Synuclere™: Development of a Small Molecule that Effectively Reduces Alpha-Synuclein Aggregation and Improves Motor Dysfunction

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Introduction/Background

Parkinson's disease (PD) is a neurodegenerative disorder that is pathologically characterized by the presence of intracytoplasmic Lewy bodies, the components of which are filaments of a 140-amino acid protein known as α-synuclein. Transgenic mice overexpressing human α-synuclein as they age demonstrate marked accumulation of α-synuclein correlating with motor dysfunction (as determined by motor function tests such as the beam traversal test and/or pole test).

ProteoTech Inc. (Kirkland, WA, USA) has been working in the amyloid disease field for over 10 years and has developed a unique library of small molecule compounds (mostly representing new chemical entities) that are specifically effective against different amyloid proteins. ProteoTech's small molecule Exebryl-1® is currently in Phase 1 human clinical trials and has been shown to effectively cause marked brain reduction of Aß load with correlative improvement in memory in different APP mouse models (see Snow et al, Oral presentation, Sun. March 15th 2009, 12:20pm "Exebryl-1®: A Novel Small Molecule Currently in Human Clinical Trials as a Disease-Modifying Drug for the Treatment of Alzheimer's Disease").

Synuclere™ represents the development of a novel small molecule that specifically targets α-synuclein aggregation in PD. Small molecule lead compounds were initially identified that caused a marked reduction in α-synuclein aggregation *in vitro* (as determined by Thioflavin T fluorometry, Congo red binding, circular dichroism spectroscopy and electron microscopy), and in cell culture studies (using neuronal cells that overexpress A53T α-synuclein and accumulate Thioflavin S-positive intraneuronal aggregates following rotenone treatment).

Seven small molecule lead compounds chosen for *in vivo* transgenic mouse model testing demonstrated non-toxic/good safety profiles, and good drugability characteristics including a) non-binding to brain receptors, transporters, and/or channels, b) no significant CyP450 enzyme inhibition, c) good levels of free drug in plasma (i.e. plasma protein binding), and d) moderate to high stability in microsomes.

Methodology

Lead Compound Studies in Human α-Synuclein Transgenic Mice

6-Month Treatment in Aged 12-Month Old Human α-Synuclein Transgenic Mice

Seven proprietary small molecule compounds (including PD-73, PD-61, PD-61-C, PD-86, PD-61-W3, PD-61-P2, and PD-31) were first administered to 12-month old human α-synuclein transgenic mice (n=12 per test compound) for 6 months by daily i.p. injections (at a dose of 50mg/kg/day)(Masliah et al, Neuron 46:857-868, 2005). A group of fourteen 12-month old human α-synuclein transgenic mice were also treated with vehicle only.

6-Week Treatment in Younger 4-5 Month Old Human α-Synuclein Transgenic Mice

In a second set of animal studies, 5 lead compounds (including PD-61-W3, PD-73, PD-73-C, PD-61-C and PD-86) were administered for 6-weeks (at 50mg/kg/day i.p.) to 4-5 month old human α-synuclein transgenic mice that in a pilot study already contained elevated α-synuclein levels in brain and motor dysfunction.

Behavioral Testing

Behavioral testing on the beam traversal test and pole test were carried out prior to treatment, and at 3 and 6-months of treatment prior to sacrifice in the first study using aged human α-synuclein transgenic mice as described above. In the second study using younger human α-synuclein transgenic mice, behavioral testing was implemented on weeks 5-6 of dosing, prior to sacrifice. In the beam traversal test, a measure of motor performance, mice were trained over two days, with 5 trials per day, to cross a narrowing beam (separated into four segments) with support ledges attached along each side, and leading to the animal's home cage. On the third day, the test was made more challenging by placing a mesh grid over the beam surface, leaving a small space of about 1cm between the grid and the surface of the beam. Animals were then videotaped over a period of 5 trials, and the time to cross, number of steps taken and number of slips were recorded by an investigator blind to drug treatment. Performance in the pole test was used to assess basal ganglia-related movement disorders in mice and was carried out as previously described (Fleming et al, Neuroscience 142:1245-1253, 2006), except only 1 day (2 trials) were performed, followed by 5 trials where time to descend the pole, turn time and total travel time was measured and recorded by an investigator blind to drug treatment.

