS award project funded by the Michael J. Fox Foundation for PD Research we are working on the identification and development of novel small molecule compounds that can penetrate the brain, and cause a clearance/disruption of neuronal α-synuclein fibrils, as well as inhibit Lewy body formation. Evidence presented here demonstrates the identification of small molecule compounds that have been found to markedly inhibit/disrupt α-synuclein fibrils in vitro and in cell-based assays.

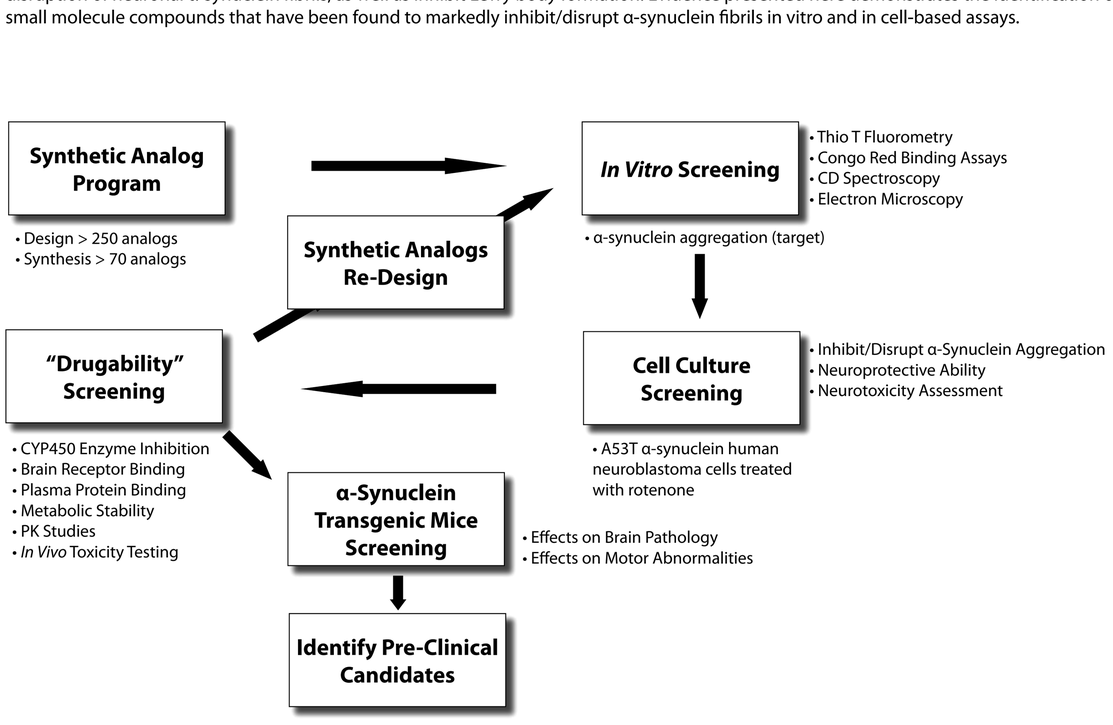


**Figure 1: ProteoTech's Small Molecule Program for Targeting α-Synuclein Aggregation in Parkinson's Disease.**

Shown is a schematic outlining ProteoTech's approach to identifying novel small molecule compounds targeting α-synuclein accumulation/aggregation in PD.

