P2-529

Identification of Exebryl-1™ and Other Novel Small Molecules as Tau Protein Aggregation Inhibitors

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Introduction

Targeting of the microtubule-associated protein tau that pathologically accumulates in the brains of patients with Alzheimer's disease (AD) and other neurodegerative diseases has emerged as a potentially effective therapeutic strategy. ProteoTech has developed a number of different *in vitro* screening technologies, and cellular and animal models that allow for identification of new and potent inhibitors of amyloidosis in general. We have also designed and synthesized a class of unique small molecule compounds, mostly representing new chemical entities (NCEs). The novel small molecule compounds have been screened for their ability to inhibit/disrupt aggregation and fibril formation of Tau by several in vitro techniques including Thioflavin S (ThioS) fluorometry, circular dichroism (CD) spectroscopy, and use of Tau-expressing cell cultures and Western blot analysis. In preliminary experiments, we discovered that a number of our unique small molecular compounds specifically inhibited tau aggregation/fibril formation *in vitro*. One such compound, Exebryl-1™, was also previously found to be a potent inhibitor of β-amyloid protein aggregation and is currently in Phase 1 human clinical trials.

Methods and Results

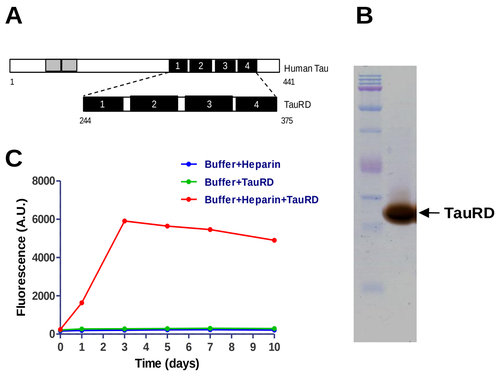

Figure 1. Preparation of Aggregated/Fibril Tau Repeat Domain (TauRD) for Screening Tau Aggregation Inhibitors. (A) The human TauRD (residues 244-375) was cloned and expressed in *E. Coli.* **(B)** The recombinant TauRD protein was purified by heat-stability treatment and cation exchange chromatography. Purity (>98%) of TauRD was assessed by SDS-PAGE and silver/Coomassie blue double staining. **(C)** Aggregation of TauRD was achieved by incubation of equal molar ratios of TauRD (10-20μM) and heparin in sodium phosphate buffer at 37°C for 3 hours to 10 days (depending upon shaking speed). Formation of β-sheet-containing TauRD aggregates was monitored by ThioS fluorometry. In the ThioS assay, ThioS binds to aggregated/fibrillar TauRD protein, producing a fluorescence enhancement at 485nm that is directly proportional to the amount of aggregated TauRD.

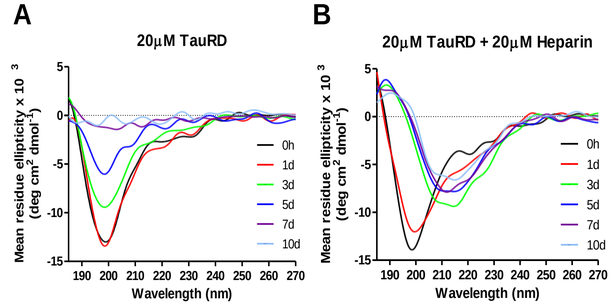

Figure 2. Circular Dichroism (CD) Spectroscopy Profiles of Heparin-Induced Conformational Changes of TauRD. (A) Non-aggregated TauRD in solution is in the state of random coil (minima at 195nm). In this study, TauRD (20μM) was incubated in sodium phosphate buffer without heparin at 37°C for 0-10 days. The CD spectra showed random coil of TauRD. Note: The reduced magnitudes reflected sample precipitation during the incubation. **(B)** When heparin (20μM) was present in the reaction mixture, TauRD underwent a conformational change from random coil to β-sheet secondary structure (minima at 218nm). The conformational changes also correlated well with enhanced ThioS fluorescence (Fig 1C). The two independent assays consistently conform the formation of heparin-induced TauRD aggregates/fibrils.

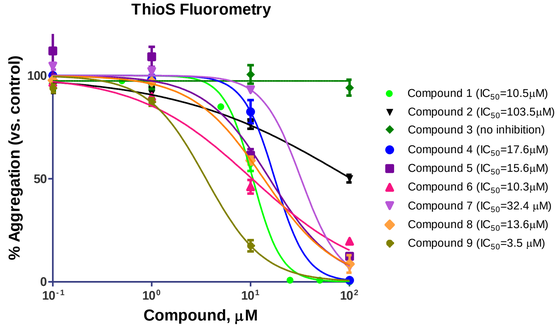

Figure 3. Inhibition of TauRD fibril Formation By ProteoTech's Novel Small Molecule Compounds. A class of novel small molecule compounds (NCEs) were screened for their ability to inhibit tau fibril formation by ThioS fluorometry. In these assays, 10 μM of TauRD was incubated with 10 μM of heparin, without or with increasing concentrations of small molecule compounds, at 37°C with shaking at 650rpm for 18 hours. TauRD fibrillization was assessed by ThioS assays. The results showed that representative ProteoTech small molecule compounds inhibited TauRD fibril formation to different extents. For instance, the negative control (Compound 3) caused no inhibition, whereas other compounds caused dose-dependent inhibition of TauRD aggregation with IC50 ranging from 3.5μM to 103.5μM. Note: Compound 1 was Exebryl-1™.

