

Identification of Hits and Leads for Neurodegenerative Diseases: Case Studies

Laboratory for Drug Discovery in Neurodegeneration
Brigham and Women's Hospital
Harvard Medical School

A Course for Academic and Industry Scientists
February 4-5, 2007



Harvard Center for Neurodegeneration and Repair

Outline

Case Studies for Alzheimer's disease and Neuroprotection

– Biochemical Assay

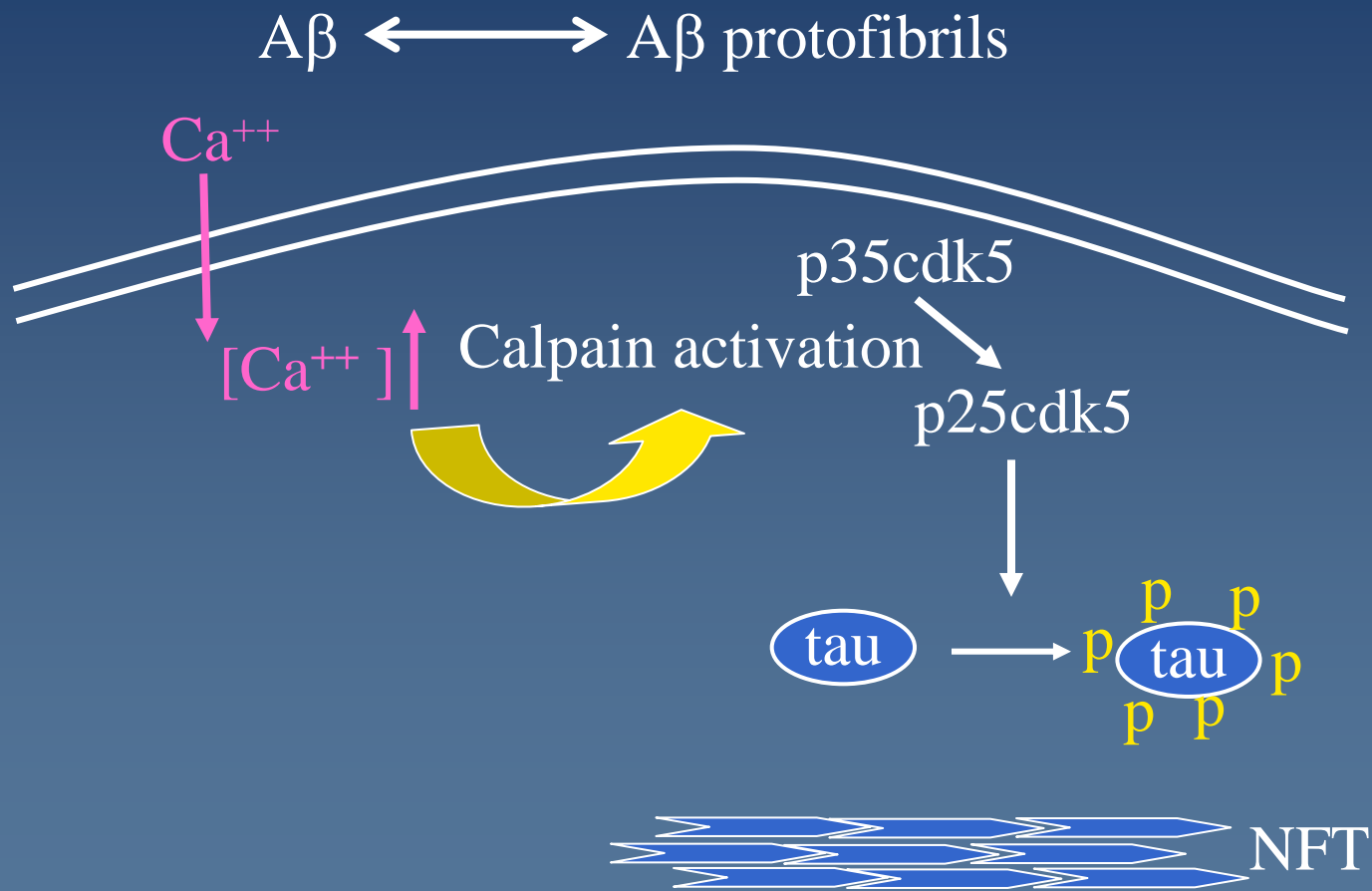
- Cdk5
- Collaboration with Dr. Ken Kosik (University of California, Santa Barbara)

– Cell based Assay

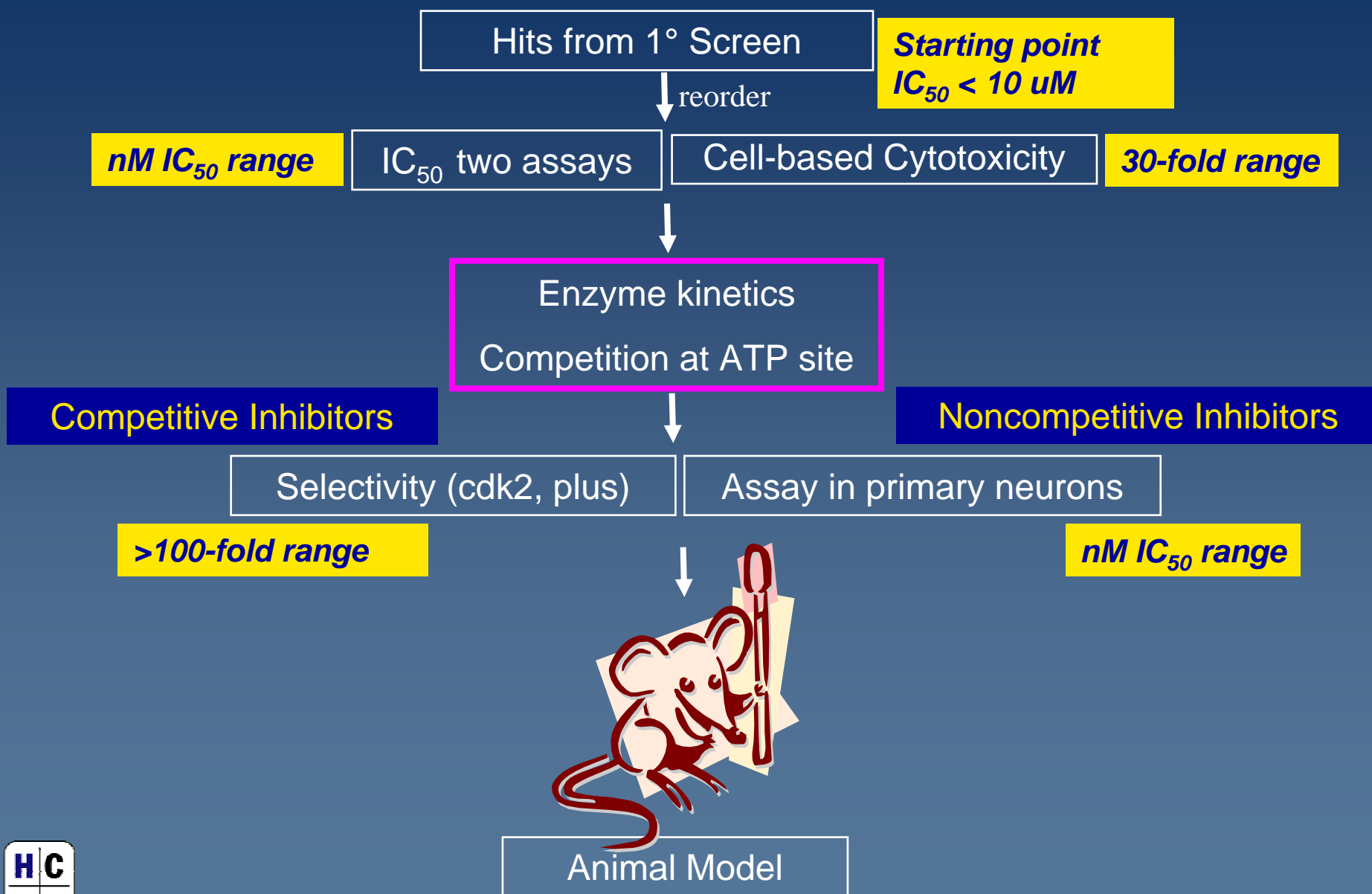
- Protection against A β toxicity
- Collaboration with Dr. Mary Lou Michaelis (University of Kansas)