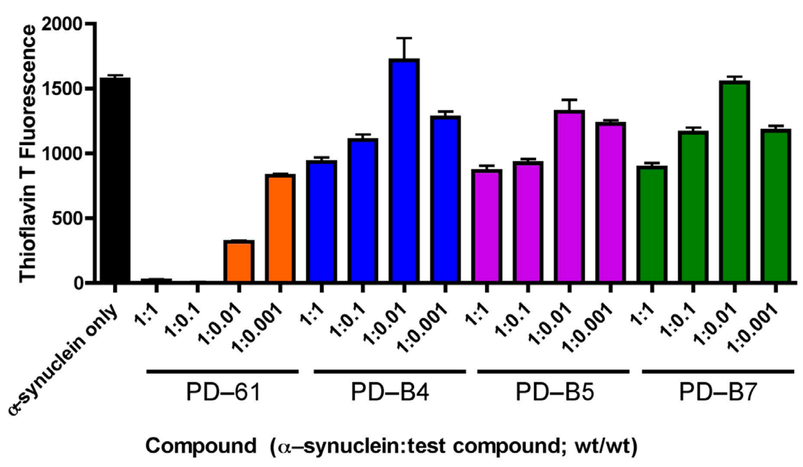


**Figure 2: Small Molecule Compound PD-61-7 Inhibits α-Synuclein Aggregation as Determined by Thioflavin T Fluorometry.**

Small molecule compound PD-61, PD-61 and 3 analogs (PD-61-B4, PD-61-B5, and PD-61-7) were tested for their ability to disaggregate pre-fibrillized α-synuclein. Following 3 days of incubation, PD-61-7demonstrated a dose-dependent disruption of α-synuclein fibrils as shown by Thioflavin T fluorescence. The other 3 analogs were not as effective.

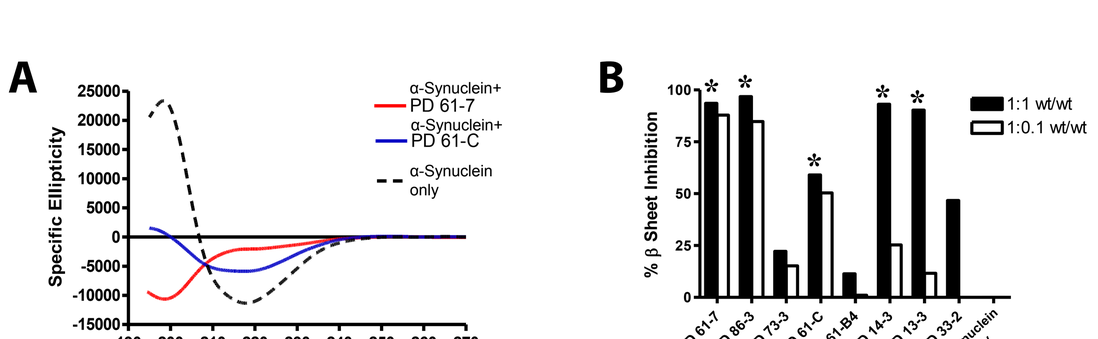


**Figure 3: ProteoTech's Small Molecule Compounds Inhibit ß-Sheet Secondary Structure as Determined by Circular Dichroism Spectroscopy.**

A) A-synuclein following 4-days of incubation at 37°C (and continuously shaking) forms a predominant ß-sheet secondary structure as demonstrated by a marked minima at 218nm (α-synuclein post-incubation) as demonstrated by CD spectroscopy. In the presence of small molecule compound PD-61-7 (α-synuclein + PD-61-7) there is a complete disaggregation/disruption of the pre-formed α-synuclein fibrils. B) Quantitation of the effects of different small molecule compounds on disaggregation of pre-formed α-synuclein fibrils demonstrates that PD-61-7, PD-86-3, PD-31-4 and PD-13-3 are effective inhibitors and exert their effects in a dose-dependent manner.




**Figure 4: Thioflavin S Fluorescence and Image Analysis Quantitation in A53T α-Synuclein-Expressing Cells.**

A) A53T mutant α-synuclein-over-expressing BeM17 cells were treated with increasing doses (1μM and 5μM) of rotenone (mitochondrial agent). Increasing doses of rotenone cause increased induction of intraneuronal α-synuclein aggregation as demonstrated by enhanced Thioflavin S fluorescence. B) Image analysis demonstrated a marked increase in Thioflavin S-positive aggregates in neuronal cytoplasm following increased treatment of rotenone (i.e. from 1μM to 5μM).

