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## **Title: Acetylcholinesterase Inhibitor Screening Assay Development: $K_m$ for AChE and IC<sub>50</sub> Determination for Donepezil and Physostigmine**

### **Summary**

In order to develop an Acetylcholinesterase inhibitor screening assay kit, we performed some initial experiments to establish appropriate assay conditions. We assessed conditions for Acetylcholinesterase from electric eel. First we titrated the AChE using 1 mM Acetylthiocholine iodide as the substrate and DTNB as the chromagen. From this titration we decided to use 0.0035 Units/reaction AChE for assay development and IC<sub>50</sub> determinations. The  $K_m$  of Acetylthiocholine iodide for AChE was then determined. We next titrated DMSO to determine the highest concentration that would be tolerated by AChE. Finally, the IC<sub>50</sub>'s for two known Acetylcholinesterase inhibitors, Donepezil and Physostigmine, were measured. We determined the  $K_m$  to be 0.425 mM and the maximum DMSO concentration to be 100 v%. Using 0.425 mM acetylthiocholine iodide, we observed an IC<sub>50</sub> = 48.3 nM for Donepezil and an IC<sub>50</sub> = 46.7 nM for Physostigmine when each inhibitor was dissolved in 20v% DMSO.

### **Results**

#### **AChE Titration**

In order to determine an appropriate amount of AChE to use for the inhibitor screening assay and for IC<sub>50</sub> determinations, we titrated AChE. The reaction was performed using 1 mM Acetylthiocholine Iodide and 0.4 mM DTNB dissolved in Assay Buffer pH 7.6. From these results we opted to use 0.0035 U/rxn AChE. At this concentration the reaction would remain linear for at least 10 minutes and the  $\Delta OD$  should be significant ( $>0.2$ ) even at the Acetylthiocholine Iodide  $K_m$ .

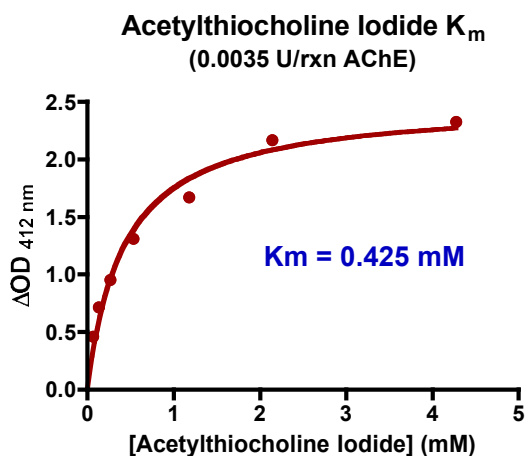
#### **$K_m$ of Acetylthiocholine Iodide**

In order to determine the  $K_m$  of Acetylthiocholine iodide for Acetylcholinesterase (electric eel), we titrated acetylthiocholine iodide from 0–4.3 mM with 0.0035 U/rxn AChE and computed  $\Delta OD$  ( $OD_{10min} - OD_{0min}$ ), which is proportional to the rate of reaction, for acetylthiocholine iodide. The  $\Delta OD$ 's were then plotted versus acetylthiocholine iodide concentration and the  $K_m$  was computed using Prism 4 (GraphPad Software Inc.). We found the  $K_m$  = 0.425 mM for Acetylthiocholine Iodide.



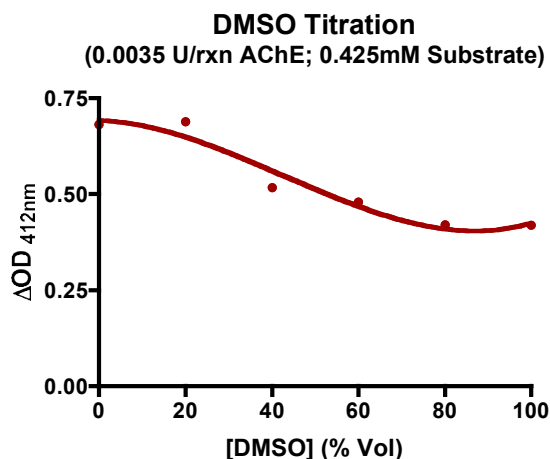
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## DMSO Titration

In order to determine tolerance for DMSO of Acetylcholinesterase, we ran a DMSO titration (from 0–100 v% for the 5  $\mu\text{L}$  of variable compound in a 200  $\mu\text{L}$  reaction). For this titration we used 0.0035 U/rxn AChE. The reaction was initiated by adding Acetylthiocholine Iodide, DTNB, and Assay buffer pH 7.6 (Final concentrations 0.425 mM Acetylthiocholine Iodide and 0.3 mM DTNB) and was allowed to proceed for 10 min. We then computed the  $\Delta OD$  ( $OD_{10\text{min}} - OD_{0\text{min}}$ ), which is proportional to the rate of reaction, for each DMSO concentration. The  $\Delta OD$ 's were then plotted versus DMSO concentration. From these results we discovered that that we can use up to 100 v% DMSO if needed, but decided to move forward with 20 v% DMSO for  $IC_{50}$  determinations.



## Donepezil $IC_{50}$ Determination

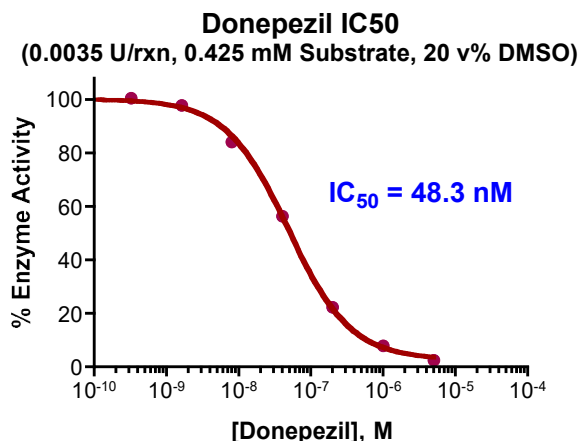
The  $IC_{50}$  determination required two steps: 1) 15 min pre-incubation of 45  $\mu\text{L}$  AChE (78 U/L) with 5  $\mu\text{L}$  Donepezil (in 20 v% DMSO), and 2) acetylcholinesterase reaction with 150  $\mu\text{L}$  Working reagent



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containing acetylthiocholine iodide, DTNB, and assay buffer for 10 min (0.425 mM [Acetylthiocholine Iodide] and 0.31 mM [DTNB] final). We titrated Donepezil from 0-25  $\mu$ M and computed the  $\Delta$ OD for each Donepezil concentration. The  $\Delta$ OD's were then plotted versus the Log [Donepezil] and the IC<sub>50</sub> was computed using Prism 4 (GraphPad Software Inc.). We observed an IC<sub>50</sub> = 48.3 nM.



### Physostigmine IC<sub>50</sub> Determination

The IC<sub>50</sub> determination required two steps: 1) 15 min pre-incubation of 45  $\mu$ L AChE (78 U/L) with 5  $\mu$ L Physostigmine (in 20 v% DMSO), and 2) acetylcholinesterase reaction with 150  $\mu$ L Working reagent containing acetylthiocholine iodide, DTNB, and assay buffer for 10 min (0.425 mM [Acetylthiocholine Iodide] and 0.31 mM [DTNB] final). We titrated Physostigmine from 0- 25  $\mu$ M and computed the  $\Delta$ OD for each Physostigmine concentration. The  $\Delta$ OD's were then plotted versus the Log [Physostigmine] and the IC<sub>50</sub> was computed using Prism 4 (GraphPad Software Inc.). We observed an IC<sub>50</sub> = 46.7 nM.