Proposed Role of cdk5 in Alzheimer's disease

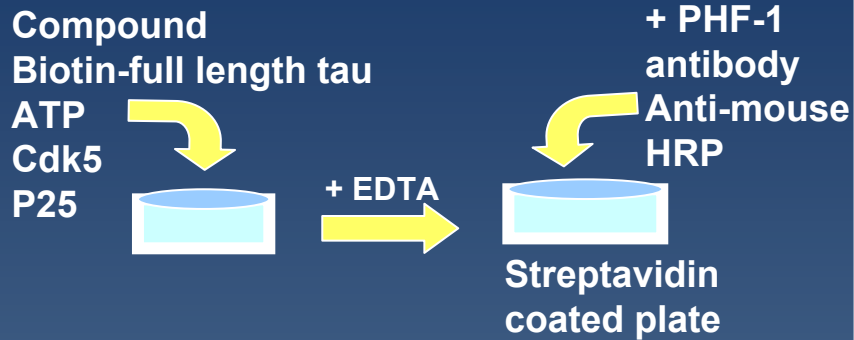


Flow Chart cdk5/p25

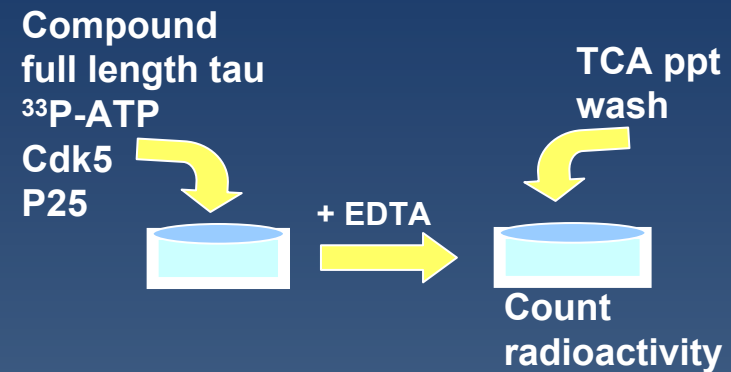


Two Assay Formats: cdk5/tau Phosphorylation

Assay 1 ELISA



Assay 2 Filter Binding



- ▶ Discover compounds that inhibit Cdk5 by mechanisms other than competing with ATP



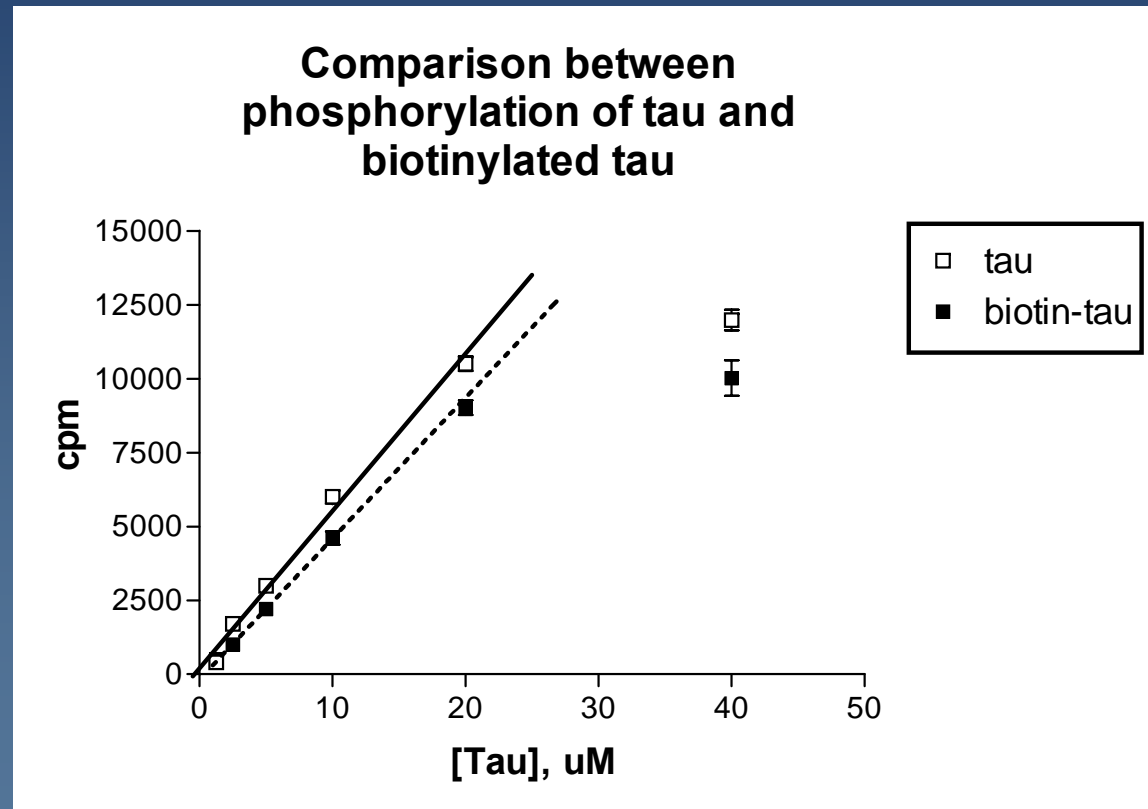
Assay Optimization

Assay reagents	component	comments
	Tau substrate	Expressed and purified at LDDN
	Biotinylated tau	Biotinylated at LDDN
	Cdk5 enzyme	Provided by collaborator
	Primary phospho-specific antibody	Gift from Peter Davies
Assay parameters	Stability	
	Reagent concentrations	Enzyme, substrates
	Buffer components	Salts, detergents, BSA, DMSO, reducing agents
	Miniaturization	
Assay Validation	Known inhibitors	Commercially available
	Performance of the assay when automated	Z' values, day-to-day variability



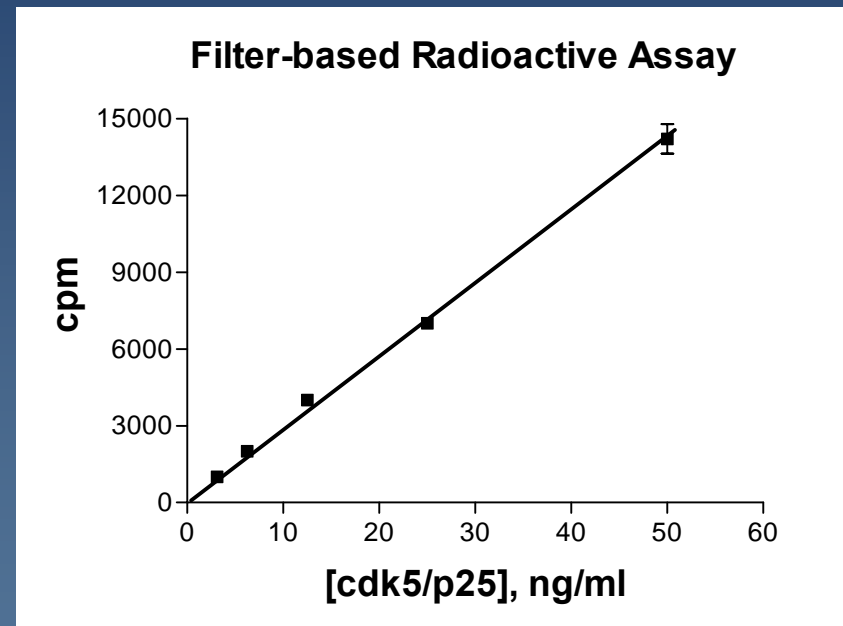
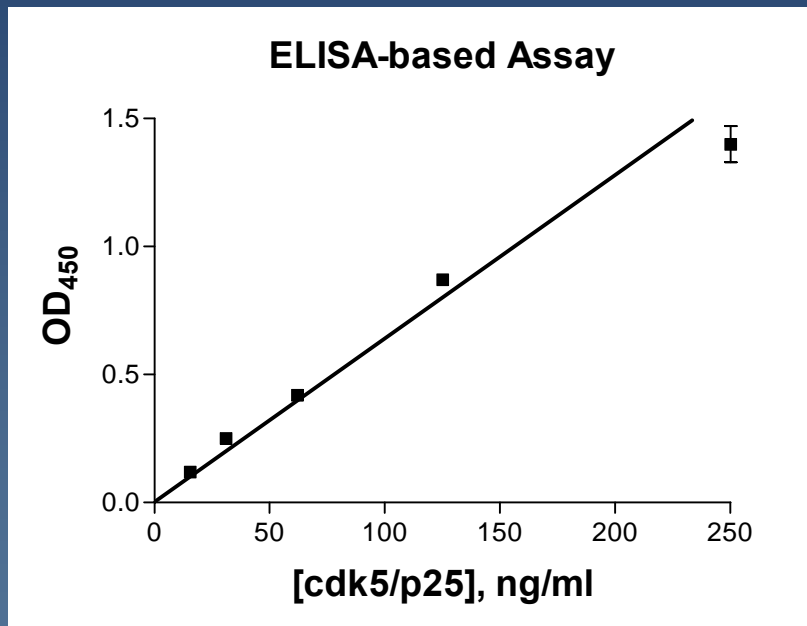
Reagents

- Biotinylation of tau
 - 44 lysines in tau, 8 biotins/mole of tau optimum
 - Verify phosphorylation activity of tau vs biotin-tau as substrate



Titration of Enzyme concentration

- ▶ Phosphorylation of tau was proportional to the enzyme concentration in both assay formats

