Development of a Reconstituted Assay to Test Casein Kinase 1δ Inhibitors to Block Alzheimer's Disease Progression

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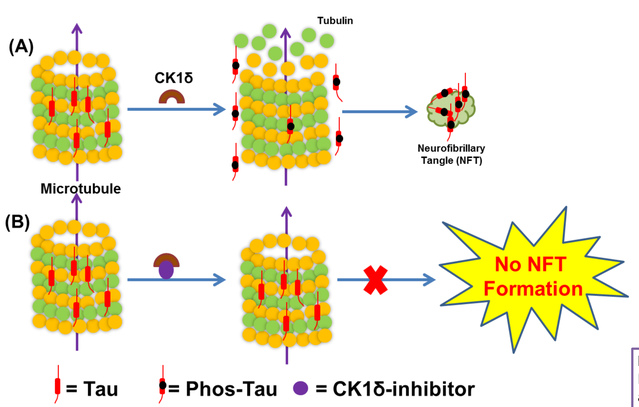
ABSTRACT

Neurofibrillary tangles (NFTs) are one of the pathological hallmarks of Alzheimer's Disease (AD). NFTs are primarily composed of hyperphosphorylated tau, which in its unphosphorylated state binds to and stabilizes the microtubule array in neurons. It is believed that tau phosphorylation is then a predisposing event in the progression of AD. Thus, the development of therapeutics that could inhibit the hyperphosphorylation of tau would potentially enable intervention to block the progression of AD. Casein kinase 1δ (CK1δ) is upregulated in AD and is also able to phosphorylate tau on a number of residues that regulate tau's affinity for microtubules, making CK1δ a prime candidate for therapeutic target. We have taken an *in silico* approach to the design of competitive inhibitors of CK1δ using a napthoquinone molecule that inhibited CK1δ selectively over 100 other disease relevant kinases as a starting point for forward design and synthesis. A series of resulting products were tested in a cellular assay and showed a dose-dependent decrease in tau phosphorylation via Western Blot of lysate from treated cells compared to untreated. However, as tau can be phosphorylated by many cellular kinases, we wanted to determine if the decreased tau phosphorylation was directly due to inhibition of CK1δ by our compounds. Thus, we have reconstituted tau phosphorylation by CK1δ in an *in vitro* assay using recombinantly expressed and purified components. We have expressed human CK1δ and tau (4R) in bacteria and have purified them to >90% homogeneity. We have shown that the tau protein is biologically active, as it shows standard, one-step binding affinity to microtubules in a pulldown assay. We have developed and optimized our *in vitro* kinase assay and observe robust, CK1δ-dependent phosphorylation of tau that is blocked by known inhibitors of CK1δ. This assay is now being used to test newly synthesized compounds designed to more effectively inhibit the kinase activity of CK1δ.

INTRODUCTION

Alzheimer's disease (AD) continues to pose a major public health burden that disproportionately impacts older African Americans in the United States. This health disparity is further exacerbated by the unmet need for a clinically effective treatment for the disease. A principal characteristic of the progression of Alzheimer's disease is the formation of abnormal phosphorylation of the protein tau in the brains of affected individuals. Tau is a microtubule binding protein that interacts with and stabilizes the microtubule network in neuronal axons. The human tau gene gives rise to six isoforms with either three or four microtubule-binding repeats. All tau isoforms contain a microtubule binding region that is positively charged (isoelectric point of 11.4 and 10.8) and has a high affinity for the negatively charged surface of microtubules. Phosphorylation of residues in this region neutralizes this positive charge, causing tau to dissociate from microtubules. While the free, cytosolic pool of tau has been shown to aggregate into NFTs, dissociation of tau also destabilizes the axonal microtubule array, leading to a loss of polarized, motor-dependent axonal transport. It was observed that casein kinase 1δ (CK1δ) and casein kinase 1ε (CK1ε) are upregulated in AD patients, suggesting that tau phosphorylation by CK1δ and CK1ε may promote dissociation of tau from microtubules. This would promote microtubule disassembly and tau aggregation into NFTs, as shown below in (A). Our goal is to generate specific inhibitors of CK1 isoforms that can block the CK1-dependent phosphorylation of tau and stabilize its interaction with axonal microtubules, as shown in (B). This has the dual benefit of stabilizing the microtubule array that is necessary for the polarized transport of cellular cargoes, as well as decreasing the probability of aggregating the free, cytosolic pool of tau into NFTs. This project was designed to develop in vitro assays to test whether our inhibitors of CK1 isoforms block CK1-dependent phosphorylation of tau and whether in doing so, the interaction of tau with microtubules is stabilized.