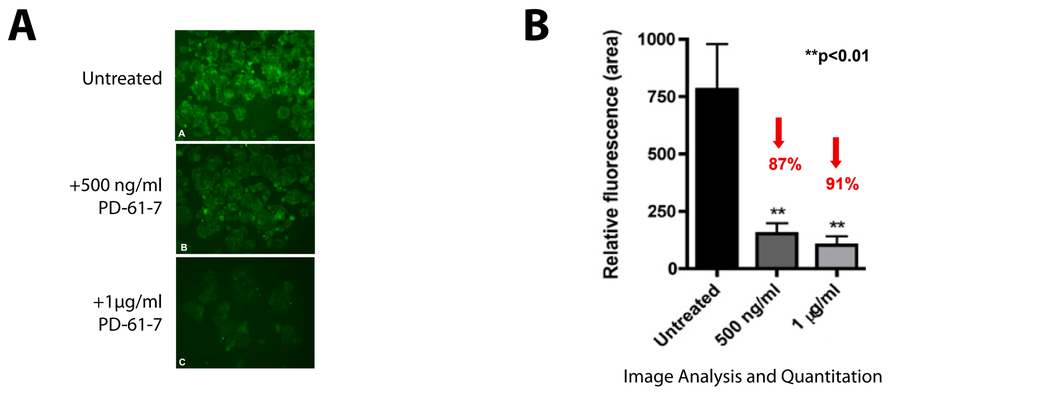


**Figure 5: PD-61-7 is a Potent Inhibitor of α-Synuclein Aggregation in A53T A-Synuclein Cells Induced with Rotenone.**

A) A53T α-synuclein BeM17 cells induced with 1μM rotenone demonstrate Thioflavin S fluorescent inclusions in neurons. B) & C) A53T α-synuclein BeM17 cells induced with 1μM rotenone and treated with 500ng/ml (Fig. B) or 1μg/ml of PD-61-7 Fig. C) demonstrate a marked decrease in α-synuclein Thioflavin S-positive inclusions. D) Image analysis and quantitation demonstrates that 500ng/ml and 1μg/ml of PD-61-7 causes a significant 87% and 91% decrease in Thioflavin-S positive α-synuclein neuronal inclusions, respectively.

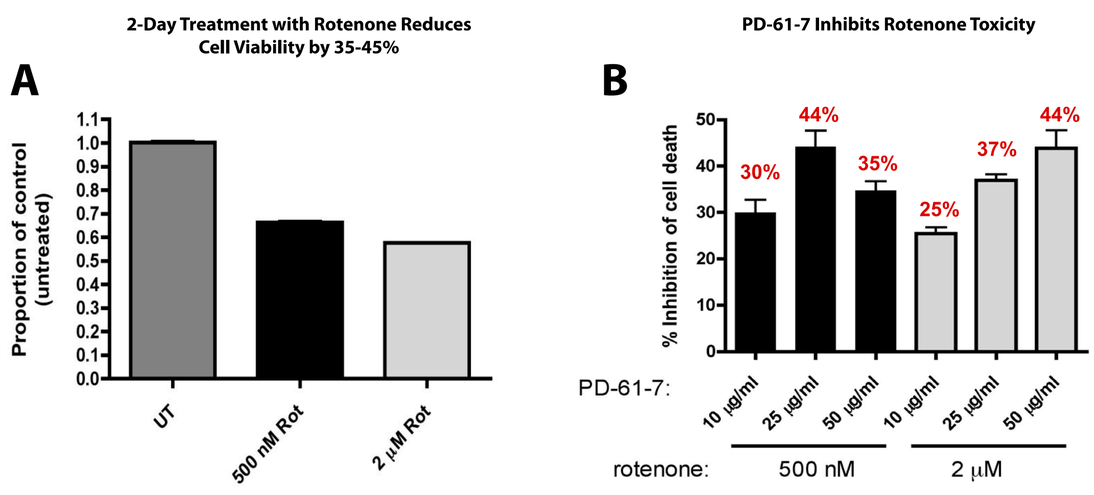


**Figure 6: PD-61-7 is a Potent Inhibitor of Toxicity in A53T α-Synuclein Cells Treated with Rotenone.**

A) An XTT assay was used to assess cell viability of A53T α-synuclein BeM17 cells treated with increasing concentrations of rotenone. A 2-day treatment with rotenone reduced cell viability by 35-45%. B) Small molecule PD-61-7 was found to inhibit rotenone toxicity by 25-44%.