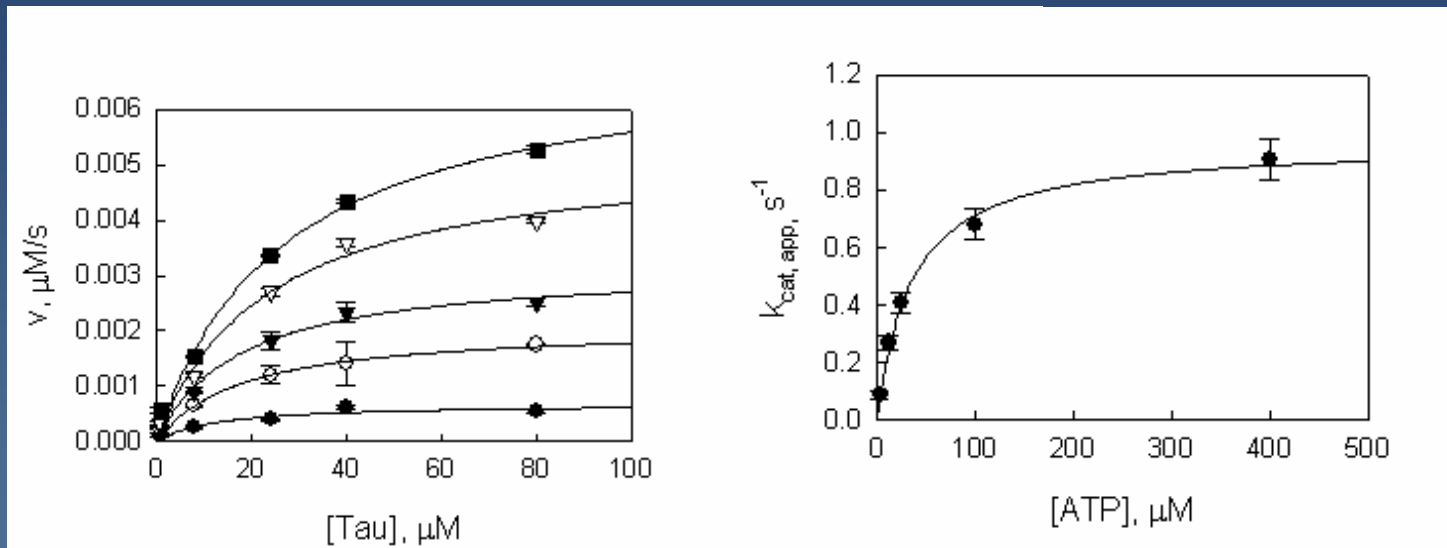


Tau concentration 94 nM



Substrate Dependent Phosphorylation

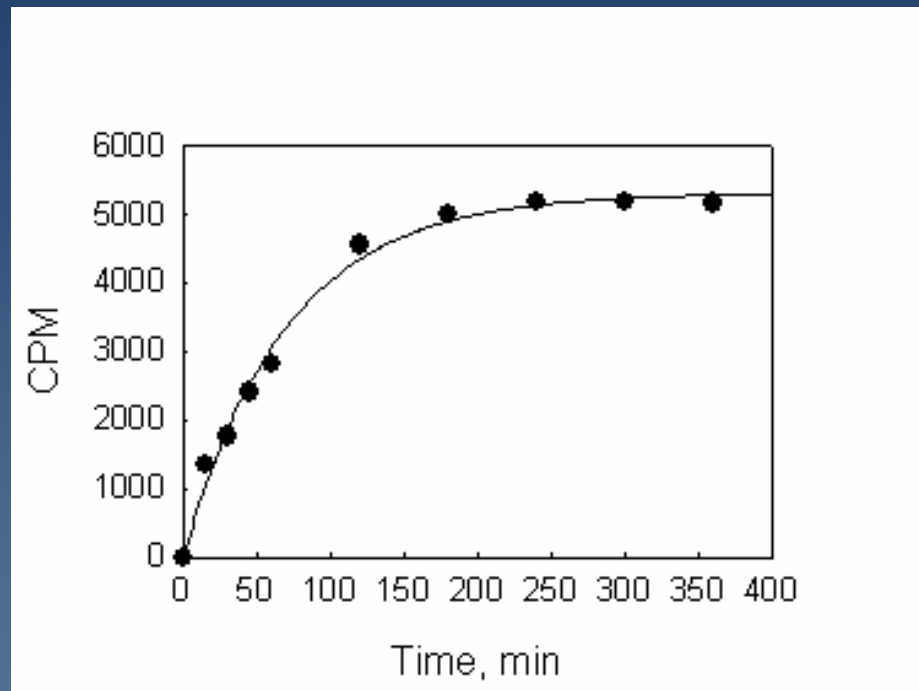
- ▶ Similar results in both assay formats



$K_m \text{ tau} = 4 \text{ uM}$
 $K_m \text{ ATP} = 35 \text{ uM}$

Time dependent phosphorylation of tau by cdk5/p25

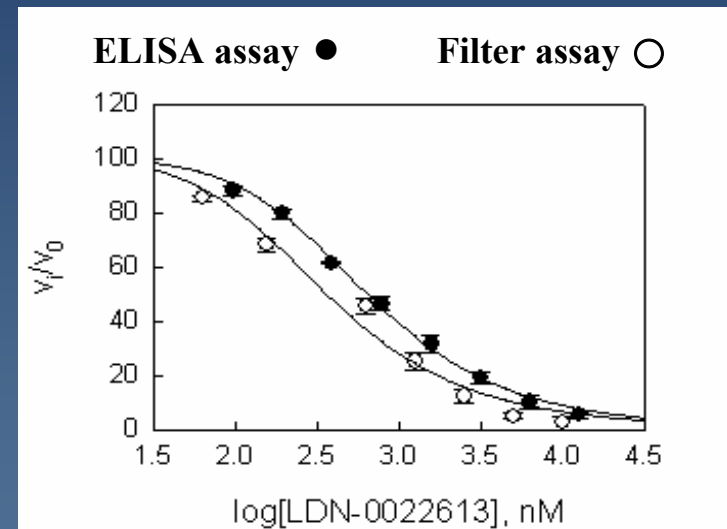
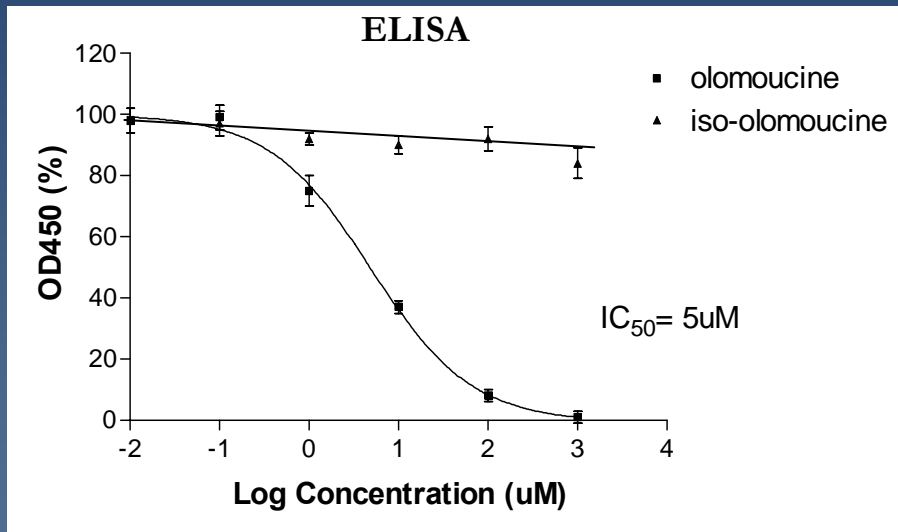
- ▶ Similar results in both assay formats



