Discovery of Inhibitors of GALNT3 by Orthogonal Pooled Screening with a Universal Glycosyltransferase Assay

Tom Zielinski[1], Melvin Reichman[2], P. Scott Donover[2], and Robert G. Lowery[1]
[1]BellBrook Labs, Madison, WI, USA, [2]Lankenau Institute for Medical Research (LIMR), Wynnwood, PA, USA

Abstract

Glycosyltransferase enzymes participate in diverse metabolic and regulatory roles by catalyzing the transfer of sugars to protein, lipid and carbohydrate acceptors as well as to other endogenous and xenobiotic molecules. Of the more than 200 human glycosyltransferases (GTs), there are over 20 distinct polypeptide N-acetylgalactosaminyltransferases (GALNTs) that catalyze the initial step of O-glycosylation by transferring GalNac to Thr or Ser residues on multiple targets, including mucins. Abnormal post-translational glycosylation of mucin is a driver of cancer-associated changes affecting growth and survival of cancer cells and their ability to invade and metastasize. GALNT3 overexpression and dysregulation has been directly linked to multiple cancers, including gastric, ovarian, pancreatic and lung, making it a compelling target for drug discovery. Development of an HTS workflow for GALNT3 is described here. Recombinant GALNT3 enzyme activity was first optimized in the Transcreener UDP^2 Assay with the donor and acceptor substrates UDP-GalNac and Mucin 10(153-165) EA2 peptide, and K_m values were determined. A pilot screen was run using the TR-FRET-based assay with a diverse, pre-filtered orthogonally pooled compound set from the Lankenau Institute for Medical Research (LIMR) Chemical Genomics Center, which allowed screening of 8,000 compounds, in duplicate, in just five 384-well plates. Hits were confirmed by dose-response measurements with the primary screening assay and then further validated with an FP based UDP^2 Assay. Finally, two confirmed hits were further evaluated for the longevity of target engagement by performing rapid dilution experiments to measure dissociation rates.

Polypeptide N-Acetylgalactosaminyltransferases (GALNTs)

- Over 20 GALNTs known, typically found in Golgi
- Catalyze the initial step of Mucin type O-glycosylation
- Abnormal Mucin glycosylation typical in cancers

GALNT3

- Expression highly regulated and mainly found in pancreas & testis[3]
- Dysregulation has been directly linked to multiple cancers, including gastric, ovarian, pancreatic and lung[4,5]

EA2 Peptide + UDP-GalNAc $\xrightarrow{\text{GALNT3}}$ UDP + GalNAc-EA2

Transcreener® UDP^2 Assays: Universal, Mix-and-Read Detection of UDP-Glycosyltransferases

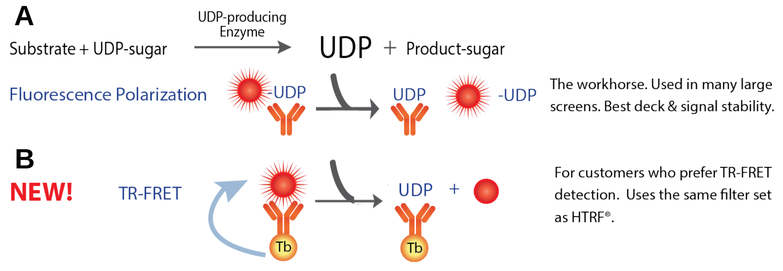


Figure 1. Transcreener UDP^2 Assay. The Detection Mixture comprises either of **A**) UDP Alexa 633 tracer bound to an UDP^2 antibody or **B**) an UDP HiLyte 647 tracer bound to an UDP^2 antibody-Tb conjugate. UDP displaces the tracer, which causes either an increase in polarization or a decrease in TR-FRET. Any UDP-Sugar can be used as a substrate, making it versatile for glycosyltransferases which produce UDP.

GALNT3 Enzyme Validation

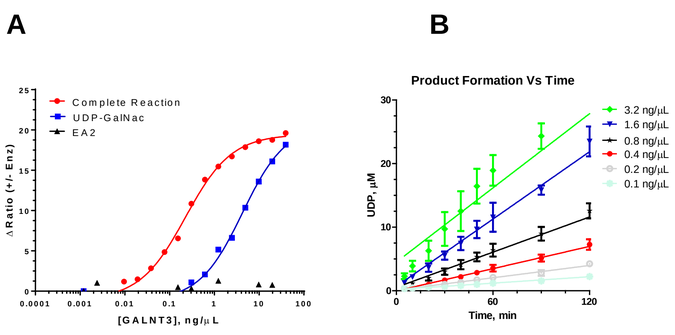
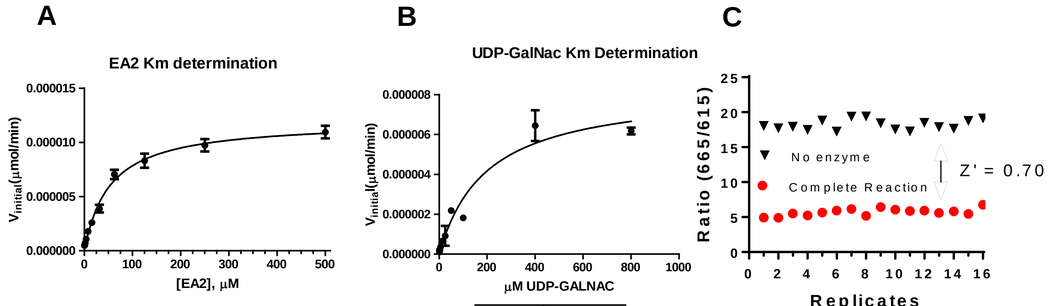


Figure 2. GALNT3 Enzyme Validation. A) GALNT3 was titrated in the presence and absence of Mucin 10-EA2 peptide and UDP-GalNac **B**) Optimal enzyme concentration was determined where there was a linear correlation of [enzyme] with UDP production. Optimal [GALNT3]: 1.5 ng/µL.