Immunohistochemistry, Western Blotting and Quantitation

Upon sacrifice of α-synuclein transgenic mice following 6-months of treatment (when animals were 18 months of age), brains were removed and bisected along the midline. The left hemibrain was fixed in 4% paraformaldehyde for immunohistochemical staining (using an affinity purified version of AB5038 rabbit polyclonal antibody against α-synuclein; Chemicon) and image analysis. The right hemibrain was bisected coronally to yield an anterior and posterior portion. Frozen hemibrains were then subjected to biochemical fractionation to separate the soluble cytosolic fractions from the insoluble, particulate (membrane) fraction. α-synuclein levels in both the particulate and cytosolic fractions were analyzed by SDS-PAGE, western blot analysis and scanning densitometry using Scion software for quantitation. For the particulate fraction the intensity of a non-specific 25kDa non-specific band was determined (as a loading control and normalizer), and for cytosolic fractions, the intensity of the α-tubulin band was determined for use as a normalizer.

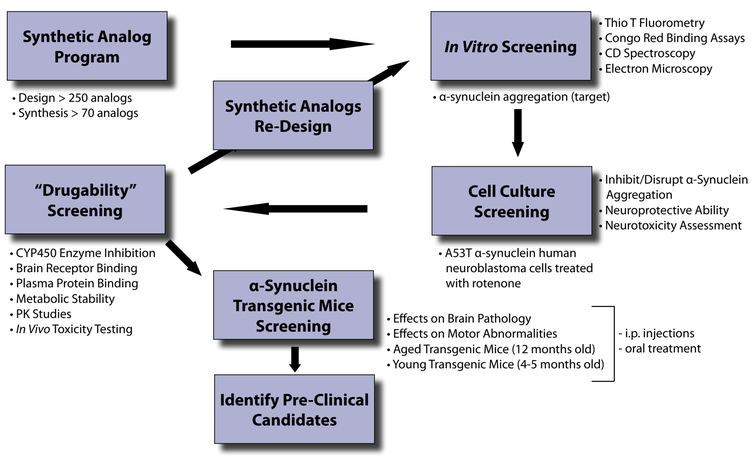


Figure 1: ProteoTech's Small Molecule Program for Targeting α-Synuclein Accumulation in Parkinson's Disease.

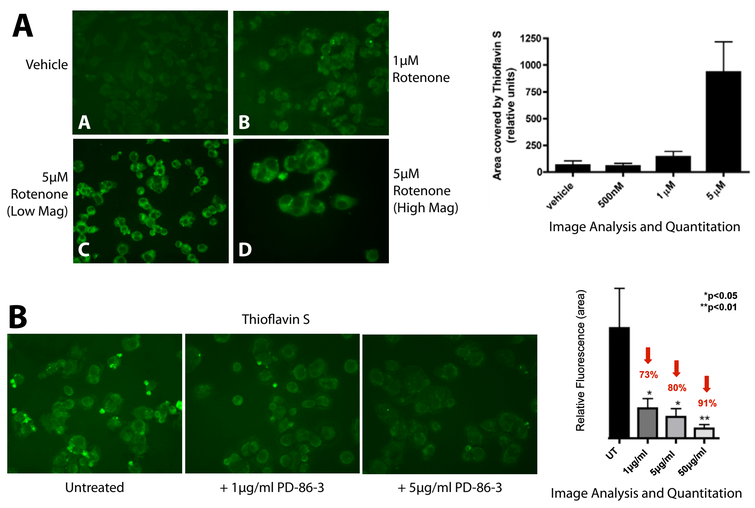


Figure 2: Use of a Cellular Model to Identify Potent Inhibitors of α-Synuclein Aggregation

A: Thioflavin S Fluorescence and Image Analysis Quantitation in A53T α-Synuclein-Expressing Cells. Human neuroblastoma BeM17 cells overexpressing A53T α-synuclein treated with increasing concentrations of rotenone (1μM to 5μM) demonstrate a dose-dependent increase in Thioflavin-S positive intraneuronal aggregates.