94 nM tau, 200 μ M ATP

Assay Validated with Known cdk5 Inhibitor

Olomoucine and our own compound shown

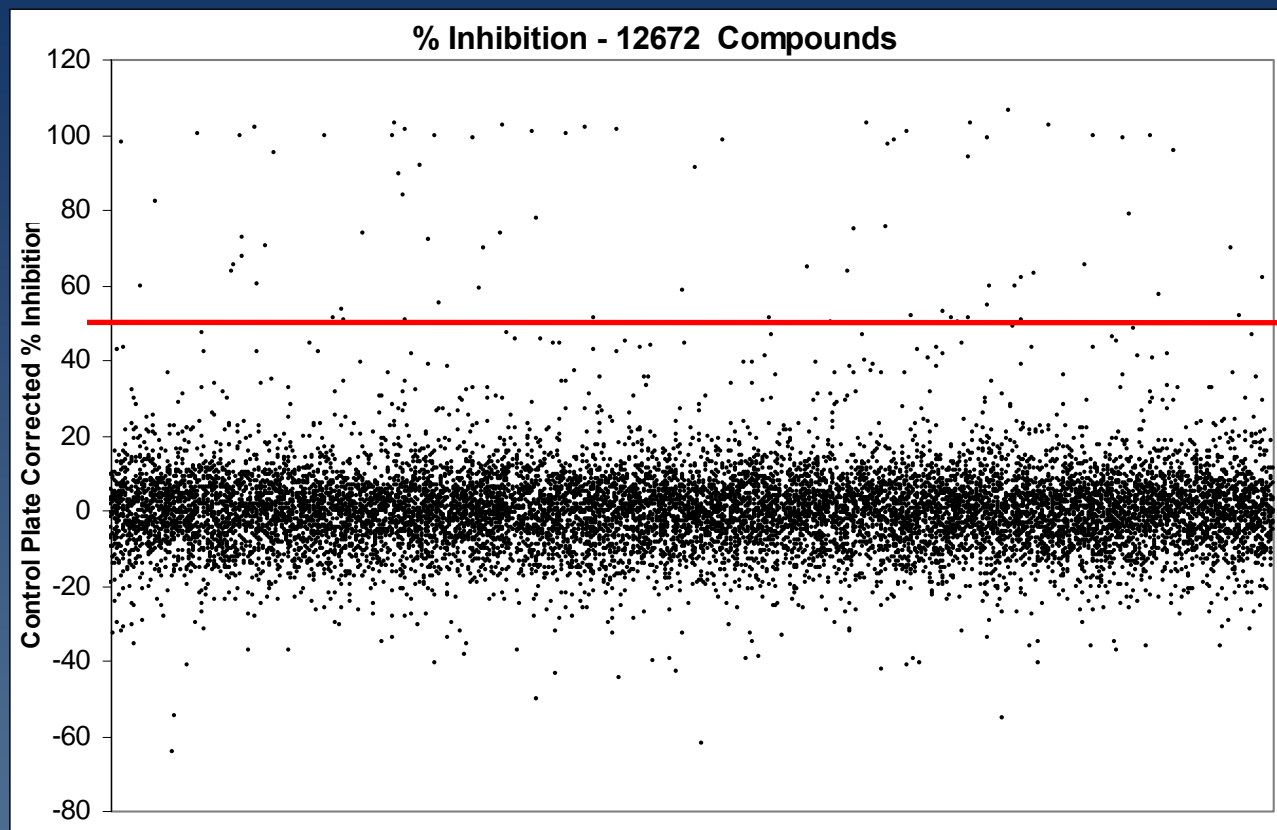


Problems Encountered

- Other kinase assays tried
 - ADP coupled enzyme assay
 - Anti-ADP antibody competition (Bellbrook)
 - 3-coupled enzyme assay (DiscoverRx)
 - Alpha screen no antibody needed with metal coated beads (Perkin Elmer)
- Many issues encountered mainly associated with using full length tau protein as substrate
- ELISA has many components, each have the chance of going bad



HTS Scatter plot of Cdk5 HTS

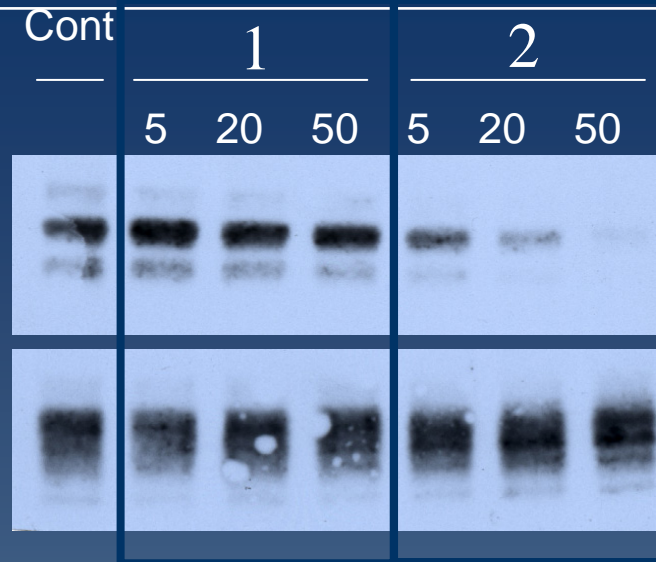


$Z' = 0.8$, Primary hit rate 0.7%, Confirmation rate 65%,
Screening concentration 10 μM
 IC_{50} 's between 0.8 – 50 μM



Cell-based Assays

IMR-32

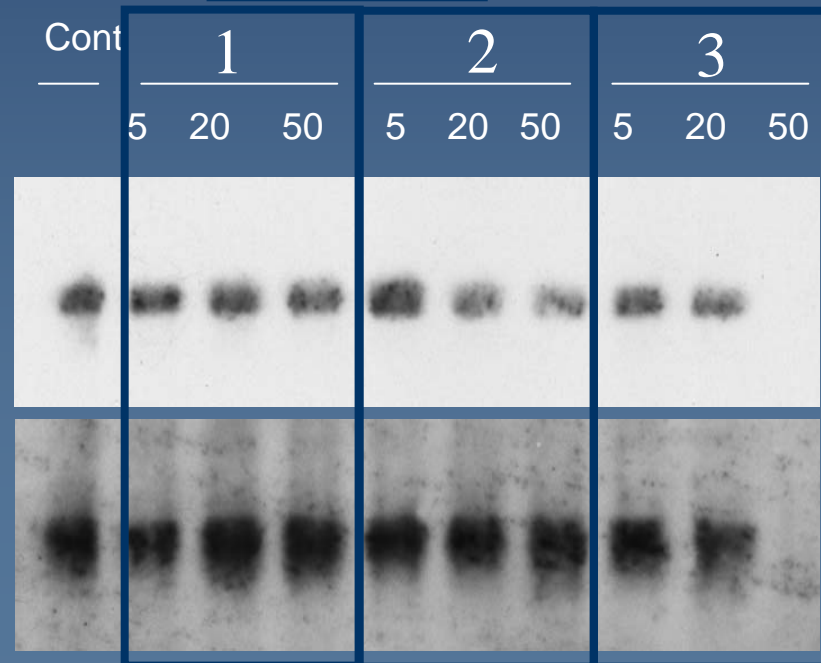


(μM)