K_m Determination of Substrates & Assay Robustness

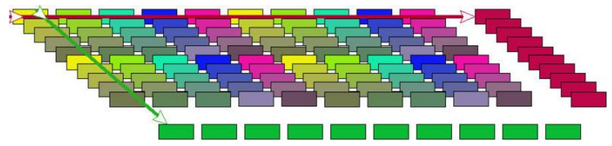


Vmax	1.198e-005
Km	52.82

Vmax	8.304e-006
Km	198.0

Figure 3. Substrate K_m Determination and Assay Robustness:. A) EA2 and **B)** UDP-GalNac Km determinations. **C)** Screening conditions were tested to determine assay robustness. Z' = 0.7

Orthogonal Pooled Screening (OPS™)



- Each well contains 10 compounds from a 104,000 compound library with many core scaffolds not found elsewhere.
- Each compound is present in two wells, amongst a unique combination of 9 other compounds
- To be tallied as a hit, the compound must show reactivity in both wells.
- The net result is a five-fold reduction in screening wells (e.g., 8,000 compounds in five 384 well plates), with n=2 corroboration.

Screen Set-up and Results

8000 Cmpds (N=2) in 5 Pre-dispensed Plates

↓

Pre-incubate with GALNT3 for 30 min at RT

↓

Start reaction with addition of substrates

↓

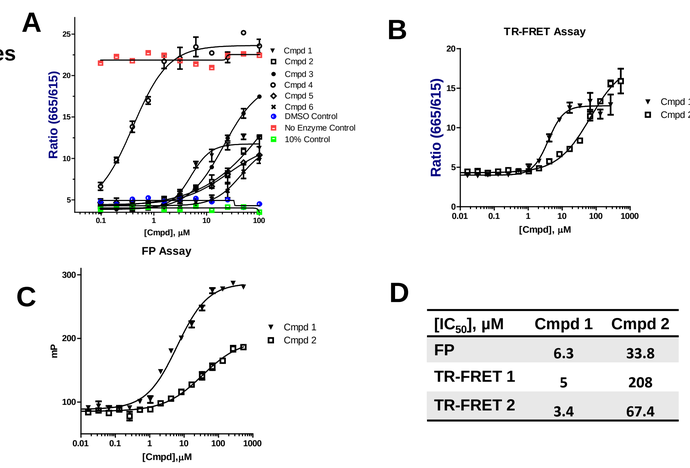
Stop Reaction after 1 hr at RT

↓

Data sent to LCGC for Deconvolution

Screen Statistics	
Z'-From DMSO Controls	0.8
Z-factor of entire Screen	0.7
Hits > 3 Std Devs	11
Hits > 2 Std Devs	3
Hits > 1 Std Devs	14

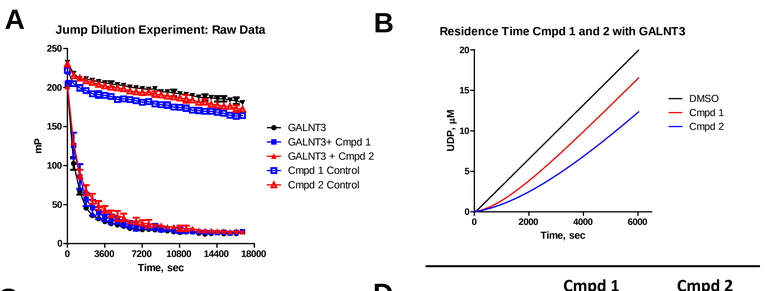
Hit Validation of Compounds



[IC_{50}], µM	Cmpd 1	Cmpd 2
FP	6.3	33.8
TR-FRET 1	5	208
TR-FRET 2	3.4	67.4

Figure 4. Hit Validation:. A) 6 compounds had a dose response, of them 2 were further validated in both **B)** TR-FRET and **C)** FP assays. **D)** IC_{50s} from 3 experiments.

Determining the Inhibitor Residence Time using a Jump Dilution Method



C

$$P = V_S t + (V_0 - V_S)\frac{(1 - e^{-kt})}{k}$$

D

	Cmpd 1	Cmpd 2
K	0.0007726	0.0003015
Tau (min)	21.6	55.3

Figure 5. Jump Dilution Method: 10X [IC_{50}] of each compound was pre-incubated with 100X [GALNT3] to form EI complex. The reaction was then diluted 100X by addition of substrates. **A)** Raw data was measured continuously & plotted as **B)** UDP vs Time. Data was fit to the integrated rate equation **C)** to determine k_{off} and **D)** residence time, Tau.

Summary

- ***GALNT3 was screened using an OPS library***
- ***2 Compounds [IC_{50}]s and residence time were determined***
- ***SAR will be performed on compounds to test scaffold variants***
- ***Hits will be profiled against different GTs***

References
3. Bennet, E.P. et al.(1996). *J.Biol.Chem.* 271:17006
4. Kitada, S. et al.(2013). *Brit.J. of Can.* 109
5. Wang, Z. et al.(2014). *Oncotarget.* 5:2

BellBrook Labs, 5500 Nobel Drive, Suite 250, Madison, WI 53711
866.313.7881 or 608.443.2400