Kinetic analysis of N-Deacetylase / N-Sulfotransferase (NDST1) inhibitors using a fluorometric coupled enzyme assay

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Introduction

NDSTs are Golgi-membrane proteins that catalyze the exchange of acetyl to sulfate groups on nascent heparan sulfate (HS) chains. This requisite reaction permits subsequent modifications by epimerases and other sulfotransferases to yield fully mature HS with diverse structures and biological functions.

The pathophysiology of lysosomal storage disorders such as Sanfilippo Syndrome are characterized by HS accumulation. In our previous work, we have demonstrated that NDST1 is a druggable target in the treatment of Sanfilippo Syndrome and engaged in in silico drug screening. Limitations in existing methodologies to quantify drug effects on NDST1 necessitated the development of a dynamic fluorometric assay. In Figure 1, the reaction components are listed in the schematic: recombinant mouse NDST1 (mNDST1), adenosine 3'-phosphoadenosine-5'-phosphosulfate (PAPS), a bacterial analogue of desulfated human HS (Escherichia coli capsular polysaccharide, K5 or heparosan), 4-methylumbelliferyl sulfate, a fluorogenic compound and a double mutant rat phenol sulfotransferase, SULT1A1 K56ER68G. Used in tandem with a new LC-MSMS method quantifying HS in patient fibroblasts, we aim to identify potential drug candidates that decrease HS levels in cells through the inhibition of NDST1.

Methodology

• 96 well format; real time reaction monitoring using Molecular Devices M2 Spectrophotometer on black Greiner Bio flat opaque bottom microplate with data fitted to Michaelis-Menten analysis
• Optimal reaction conditions for the coupled enzyme assay: phosphate buffer (pH 8.2), 37°C, 100 µl total reaction volume, 30 min
• IC50s were calculated for select drugs identified by in silico studies
• Comparison of HS levels in healthy and Sanfilippo patient derived fibroblasts through a new LC-MSMS method
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SULT1A1 K56ER68G efficiently catalyzes the regeneration of PAPS and the formation of MU

Figure 2: Characterizing the kinetics of the PAPS regeneration reaction. A) Generating an MU standard curve, it was determined that for every nanomole of MU formed by the SULT1A1 K56ER68G reaction, there is an increase of 45.01 relative fluorescence units. B) In a concentration-dependent manner, the double mutant rat phenol sulfotransferase formed PAPS and MU with a concomitant increase in fluorescence that was linear for the 30-minute reaction time course. C) Michaelis-Menten analysis reveals that SULT1A1 K56ER78G has saturation kinetics and a $K_M$ of 38.8 μM and a $V_{max}$ of 4.57 ± 0.59 nmol/min/μg enzyme.

The coupled enzyme assay allows for kinetic characterization of mNDST1 enzyme activity

Figure 3: Kinetic characterization of the mNDST1 enzyme with the coupled assay. Reactions carried out with variable concentrations of K5 (A) and PAPS (B) allowed for the calculation $K_M$ of 34.8 μM (C) & $K_M$ of 10.7 μM (D). The assay ran under steady state conditions with saturated substrate concentrations calculated a specific activity of mNDST1 at 394 pmol/min/μg enzyme. This data demonstrates the facility of monitoring changes in enzyme activity due to drug effects in subsequent inhibitor tests.
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Assessment of mNDST1 drug candidates using the coupled assay and LC-MSMS quantitation of HS

Figure 4: Examining the impact of drug treatment on the activity of NDST1. (A) In order to validate the efficacy of our assay, a selection of drugs, previously identified from an in silico screen as potential inhibitors of NDST1, were tested using the coupled assay. Excluding drugs that had cross-reactivity with SULT1A1 K56ER68G, we were able to calculate an IC50 curve for select drugs characterizing their inhibitory effects on the activity of NDST1. (B) We have recently devised a new LC-MSMS method capable of measuring heparan sulfate levels in patient derived fibroblasts.

Key Conclusions

1. Previous methods of quantifying NDST1 activity were onerous and technically complex, we demonstrate the facility of characterizing the inhibitory effects of select drugs on NDST1 using a fluorometric coupled enzyme assay with an aim to identify potential therapeutics for Sanfilippo syndrome.

2. Using an in vitro enzyme assay, kinetic parameters such as the Michaelis constant (Km) and maximum reaction velocity can be easily calculated to identify the mechanism of inhibition to optimize therapy.

3. The optimal conditions for the NDST1-SULT1A1 K56ER68G coupled assay involves: a 30-minute reaction course at 37°C, phosphate buffer (pH 8.2), 37°C, & 100 μl total reaction volume.

4. These findings will be further validated through more extensive inhibitor analysis and LC-MSMS quantitation of HS levels in fibroblasts cells derived from healthy controls and Sanfillipo patients to best identify drugs that mitigate HS accumulation to treat Sanfilippo Syndrome.

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