PHF-1

Total Tau Ab

Primary
Neurons



PHF-1

Total Tau Ab

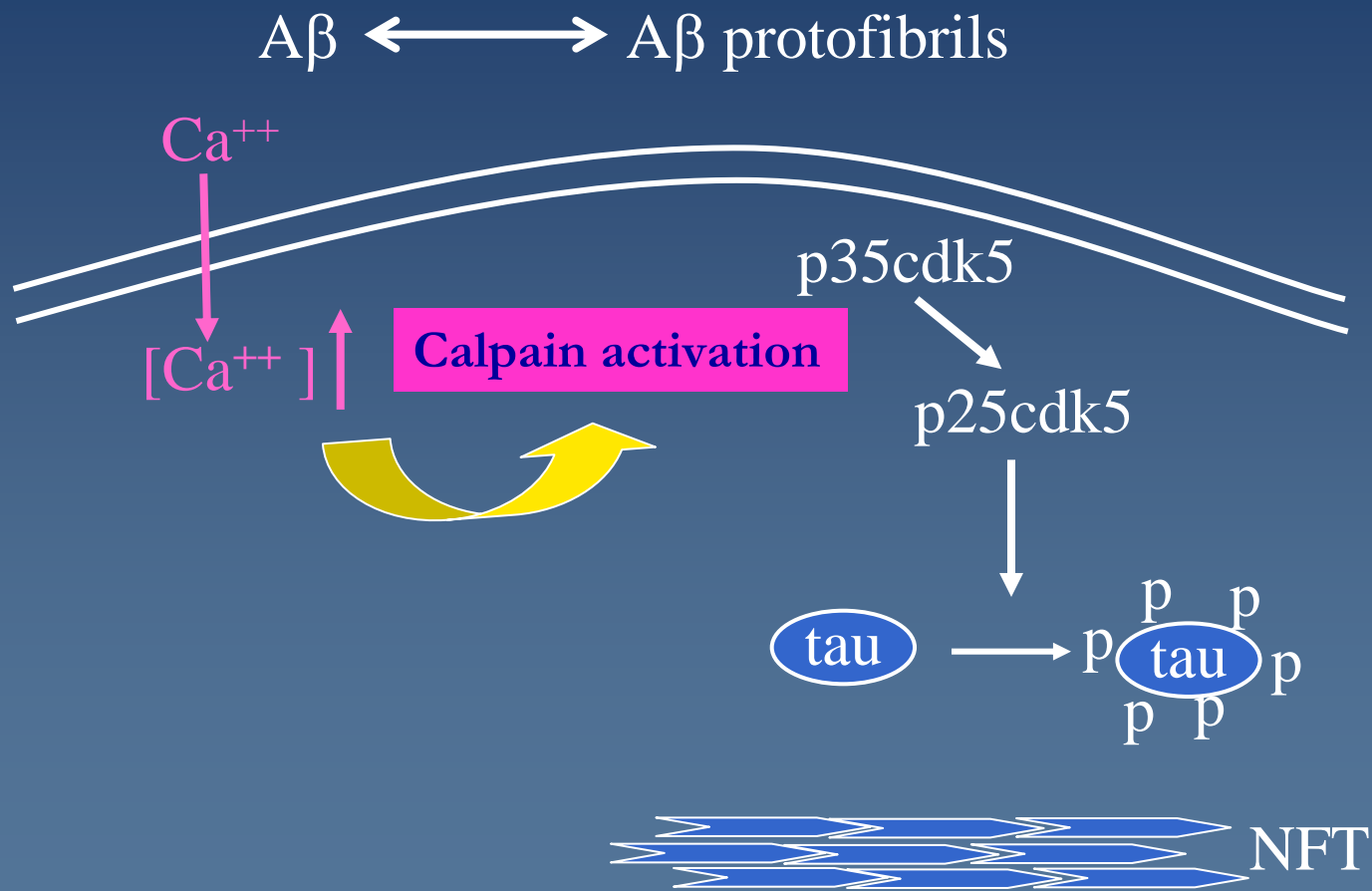


Case Study 2

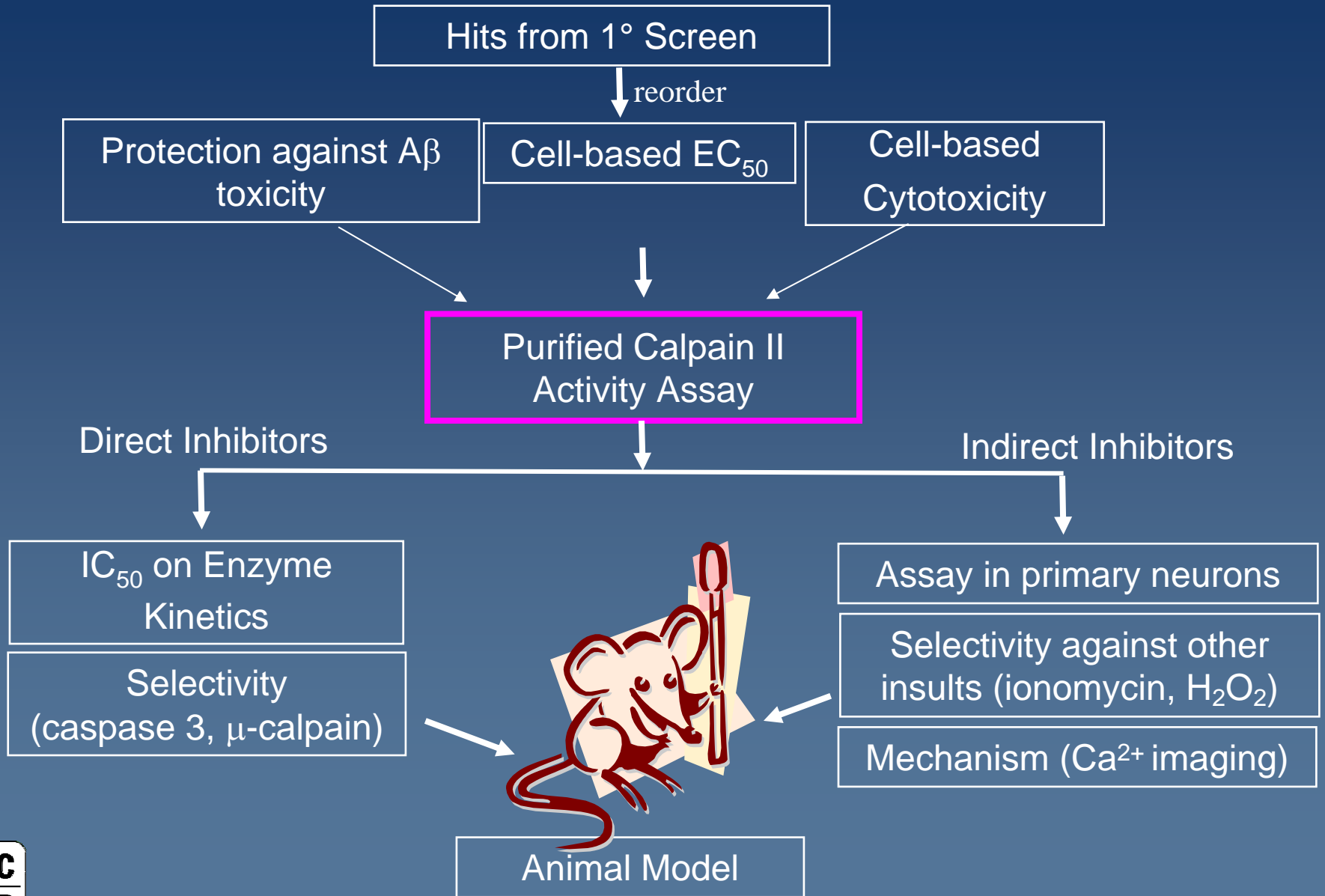
Neuroprotection against A β Toxicity by the Inhibition of Calpain Activation



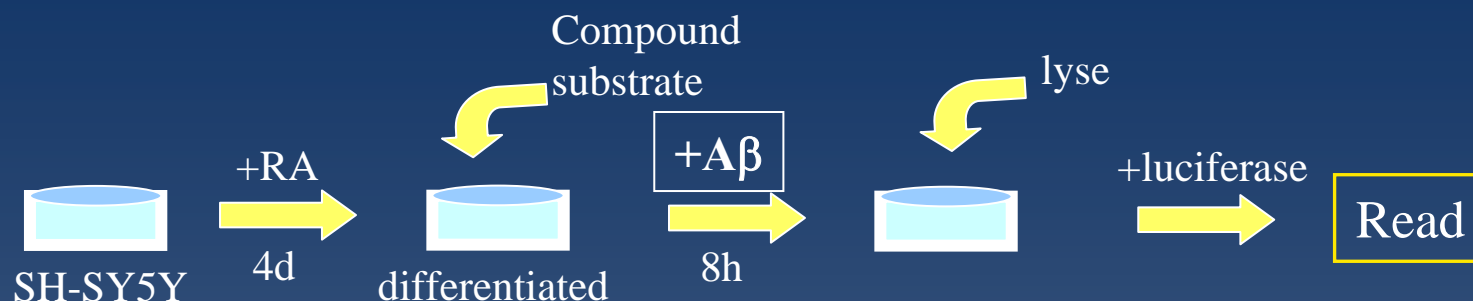
Proposed Role of Calpain in Alzheimer's disease Model of A β Toxicity



Flow Chart



Inhibitors of A β induced Calpain Activation Assay



Protocol:

- Plate 10,000 cells per well in medium containing 1% serum
- Day 1 after plating, treat with 10 μ M RA for 4 days to differentiate
- 30 minute incubation with compound
- 30 minute incubation with substrate
- 8 h exposure to A β ₂₅₋₃₅
- Lyse cells with 0.9% Triton-X-100 containing 100 μ M MDL-28170
- Incubate with detection reagent (luciferase) for 15 minutes prior to reading plate

- Calpain substrate: Suc-LLVY-aminoluciferin
- Positive control for inhibition: 50 μ M MDL-28170

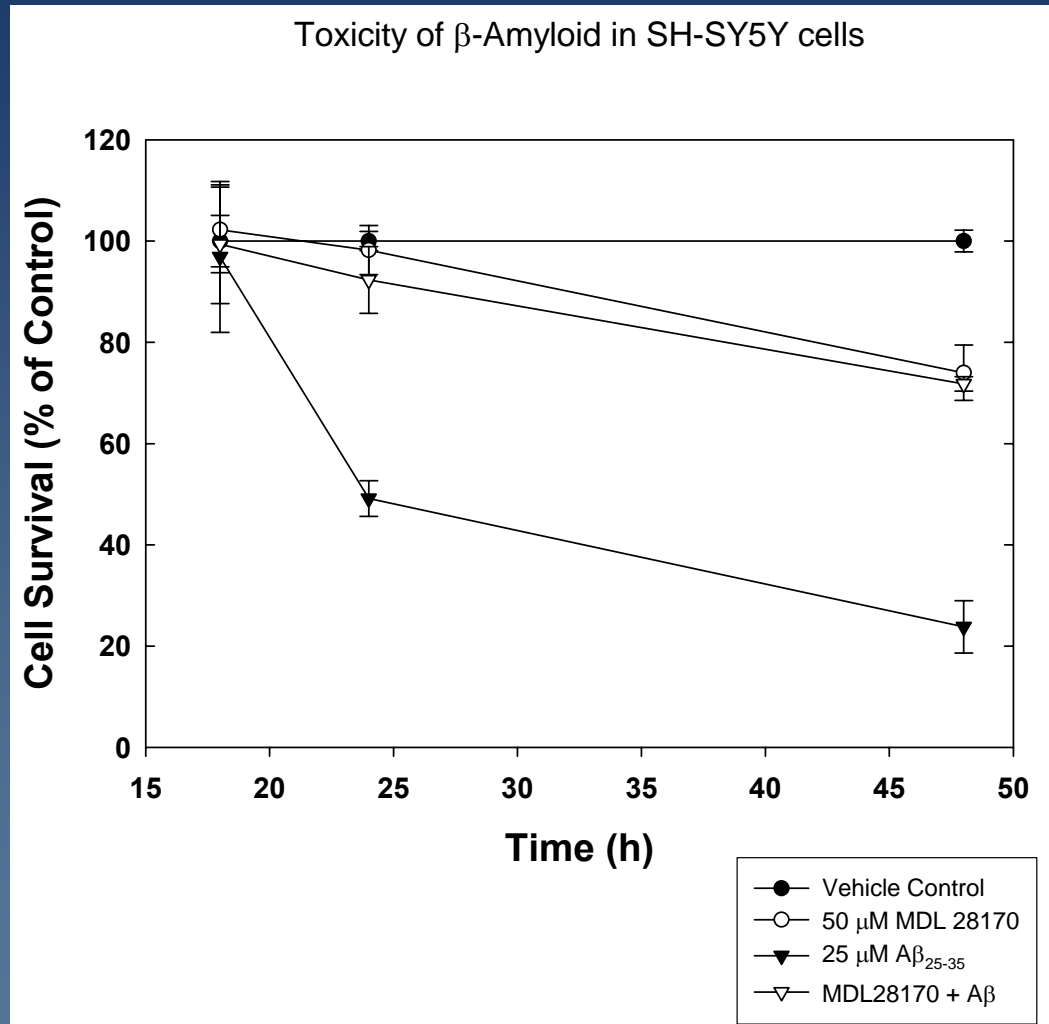


Steps to Develop the Assay

- **Establish cell line and time course**
 - Assay had been established in primary neuronal cultures
 - Needed to select cell line to use for primary screen
 - N2A, SH-SY5Y, IMR-32, PC-12)