RESULTS

PROTEIN PURIFICATION

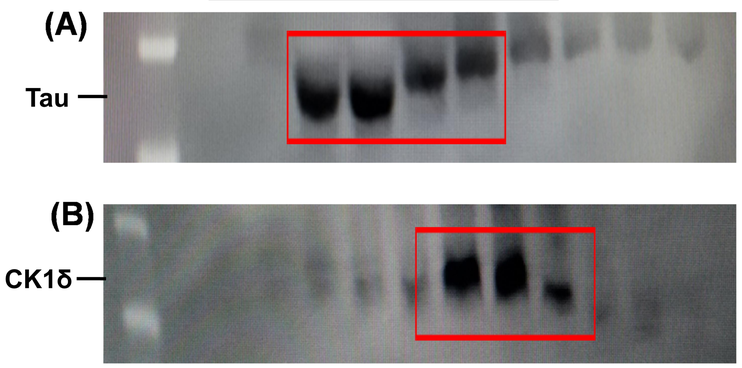


Fig. 1. Expression and purification of recombinant Human-Tau and CK1δ. Proteins were expressed in bacteria and purified via a two-step process using affinity and gel filtration chromatography. Images shown are representative Western blots of fractions from the final gel filtration elution probed with an anti-tau antibody (A), and an anti-CK1δ antibody (B). Boxes show the fractions that were enriched with the protein of interest that were subsequently concentrated for experimental use in our assay.

in vitro KINASE ASSAY DEVELOPMENT

Based on previously published *in vitro* kinase assays, we utilized a kinase assay buffer containing 10 mM HEPES , 5 mM $MgCl_2$, 1 mM EGTA and 1 mM DTT at pH 7.4. To ensure saturating ATP concentration, we added 20 μM ATP to the reaction mixture. The experiments below were designed to optimize the concentration of CK1δ necessary to fully phosphorylate tau, and the time necessary to fully phosphorylate tau.

CK1δ CONCENTRATION OPTIMIZATION

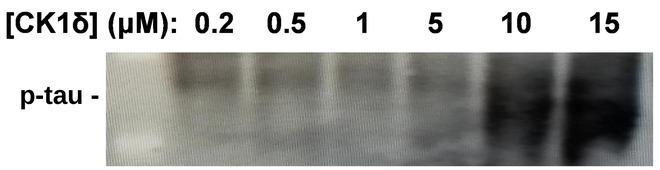


Fig. 2. Measurement of threshold concentration of CK1δ required in the *in vitro* kinase assay. Different concentrations of CK1δ (as indicated) were mixed with 20 μM Mg-ATP and 10 μM Tau in kinase assay buffer. The reaction mixtures were incubated overnight at room temperature. The reaction was stopped by adding gel sample buffer and boiling. Image shown is a western blot probed with an antibody targeting p-Tau (Ser199).

REACTION TIME OPTIMIZATION

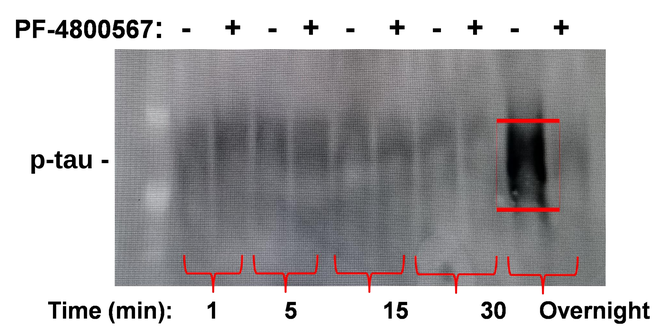


Fig. 3. Kinetics assay of measurement of time required to complete the kinase assay. 10 μM CK1δ, 20 μM Mg-ATP, and 10 μM Tau, with/without 3 μM PF-4800567 (CK1δ inhibitor) were incubated in kinase assay buffer at room temperature for the time indicated. The reaction was stopped by adding gel sample buffer and boiling. Image shown is a western blot probed with an antibody targeting p-Tau (Ser199).