| | PD-61 |
|---|---|
| **Receptor/Transporter/Channel Binding** | **Percent Inhibition** |
| Adenosine A1 | 0 |
| Adenosine A2A | 13 |
| Adrenergic alpha 1A | -10 |
| Adrenergic alpha 2A | -5 |
| Adrenergic beta 1 | 10 |
| Adrenergic beta 2 | -6 |
| L-type Ca Channel | -2 |
| Dopamine D1 | 11 |
| Dopamine D2L | -11 |
| GABA, Mucimol, Central | 8 |
| Glucocorticoid | 5 |
| Glutamate-NMDA | 14 |
| Histamine H1 | 65 |
| Imidazoline I2, Central | 3 |
| Muscarinic M2 | 12 |
| Nicotinic Acetylcholine | 5 |
| Opiate Mu (OP3) | -11 |
| Phorbol Ester | -13 |
| K Channel (K ATP) | 7 |
| K Channel (HERG) | -4 |
| Sigma 1 | 5 |
| Sigma 2 | 13 |
| Sodium Channel, Site 2 | 10 |
| NET (Norepinephrine) Transport | 9 |
| SERT (Serotonin) Transport | -7 |
| Dopamine Trasporter, VMAT, and Multidrug Resistance | not done |
| **In Vitro Drugability Testing** | **Percent Inhibition** |
| Cytochrome P450 Inhibition (1A2) | No Significant Inhibition (<50%) |
| (2C19) | No Significant Inhibition (<50%) |
| (2C9) | No Significant Inhibition (<50%) |
| (2D6) | No Significant Inhibition (<50%) |
| (3A4) | No Significant Inhibition (<50%) |
| **Plasma Protein Binding (% Free)** | **10-11%** |
| **Stability in Human Microsomes (% Remaining)** | **Moderate (55%)** |
| **Stability in CD1-Mouse Microsomes (% Remaining)** | **Moderate (55%)** |

**Figure 7: PD-61-7 Has a Good "Drugability" Profile.**

A variety of "drugability" testing at MDS Labs was implemented with PD-61-7 including receptor/transporter/channel binding, Cytochrome P450 inhibition testing, plasma protein binding assays, stability in microsomes and in vivo toxicity testing in rodents. PD-61-7 was found to have a good drugability profile in that it did not bind to other brain receptors/transporters (except the histamine H1 receptor which needs to be confirmed); showed no significant inhibition of CYP450 enzymes; had 10-11% free fraction in plasma; showed moderate stability in human and mouse microsomes; showed no in vivo toxicity in rodent following repeated dose studies (not shown).

# Conclusions

**1. A variety of in vitro assays (Thioflavin T fluorometry; Congo red binding; CD spectroscopy) and cell-based assays has screened a unique small molecule library of ProteoTech compounds to identify unique and potent inhibitors/disrupters of α-synuclein aggregation.**

**2. Four compounds (PD-61-7); PD-86-3; PD-73-3; PD-61-C) that demonstrate potent inhibition of α-synuclein aggregation, are non-toxic and good drugability profile, have been identified.**

**3. These 4 compounds are currently being evaluated for their potential to reduce α-synuclein aggregation in brain and improve motor dysfunction in Parkinson's disease transgenic mice (overexpressing human α-synuclein under Thy-1 promoter).**

**4. We have also designed and identified 7 new small molecule analogs (of PD-61) that are potent inhibitors of α-synuclein aggregation in vitro and in cell-based assays, and are currently being assessed in "drugability" and in vivo toxicity screening assays.**

**5. These studies are postulated to help identify a pre-clinical candidate that is anticipated to represent a new breakthrough disease-modifying treatment of Parkinson's disease and related disorders.**

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