Time Course of Death with A β in SH-SY5Y Cells

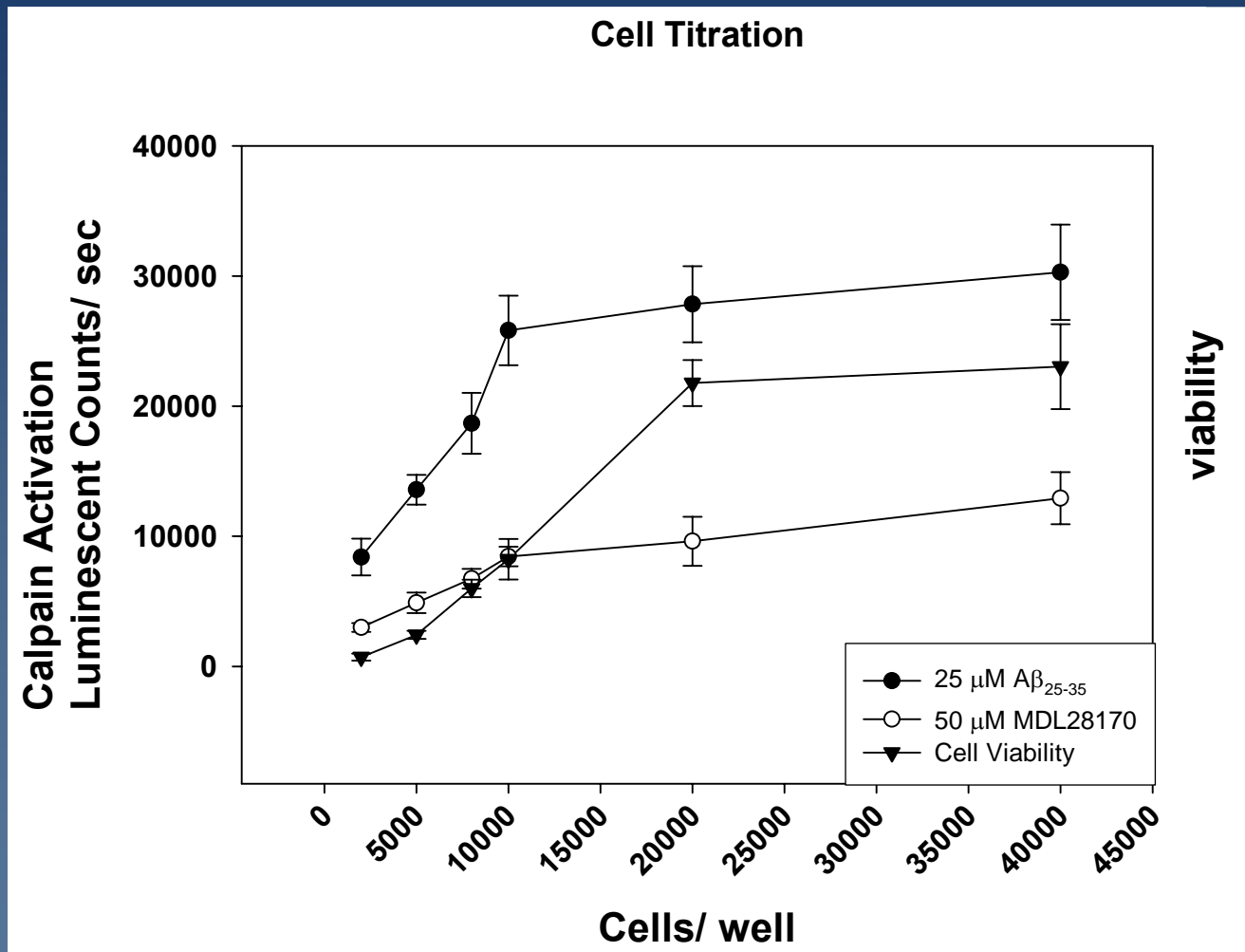


Steps to Develop the Assay

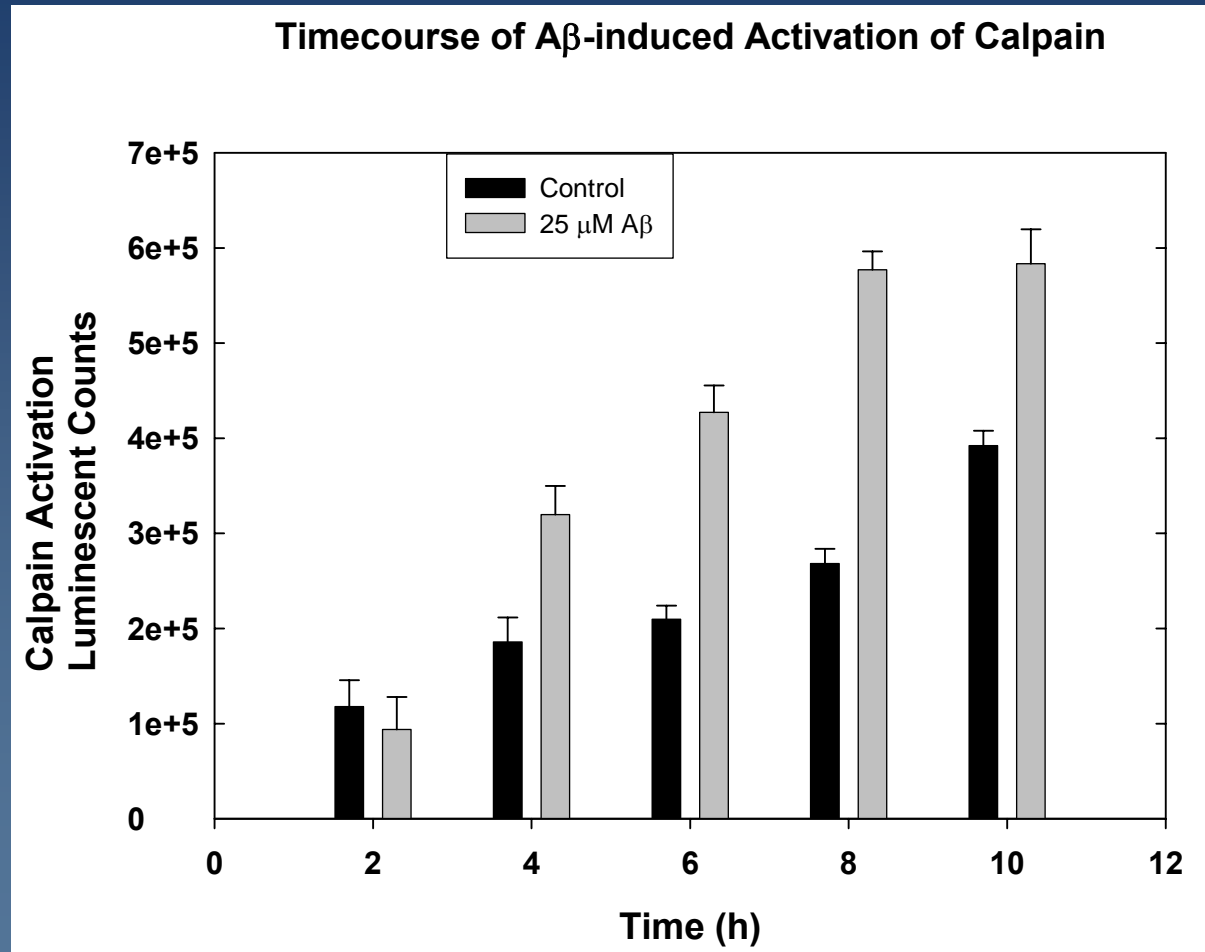
- Establish cell line and time course
 - Assay had been established in primary neuronal cultures
 - Needed to select cell line to use for primary screen
 - N2A, SH-SY5Y, IMR-32, PC-12)
- Other Parameters
 - Cell Titration
 - Time course
 - Substrate specificity
 - Validation with known inhibitors



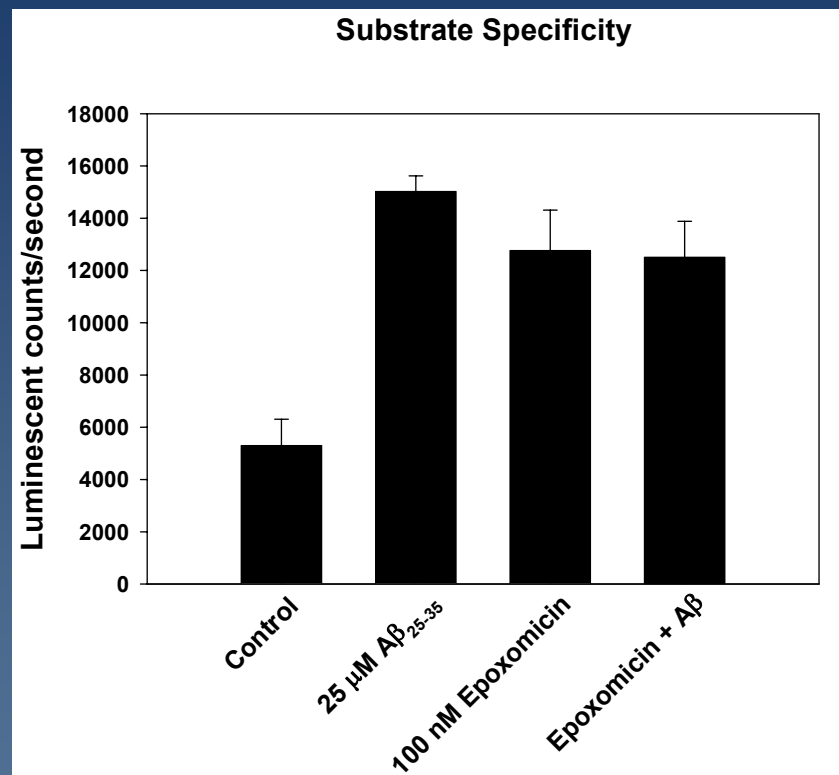
Cell Titration of Differentiated SH-SY5Y Cells