B: Dose-Dependent Inhibition of α-Synuclein Aggregation/Fibril Formation by the Small Molecule PD-86-3 in Human Neuroblastoma Cells Overexpressing A53T α-Synuclein Induced with Rotenone. PD-86-3 treatment was very effective in inhibiting α-synuclein aggregation/fibrillogenesis in a dose-dependent manner as demonstrated by a reduction in Thioflavin S fluorescence (80% reduction at 5μg/ml). This lead compound was 1 of 7 compounds chosen for further evaluation in α-synuclein transgenic studies.

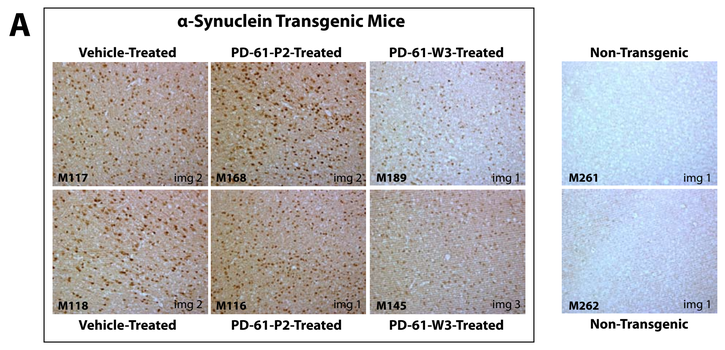
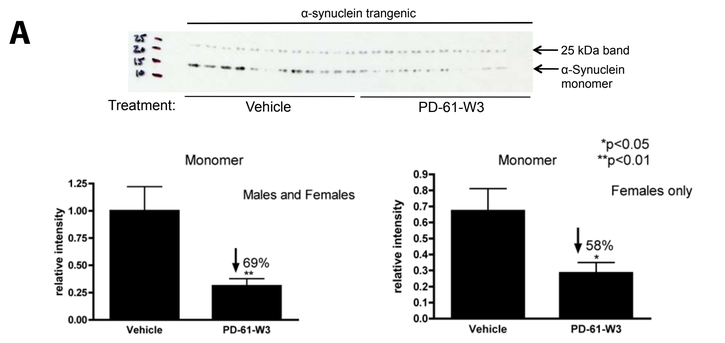


Figure 3: Synuclere™ (PD-61-W3) Markedly Reduces α-Synuclein Accumulation in Cortex of 18 Month-Old α-Synuclein Transgenic Mice as Demonstrated by Immunohistochemistry and Image Analysis Quantitation

Immunostaining of α-synuclein deposits that accumulate in cortex of 18-month old α-synuclein transgenic mice is shown using a polyclonal antibody against α-synuclein. Whereas non-transgenic brains demonstrate no real α-synuclein immunoreactivity, 18-month old saline (vehicle)-treated α-synuclein transgenic mice demonstrate punctate intraneuronal deposits **(Fig. A)**. Whereas PD-61-P2 had little effect in reducing intraneuronal α-synuclein, 6-months of PD-61-W3 treatment caused a significant (p<0.001) **81% reduction** in α-synuclein accumulation as determined by immunostaining and image analysis **(Fig. B)**.



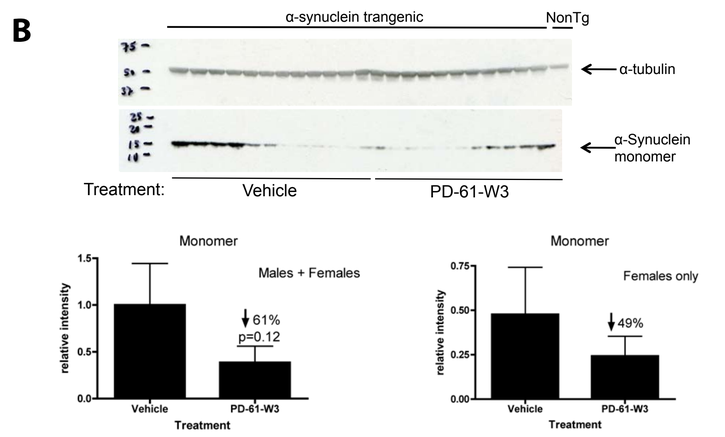


Figure 4: Synuclere™ (PD-61-W3) Markedly Reduces α-Synuclein Accumulation in the Particulate and Cytosolic Fractions of the Anterior Brain in 18 Month-Old α-Synuclein Transgenic Mice as Demonstrated by Western Blotting and Quantitation

Most significant reductions in α-synuclein levels by PD-61-W3 treatment were observed in the anterior portion of the brain (which contains the striatum, a site of extensive Lewy body pathology in PD). 6-months of PD-61-W3 treatment significantly reduced α-synuclein monomeric levels (which is believed to represent aggregated α-synuclein following SDS-PAGE) in the particulate fraction by an average of 69% **(Fig. A)**, and tended to reduce α-synuclein monomer by 61% in the cytosolic fraction **(Fig. B)**.

