Transcreener® High Throughput Screening Assays for Glycosyltransferases

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Abstract

Identifying glycosyltransferase inhibitors remains a challenging task in drug discovery, because for many enzymes in this diverse class, including glucosyl-, fucosyl- and sialyltransferases, screening methods have been limited to expensive and cumbersome radiolabeled assays or HPLC/MS analyses. Over the last few years a suite of Transcreener® glycosyltransferase assays have been developed based on the simple and direct immunodetection of nucleotides, which are easily adaptable to automation and systemized high throughput screening protocols. Transcreener fluorescence polarization assays require one additional step for product detection, utilize a far red fluorescent tracer to minimize compound interference, and provide a stable signal for overnight plate reads. Here we report HTS-compatible assay windows (>90 mP units) at <10% substrate conversion for three enzyme systems (UDP-glucose/UDP, GDP-fucose/GDP, and CMP-NAN/CMP) using standard protocols. Optimal HTS assay conditions including minimum substrate and enzyme requirements for glucosylceramide synthase (GCS), one of the key enzymes targeted for drug therapy associated with Gaucher’s disease and related disorders, Tay-Sachs, Fabry and Sandhoff’s disease, will be presented.

Figure 1. Transcreener Assay Principle

Transcreener assays rely on the homogeneous fluorescent detection of the invariant reaction product of a group transfer reaction. The assay antibodies differentiate between nucleotides on the basis of small structural differences, such as a single phosphate group, with 100-fold or greater selectivity. There is only one “donor” product for each type of group transfer reaction; so a single set of Transcreener detection reagents can be used for all family members, regardless of the acceptor substrate. This enables screening of any isoform in a family of group transfer enzymes, with any acceptor substrate, using the same assay reagents. For the purposes of this discussion the nucleotide product is either UDP, GDP, or CMP. Transcreener assays are available for ADP, AMP and GMP as well.

Figure 2. A Suite of Transcreener® Assays for Glycosyltransferases

Transcreener UDP Assay

UDP-producing Enzymes galactosyltransferase glucuronolysyltransferase N-acetylgalcosamyltransferase N-acetylglucosamyltransferase xylosyltransferase

Transcreener GDP Assay

GDP-producing Enzymes fucosyltransferase mannosyltransferase

Transcreener AMP/GMP Assay

CMP-producing Enzymes sialyltransferase

There are over 200 glycosyltransferases encoded in the human genome, and most use uridine diphosphate (UDP) - activated sugars as the donor. From a functional standpoint, the reactions they catalyze can be divided into three major types, biosynthesis of oligosaccharides or polysaccharides such as starch or hyalurcan, posttranslational modification of proteins, and glucuronidation of small molecules, including endogenous hormones and xenobiotics. All three types of glycosyltransferases are of interest in drug discovery. The role of the approximately 15 hepatic UDP-glucuronosyltransferases (UGTs) in drug metabolism has been an area of focus for several years. More recently, protein glycosyltransferases and those involved in synthesis of biopolymers have come under focus as therapeutic targets, for cancer and lysosomal storage diseases. Bacterial glycosyltransferases involved in cell wall synthesis are also being targeted for development of new anti-infectives.

Mammalian fucosyltransferases (FucT) catalyze the transfer of fucose from GDP-fucose to oligosaccharides chains conjugated to proteins or lipids. Based on their sequences and linker type, vertebrate fucosyltransferases are classified into three main groups: αL-αC4- and αL-αC6-fucosyltransferases. At least nine human fucosyltransferases have been cloned and characterized. In the cell fucosylation is involved in cell adhesion during development, inflammatory response, leukocyte trafficking, and fertilization. Alterations in fucosylation patterns are related to malignancy and chronic diseases. The human blood group antigens and Leu systems are determined by the sequential action of glycosyltransferases, most notably fucosyltransferases. Twenty distinct sialyltransferases, enzymes that transfer sialic acid from CMP-neuraminic to the glycan moiety of glycoconjugates, have been identified in both human and marine genomes. Despite low overall identities, they share four conserved peptide motifs: (L (large), S (small), motif III, and motif VS (very small)) that are hallmarks for sialyltransferase identification.

Figure 3. Sensitive and Robust Detection

Transcreener Assay standard curves mimic a typical enzyme reaction (as UDP is produced, UDP-sugar is depleted) by keeping the total nucleotide concentration constant. Standard samples (15 μL) were prepared in 50 mM HEPES pH 7.5, 5 mM MnCl2, 20 mM KCl, 80 mM EGTA, 1% DMSO, 2 nM UDP, DyLight™ 632 Tracer, and UDP Antibody. Polarization (mP) was measured with a PolarStar Plus instrument.

Figure 4. Glucosylceramide Synthase (GCS) Activity and Inhibition by PDMP

Dose-dependent inhibition of UDP production by glucosylceramide synthase with the known GCS inhibitor PDMP was demonstrated utilizing the Transcreener UDP Far Red Assay kit. 2 μL/well inhibitor was pre-incubated with 10 µL enzyme mixture (n = 2-4, 1.5 µg/mL GCS, 50 mM HEPES pH 7.5, 5 mM MnCl2, 20 mM KCl, 80 mM EGTA, 1% DMSO, 10 µM UDP, and antibody, and 2 nM UDP DyLight™ 632 Tracer). The reactions were then started with the addition of 8 µL UDP-Glucose +/- 1.5 µg/mL C-8 Ceramide or C-8 Ceramide alone. Polarization (mP) was measured with a PolarStar Plus. The data for this plot was generated after 2 hours at 37°C.

Acknowledgements

Terry Butters (Glycobiology Institute, Oxford University) kindly provided the glucosylceramide synthase.

The UDP assay development was supported by NIH SBIR grant 5R44GM095452.

GDP assay development is supported by NIH Grant R43NS051982.

Transcreener® HTS Assay Platform far red FP

Nucleotide Donor + Acceptor

Tracer

Nucleotide Product

Acceptor

Tracer

% Conversion of UDP-Donor to Substrate

20 µM UDP/GTP-D Glucose

100 µM UDP/GTP-Glucosamine AC

50 µM UDP/GDP-Galactose

An equal volume of pre-equilibrated GDP Detection Mixture (20 mM HEPES pH 7.5, 4 mM MgCl2, 2 mM EGTA, and 1% DMSO) was added to GDP standards prepared in 50 mM HEPES (pH 7.5), 4 mM MgCl2, 2 mM EGTA, and 1% DMSO. After one hour equilibration the microtiter plates were read on a Tecan Safire instrument.

Transcreener AMP/GMP Assay components were used to generate a CMP/GMP-NAN standard curve. CMP/GMP-NAN standards were prepared in 10 μL 15 mM Sodium Cacodylate, 6 mM Tris(3-aminopropane) dihydrochloride, 0.5 mM Lac Ser and 0.01% Brij-35. To these samples, 10 μL of AMP/GMP Antibody and 50 mM HEPES (pH 7.5), 4 mM MgCl2, 2 mM EGTA, and 1% DMSO were added. After one hour equilibration the microtiter plates were read on a Tecan Safire instrument.

IC50 = 22 μM

IC50 = 40 μM

IC50 = 100 μM

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