INHIBITION OF CK1δ-DEPENDENT PHOSPHORYLATION OF TAU

WITH PF-4800567

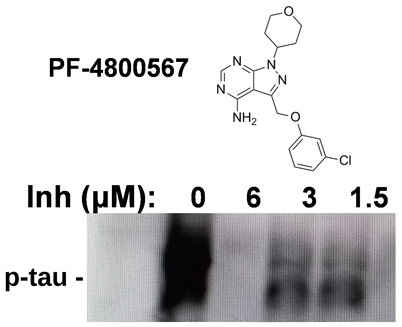


Fig. 4. Inhibition of tau phosphorylation by CK1δ. Recombinant CK1δ, Mg-ATP, tau, and different concentrations (6, 3, 1.5, and 0 μM) of CK1δ inhibitor PF-4800567 were incubated overnight at room temperature in kinase assay buffer. Then recombinant human Tau was added and the mixture was incubated overnight at room temperature. The reaction was stopped by adding gel sample buffer and boiling. Image shown is a western blot probed with an antibody targeting p-Tau (Ser199).

WITH C-3, C-11, AND C-12

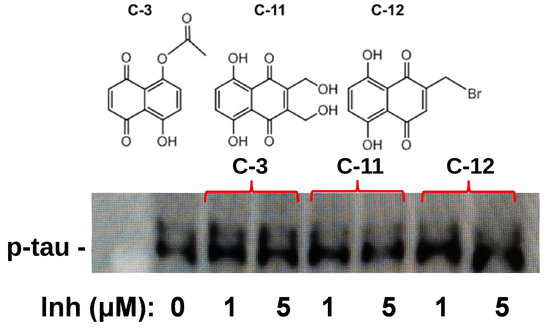


Fig. 5. Testing the inhibition of tau phosphorylation by CK1δ with our novel compounds. Recombinant CK1δ, Mg-ATP, tau, and two concentrations of our designed CK1δ inhibitors (C-3, C-11 , and C-12) were treated as in Figure 4. Data shown is the first attempt at this experiment and needs troubleshooting to optimize the relative concentration of inhibitor, ATP and protein. As these inhibitors were previously shown to inhibit CK1δ, we are using these results to further optimize out *in vitro* assay.

MICROTUBULE AFFINITY ASSAY

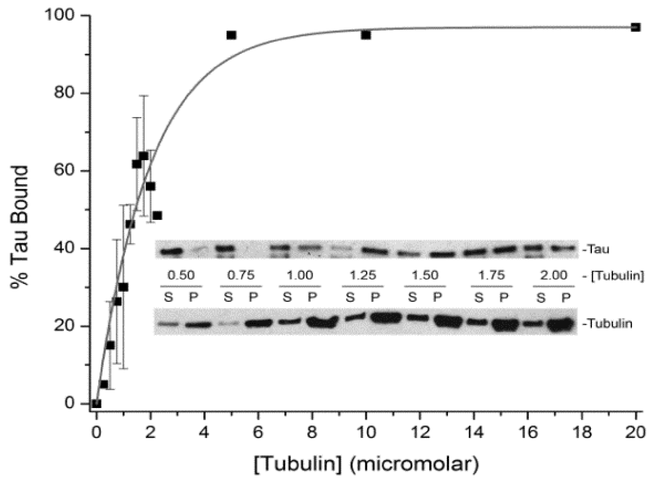


Fig. 6. Determining tau-MT affinity using a microtubule affinity assay. Microtubule (MT) affinity assays were used to study the affinity of interactions between Tau protein and MTs. To test the efficiency of our compound to not only inhibit tau phosphorylation but also to stabilize the affinity of tau for the MT polymer, we performed MT affinity assays with the products of our in vitro kinase assay. These assays are based on the differential centrifugation of MTs and tau through a 60% glycerol cushion. In these assays, tau (or tau subjected to our *in vitro* kinase assay) was mixed with different concentrations of MTs, incubated for 15 minutes, and subjected to ultracentrifugation at 70k rpm. At this speed, unbound tau remains in the supernatant fraction, while tau that bound to MTs was pelleted with MTs. Supernatant and pellet fractions were separated, resuspended in equal volumes, and subjected to quantitative western blot analysis to determined the percent overall tau bound. Inset contains a sample western blot probing for tau and tubulin in the supernatant (S) and pellet (P) for each concentration of tubulin as indicated. Band densitometry was performed in ImageJ to determine the fraction of tubulin bound (signal intensity in the supernatant divided by the sum of supernatant and pellet). Percent tau bound was plotted against concentration and a curve was applied as a best-fit exponential to the data in Origin 8. Error bars show the SD.

CONCLUSIONS

- **Optimized conditions for the reconstitution of tau phosphorylation by CK1δ, including optimal concentrations of reagents and incubation times**
- **Tau phosphorylation by CK1δ is blocked by a well-characterized, non-specific inhibitor of CK1δ, showing that our assay can be used to test for novel CK1δ inhibitors**

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