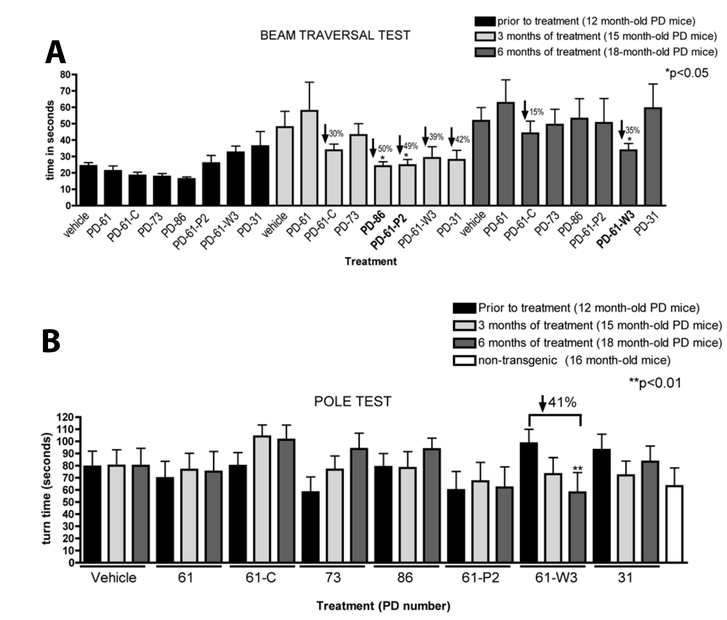


Figure 5: Synuclere™ (PD-61-W3) Improves Motor Dysfunction in 18 Month-Old α-Synuclein Transgenic Mice as Demonstrated by the Beam Traversal and Pole Tests

A: Shown are beam traversal test results in 12-month old α-synuclein transgenic mice prior to treatment, and following 3 and 6-months of treatment with one of 7 lead compounds. Whereas 5 compounds delayed the onset of maximal deficits in the beam traversal test following 3-months of treatment, only Synuclere™ (PD-61-W3) prevented the onset of motor deficits at both 3-months (**by 39%** in 15-month old α-synuclein transgenic mice) and 6-months (**by 35%** in 18-month old α-synuclein transgenic mice) following treatment. **B:** Shown are pole test results in 12-month old α-synuclein transgenic mice prior to treatment, and following 3 and 6-months of treatment with 7 lead compounds. Only Synuclere™ (PD-61-W3) treatment markedly improved performance in the pole test by a significant (p<0.01) **41%** over the 6-month treatment period.

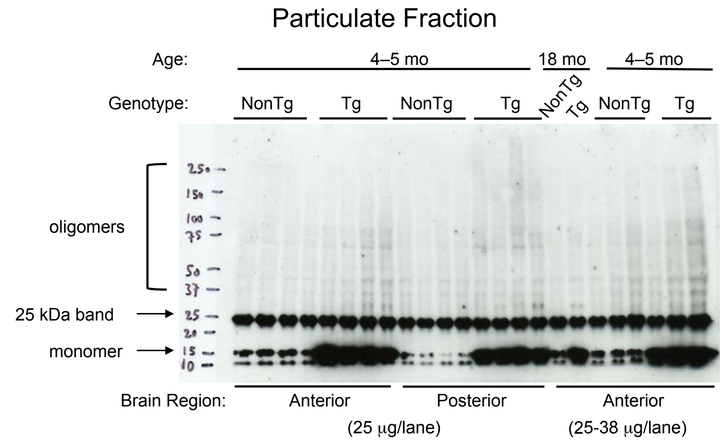


Figure 6: 4-5 Month Old α-Synuclein Transgenic Mouse Brains Have Similar Patterns of Monomers and Oligomers as 18-Month Old Mice as Assessed by Western Blot Analysis

Western blot analyses from the particulate fraction of the anterior and posterior portion of brain shows robust α-synuclein monomer levels (likely representing aggregated α-synuclein reduced following SDS-PAGE) that are greatly increased in transgenic mice relative to non-transgenic mice. In addition, it is clear that α-synuclein transgenic mice 4-5 months old already have robust levels of α-synuclein in brain, similar to levels observed in 18-month old transgenic mice.