Time Course for Calpain Activation



Substrate Specificity Control

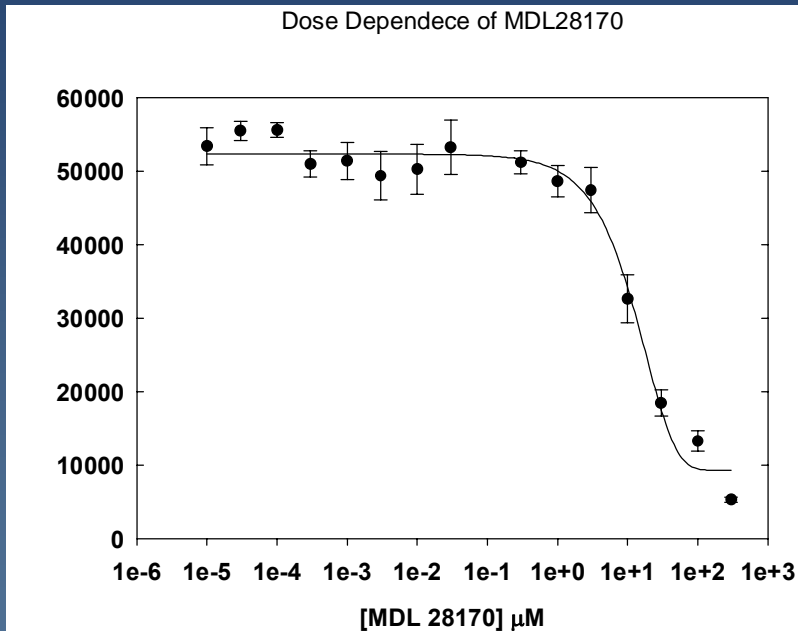


► Epoxomicin is a proteasome inhibitor



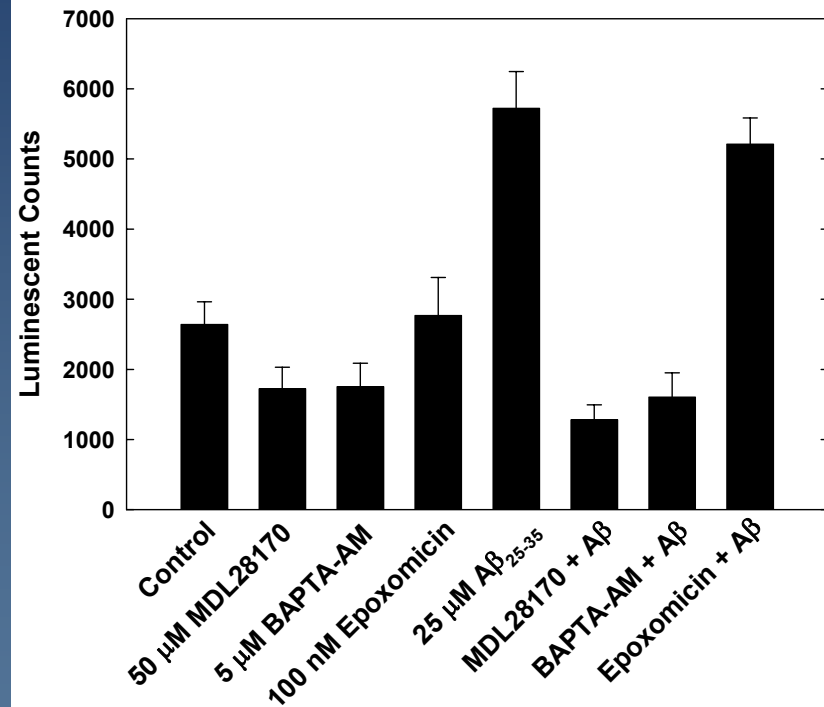
Validation of the Assay with Known Inhibitors