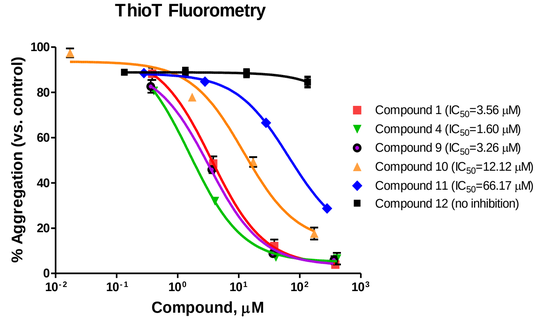

Figure 4. Disruption/Inhibition of Tau441 Protein Fibril Formation By ProteoTech Novel Small Molecules. Select small molecule compounds were also tested for their ability to inhibit/disrupt fibril formation of human full-length tau (Tau441) (See Fig. 1A). In this study, 20μM of Tau441 was pre-fibrillized by incubation with 50 μM of heparin at 37°C for 8 days. Compounds at approximately 0.1-100 μM were then added into the reaction mixture containing 2μM of pre-fibrillized tau, and incubated for an additional 3 days before ThioT fluorometry. The results showed that representative ProteoTech compounds also disrupted/inhibited fibril formation of the full-length tau. For instance, the negative control (Compound 12) caused no inhibition, whereas other compounds caused dose-dependent inhibition of TauRD aggregation with IC50 ranging from 1.6μM to 66.2μM. Note: Compound 1 was Exebryl-1™.

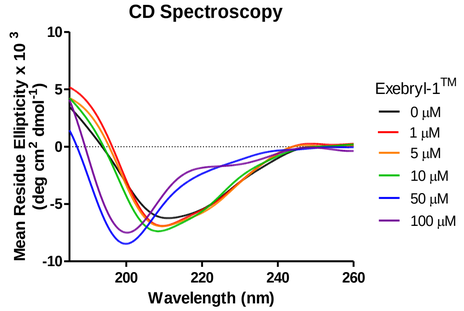

Figure 5. Exebryl-1™ Inhibits Conformational Changes of TauRD to β-sheet Secondary Structure Measured by CD Spectroscopy. To better understand the mechanisms of inhibition/disruption of tau fibril formation by the novel small compounds, CD spectroscopy was performed to analyze the effects of compounds on formation of ß-sheet secondary structure of TauRD. In this study, aggregation reactions containing TauRD and heparin at 10μM each were incubated without or with increasing concentrations of Exebryl-1™ for 3 hrs. Samples were then collected for CD spectroscopy. The CD spectra revealed dose-dependent blockage of the conformational change of TauRD from random coil (minima at 195nm) to ß-sheet-like structure (associated with Tau aggregation/fibrillization; Figs 1C & 2) by Exebryl-1™.

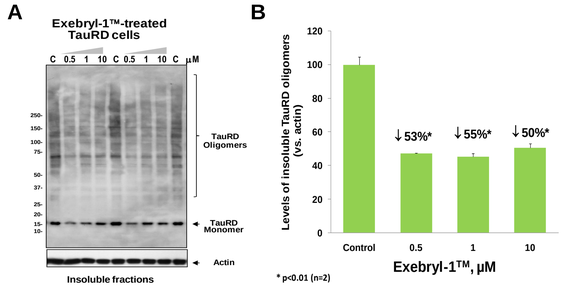

Figure 6. Inhibition of TauRD Oligomer Aggregation by Exebryl-1™ in Cell Culture Studies. Select small molecules were further tested for their ability to affect intracellular tau aggregation in a cell model that expresses inducible TauRD. **(A)** TREx293-TauRD cells were treated with increasing concentrations of Exebryl-1™ for 48 hrs. Cell lysate fractions that were sequentially extracted in buffers containing Triton X-100 (soluble) and SDS (insoluble) were analyzed by Western blotting. **(B)** Quantitative densitometry analysis of (A) indicates that the treatment led to a significant (p<0.01) 50-55% reduction in levels of insoluble TauRD oligomers. A trend of reduction in levels of soluble TauRD oligomers was also observed (not shown).

Summary

1. A class of ProteoTech's novel small molecule compounds were screened for their ability to inhibit/disrupt tau fibril formation by ThioS fluorometry, and CD spectroscopy. Select compounds were also tested in a TauRD-expressing cellular model.
2. Based on ThioS assays, a number of the novel small molecules, including Exebryl-1™, caused a dose-dependent inhibition of TauRD (and/or Tau441) fibril formation, with IC_{50} ranging from 1.6μM-103.5μM.
3. Western blot analyses demonstrated that Exebryl-1™ also reduced levels of insoluble TauRD oligomers in TauRD-expressing cell cultures.
4. CD spectroscopy confirmed that Exebryl-1™ was able to block the conformational change of TauRD from random coil to ß-sheet secondary structure.
5. Modes of action: There are two possible modes of action for these compounds. One possibility is that the compounds bind to the stacking region of tau while maintaining β-sheet conformation but not allowing tau-tau stacking. Alternatively, the compounds bind and alter tau conformation into unstructured amorphous material, thereby inhibiting fibril formation. Our current evidence from Exebryl-1™ suggests that the 2nd possibility may be the major mode of action.

Conclusions

Exebryl-1™ is a novel small molecule that appears to inhibit both Aβ and tau protein aggregation. The studies suggest that Exebryl-1™ may be the first drug to be developed, which is able to reduce accumulations of both amyloid plaques and neurofibrillary tangles in AD brains. Exebryl-1™ is currently in Phase 1 human clinical trials and is being developed for the treatment of Alzheimer's disease.

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