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Title: PTP1b Activity Assay Control Tests: K_m for Full Length and Truncated PTP1b and IC50 Determination for Sodium Vanadate (Na_3VO_4)

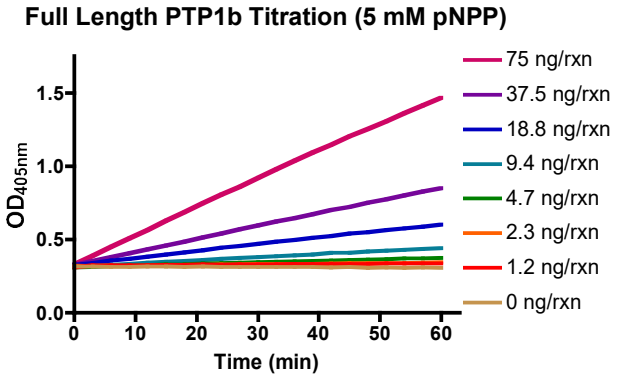
Summary

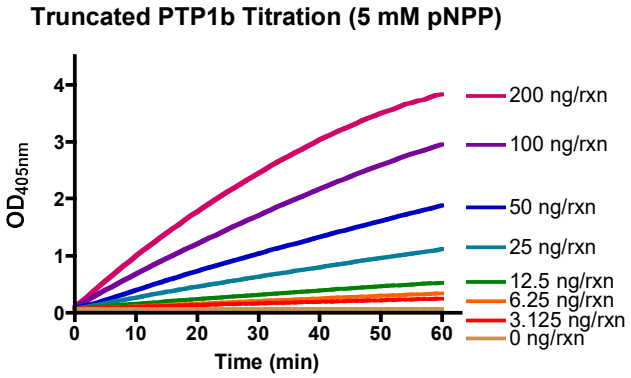
Before determining the IC50's for ten potential PTP1b inhibitors, we performed some initial experiments to establish appropriate assay conditions. We assessed conditions for a full length human PTP1b and a truncated human PTP1b (Glu2-Asn321). First we titrated the PTP1b's using 5 mM pNPP as the substrate. From these titrations we decided to use 50 ng/reaction for the full length PTP1b and 40 ng/rxn for the truncated PTP1b. We next titrated DMSO to determine the highest concentration that would be tolerated by each PTP1b. The K_m of pNPP for each PTP1b was then measured in the presence of the highest tolerable DMSO concentration. Finally, the IC50 for a known tyrosine phosphatase inhibitor, Na_3VO_4 was measured for each PTP1b. For the full length PTP1b, we determined the maximum DMSO concentration to be 15 v% and the K_m to be 0.7 ± 0.04 mM. For the truncated PTP1b, we determined the maximum DMSO concentration to be 7.5 v% and the K_m to be 1.3 ± 0.1 mM. Using 0.7 mM pNPP, we observed an IC50 = 19.3 ± 1.1 µM for Na_3VO_4 for the full length PTP1b. Using 1 mM pNPP, we observed an IC50 = 54.5 ± 1.1 µM for Na_3VO_4 for the truncated PTP1b.

Results

PTP1b Titrations

In order to determine an appropriate amount of PTP1b to use for the IC50 determinations, we titrated each PTP1b. The full length PTP1b was titrated from 75 ng/rxn and the truncated PTP1b was titrated from 200 ng/rxn. Both titrations were performed using 5 mM pNPP as the substrate. The full length PTP1b titration was performed in a 384 well plate to maximize the absorbance signal. (Due to high variation between replicates, we decided to switch to 96 well plates for the IC 50 analysis and all of the truncated PTP1b analyses.)





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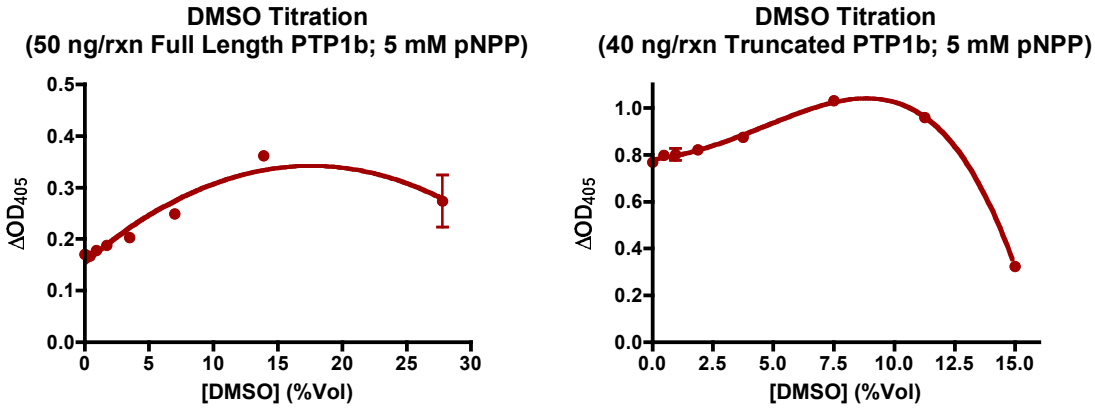
From these results we opted to use 50 ng/rxn for the full length PTP1b and 40 ng/rxn for the truncated PTP1b. At these concentrations the reaction would remain linear for at least 1 hr and the ΔOD should be significant (>0.2) even at the pNPP K_m.