Dose Dependence of MDL28170

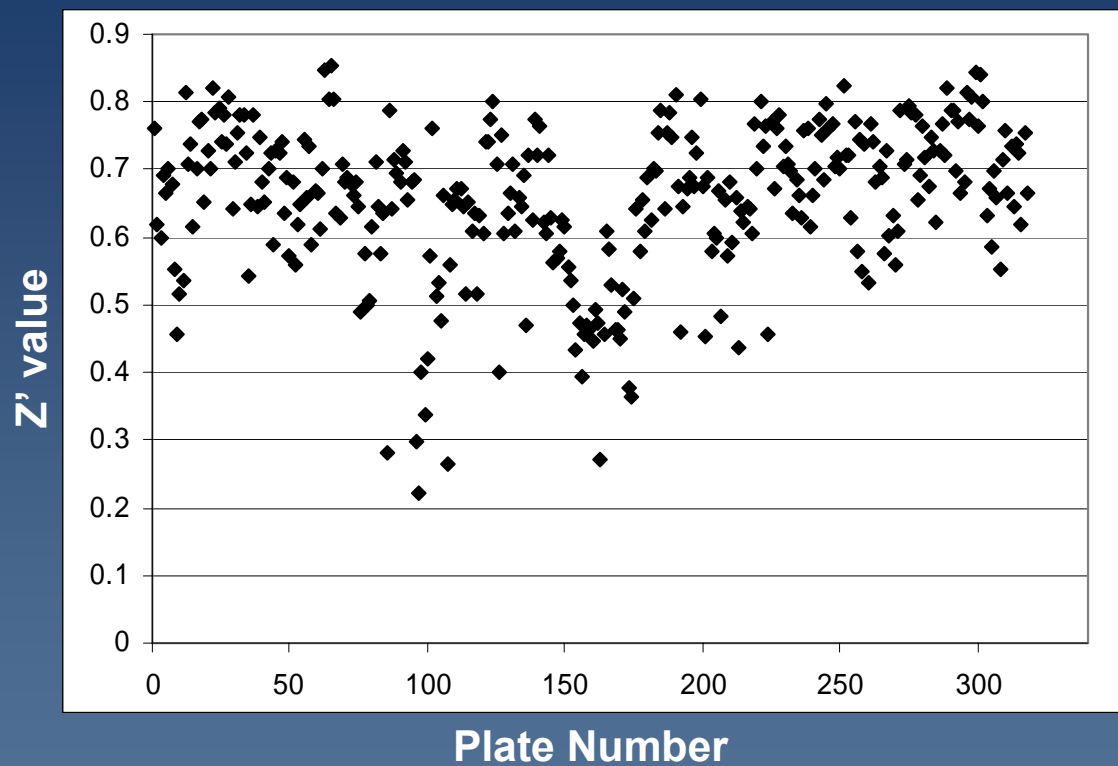


$$EC_{50} = 14 \pm 2.4 \mu M$$

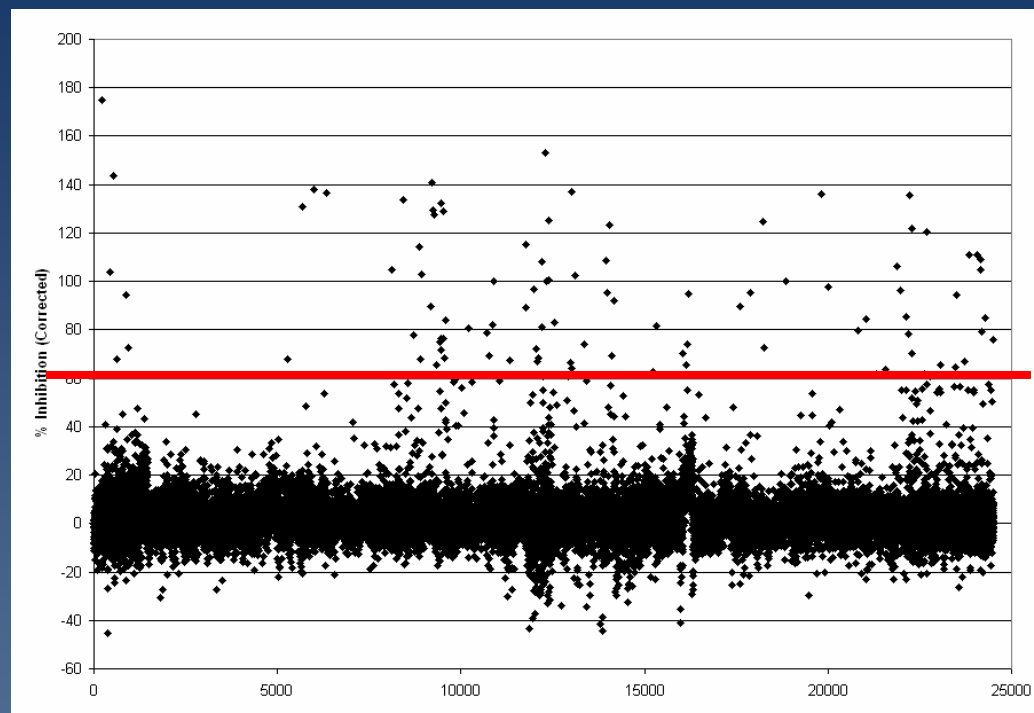
Validation with inhibitors



Z' values for the Screen



Data from the Screen

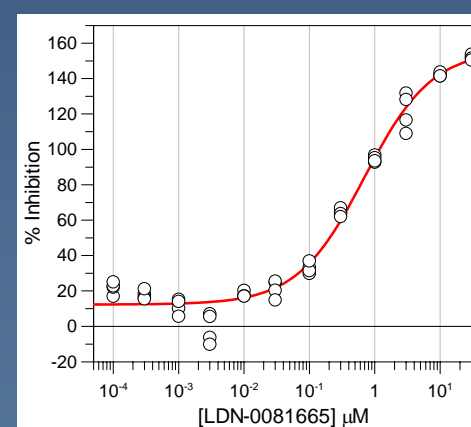
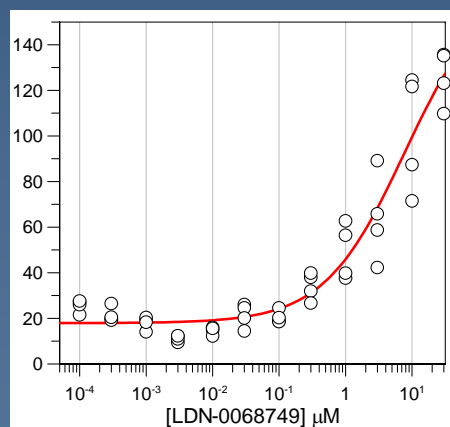
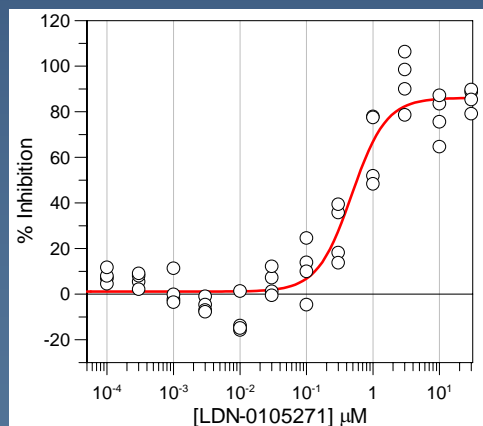
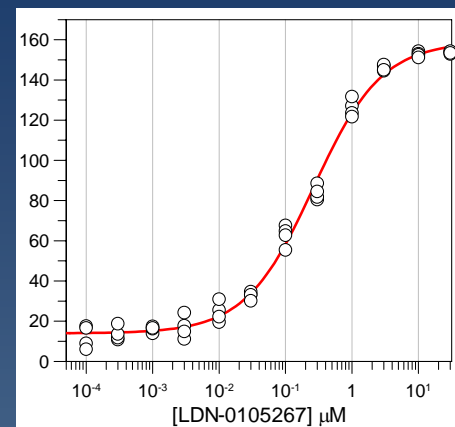
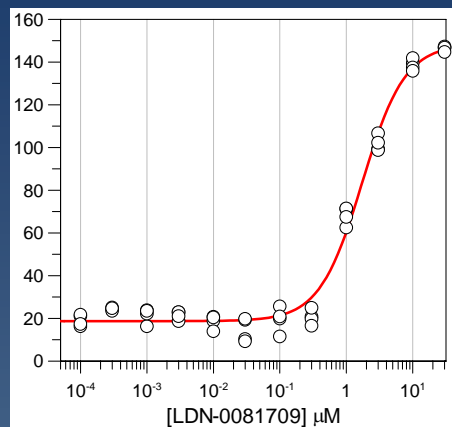
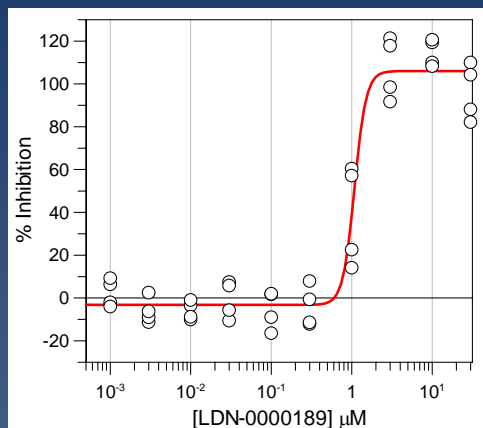


Hit Selection and Validation

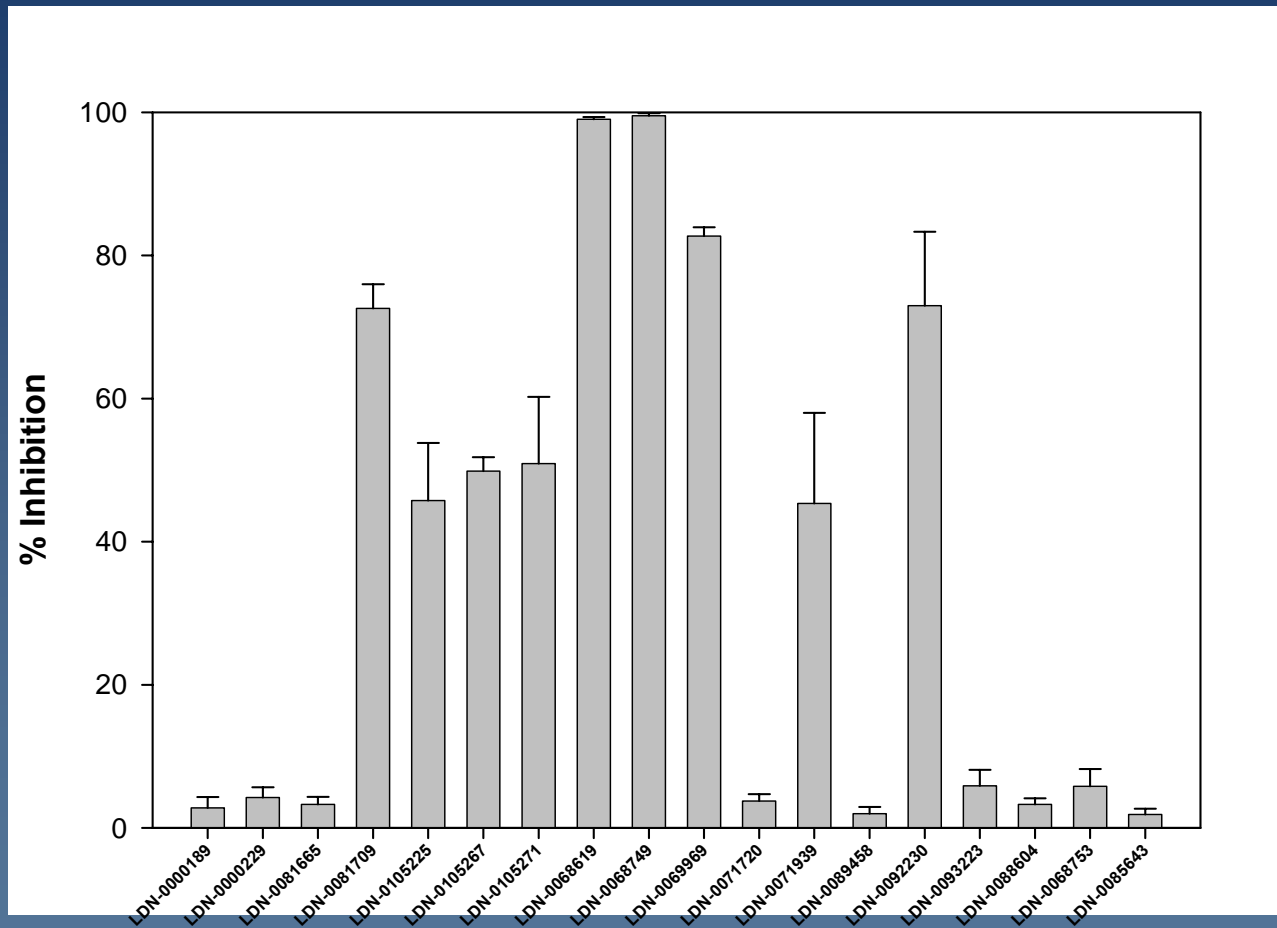
- **Compounds exhibiting $\geq 60\%$ inhibition in primary screen were re-tested at 0.1, 1, and 5 μM concentrations**
 - 353 compounds-0.29% hit rate
 - 144 compounds re-tested-80% reproduced
- **Simultaneous cytotoxicity assay performed**
 - 50% were toxic
- **Compounds selected for further studies based on chemical structure, potency, and lack of toxicity**
 - Several series of compounds with $\text{EC}_{50} = 0.033 - 3.3 \mu\text{M}$
 - The inhibitors demonstrate neuroprotection against $\text{A}\beta$ induced toxicity



Examples of Concentration Response Curves



Direct vs Indirect Calpain Inhibitors



Laboratory for Drug Discovery in Neurodegeneration

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(a) Partners Healthcare (b) Harvard Med School