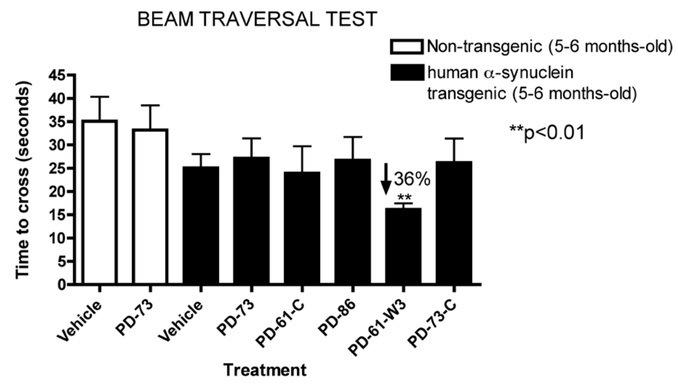


Figure 7: Synuclere™ (PD-61-W3) Improves Motor Dysfunction in Younger (4-5 Month Old) α-Synuclein Transgenic Mice as Demonstrated by the Beam Traversal Test

Shown are beam traversal test results in 4-5-month old α-synuclein transgenic mice either vehicle-treated or treated with one of 5 lead compounds. Only Synuclere™ (PD-61-W3) significantly (p<0.01) improved (by 36%) motor deficits in α-synuclein transgenic mice following only 6-weeks of treatment, as determined by the beam traversal test. Western blot analysis also demonstrated that Synuclere™ (PD-61-W3) significantly reduced α-synuclein levels in brain following only 6-weeks of treatment (not shown), correlating with the motor dysfunction improvements.

Synuclere™ Summary of *In Vitro* and *In Vivo* Data

1) *In vitro* Screening (Thio T fluorometry; Congo red binding; CD spectroscopy):
   - Potent Inhibitor of α-synuclein aggregation
2) Cell Based Assay Screening (Thio S fluorescence; XTT assays)
   - Potent Inhibitor of α-synuclein aggregation (by 60-74%)
   - Good neuroprotection against rotenone (by 25%)
3) Drugability Screening (CyP450; brain receptor binding; % free fraction; stability; PK; tox)
   - No significant CyP450 inhibition
   - No brain receptor/transporter/ion channel binding
   - High free fraction (28%) in protein binding assay
   - Moderate to high stability in microsomes (72% in human; 87% in mouse)
   - Reasonable PK parameters
   - Non-toxic *in vivo*
4) *In Vivo* Effects in α-Synuclein Transgenic Mice
   - Marked reduction in brain α-synuclein levels (~58-69%) in older 18-month PD transgenic mice (following 6 months of treatment)
   - Marked improvement in motor function as assessed by beam traversal test (35-39%) and pole test (41%) in older 18-month PD transgenic mice (following 3-6 months of treatment)
   - Marked improvement in motor function as assessed by beam traversal test (36%) in younger 5-month of PD transgenic mice (following only 6 weeks of treatment)

Conclusions

Synuclere™ : A Novel Disease-Modifying Small Molecule for the Treatment of α-Synuclein Aggregation and Accumulation in Parkinson's Disease

Synuclere™ (PD-61-W3)

- **Causes a marked reduction of α-synuclein aggregation *in vitro* and in cell culture studies**
- **Causes a marked improvement in motor deficits in α-synuclein transgenic mice (following 6-months of treatment in older 18-month old mice and following 6-weeks of treatment in younger 4-5 month old mice)**
- **Causes a marked reduction of α-synuclein aggregates in α-synuclein transgenic mouse brain**
- **Is non-toxic and has a good safety profile**
- **Has good "drugability" characteristics**
- **IND enabling studies projected for the next year**

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