DMSO Titration

In order to determine tolerance for DMSO of each of the PTP1b's, we ran DMSO titrations for each PTP1b (from 0–27 v% for the full length PTP1b and from 0-15 v% for the truncated PTP1b). For these titrations we used 50 ng per reaction for the full length PTP1b and 40 ng truncated PTP1b.



The reactions were initiated by adding 5 mM pNPP and were allowed to proceed for 60 min. We then computed the ΔOD (OD_{60min} – OD_{0min}), which is proportional to the rate of reaction, for each DMSO concentration. The ΔOD's were then plotted versus DMSO concentration. From these results we discovered that DMSO actually enhanced the activity of each PTP1b up to a certain concentration. For the full length PTP1b, we decided to move forward with 15 v% DMSO and for the truncated PTP1b, we decided on 7.5 v% DMSO.

K_m of pNPP

In order to determine the K_m of pNPP for the PTP1b's, we titrated pNPP from 0–20 mM with either 50 ng full length PTP1b or 40 ng truncated PTP1b and computed ΔOD (OD_{60min} – OD_{0min}), which is proportional to the rate of reaction, for each pNPP concentration. The ΔOD's were then plotted versus pNPP and the K_m was computed using Prism 4 (GraphPad Software Inc.). The full length PTP1b K_m determination was performed in the presence of 15 v% DMSO and truncated PTP1b K_m determination was performed in the presence of 7.5 v% DMSO. We found the K_m = 0.7 ± 0.04 mM for the full length PTP1b and the K_m = 1.3 ± 0.1 mM for the truncated PTP1b.

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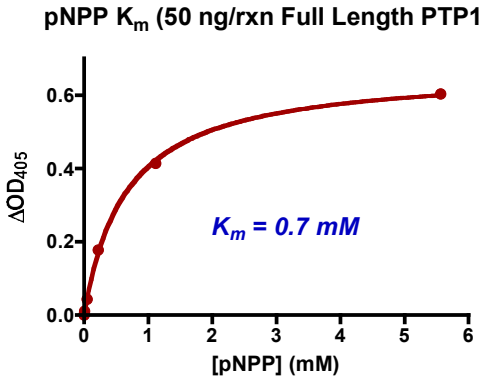

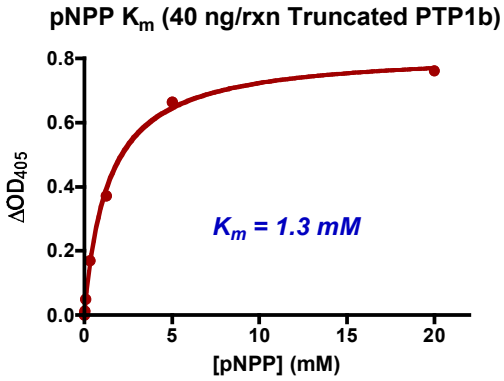

Sodium Vanadate (Na_3VO_4) IC50 Determination

The IC50 determination required two steps: 1) 30 min pre-incubation of 25 µL PTP1b (50 ng full length PTP1b or 40 ng truncated PTP1b) with 15 µL Na_3VO_4 (in 100% DMSO for full length PTP1b or 50 v% DMSO for truncated PTP1b), and 2) phosphatase reaction with 60 µL pNPP (0.7 mM final [pNPP] for full length PTP1b and 1 mM final [pNPP] for truncated PTP1b) for 60 min. We titrated Na_3VO_4 from 0-1000 µM and computed the ΔOD for each Na_3VO_4 concentration. The ΔOD's were then plotted versus the Log [Na_3VO_4] and the IC50 was computed using Prism 4 (GraphPad Software Inc.). We observed an IC50 = 19.3 ± 1.1 µM for the full length PTP1b and an IC50 = 54.5 ± 1.1 µM for the truncated PTP1b.