Glycosyltransferase Inhibitors

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High-Throughput Screening for Inhibitors of Sialyl- and Fucosyltransferases**

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Sialyl- and fucosyltransferases decorate glycans of glycoproteins and glycolipids with terminal sialic acid and fucose residues, creating diverse structures recognized as ligands by pathogens and mammalian glycan-binding proteins.[1] Their synthesis is carried out by the regulated expression of 20 sialyltransferase (ST) and 14 fucosyltransferase (FUT) genes that are highly conserved in mammalian species; each transferase exhibits high specificity for their donor and acceptor substrates, giving rise to terminal sequences expressed in a cell-type-specific manner. Although phenotypes of knock-out mice have validated several of these enzymes as drug targets for the treatment of autoimmune disorders (ST6Gal I and ST3Gal I) and chronic inflammation (FUT7),[1b] no small-molecule inhibitors have emerged to date.

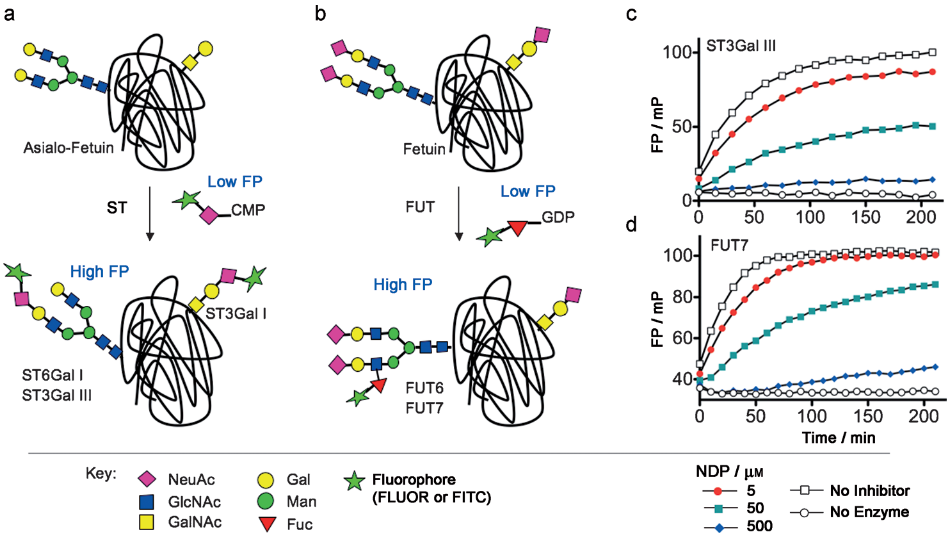

Figure 1. Fluorescence-polarization-based assays for STs and FUTs. a) ST assay based on transfer of $^{\text{FITC}}$NeuAc from CMP-$^{\text{FITC}}$NeuAc to N-linked (mediated by ST6Gal I and ST3Gal III) and O-linked (mediated by ST3Gal I) glycans of asialo-fetuin. b) FUT assay based on transfer of a fluorescent fucose analogue from GDP-$^{\text{FLUOR}}$Fuc to an N-glycan on fetuin. c) Time-course FP experiments of ST3Gal-III-mediated transfer of $^{\text{FITC}}$NeuAc to asialo-fetuin produces increased FP over time and is inhibited by the known competitive inhibitor, CDP. d) Time course of the FUT7 FP assay using GDP-$^{\text{FLUOR}}$Fuc, fetuin, and FUT7, and inhibition by the known competitive inhibitor GDP. FITC = fluorescein isothiocyanate, NeuAc = sialic acid, CMP = cytidine monophosphate, CDP, GDP = cytidine and guanosine diphosphate, FLUOR = fluorescein, NDP = CDP or GDP, Gal = galactose, GalNAc = *N*-acetlygalactosamine, GlcNAc = *N*-acetylglucosamine, Fuc = fucose, Man = mannose.

A limitation in identifying glycosyltransferase (GT) inhibitors has been the lack of simple and robust assays for high-throughput screening (HTS) of compound libraries. Although a number of approaches have been used to screen for GT inhibitors, they comprise non-homogeneous multistep formats or are designed for screening a few specific enzymes.[2] Herein we report a fluorescence-polarization (FP)-based assay system that is broadly applicable to members of the ST and FUT families. STs and FUTs are well-documented to utilize nucleotide sugar donor substrates modified with bulky substituents on the sialic acid at C-9 or on fucose at C-6.[3] Therefore we reasoned that transfer of a sugar with a fluorescent tag to a suitable glycoprotein acceptor substrate would form the basis of a homogeneous FP assay owing to the large difference in molecular size between the fluorescent substrate and the glycoprotein products (Figure 1).[4]

To test this assay strategy we synthesized fluorescein-containing analogues of the corresponding nucleotide sugar substrates CMP-NeuAc and GDP-Fucose (see Schemes 1 and 2, and the Supporting Information). The glycoprotein fetuin (ca. 50 kDa) was chosen as the acceptor substrate, because it is commercially available and contains well-characterized N-linked and O-linked glycans that are recognized as acceptor substrates by nearly all STs and FUTs.[5] As shown in Figure 1c and Figure S1 in the Supporting Information, transfer of $^{\text{FITC}}$NeuAc to asialo-fetuin by ST3Gal III, ST6Gal I, or ST3Gal I STs results in a time-dependent increase in FP, which can be inhibited in a dose-dependent manner by the competitive inhibitor, CDP. Similarly, transfer of $^{\text{FLUOR}}$Fuc by either FUT6 or FUT7 to native fetuin also results in a robust increase in FP, which can be inhibited by

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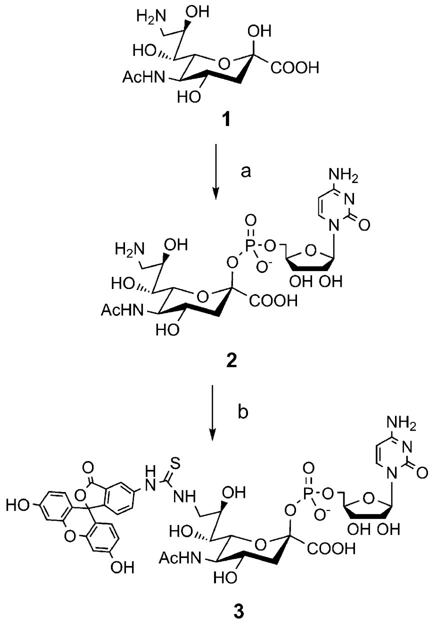
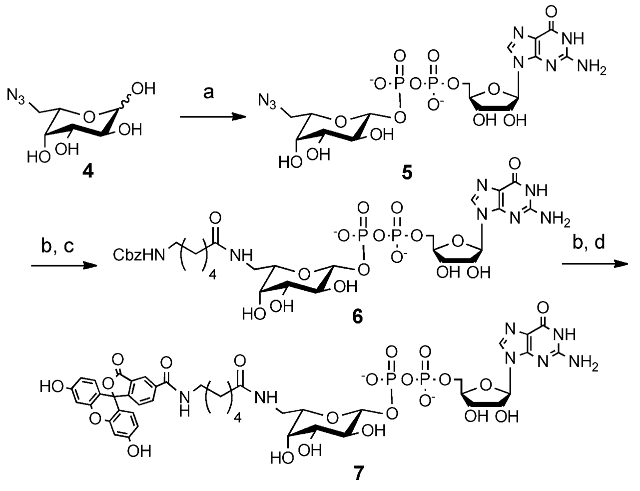
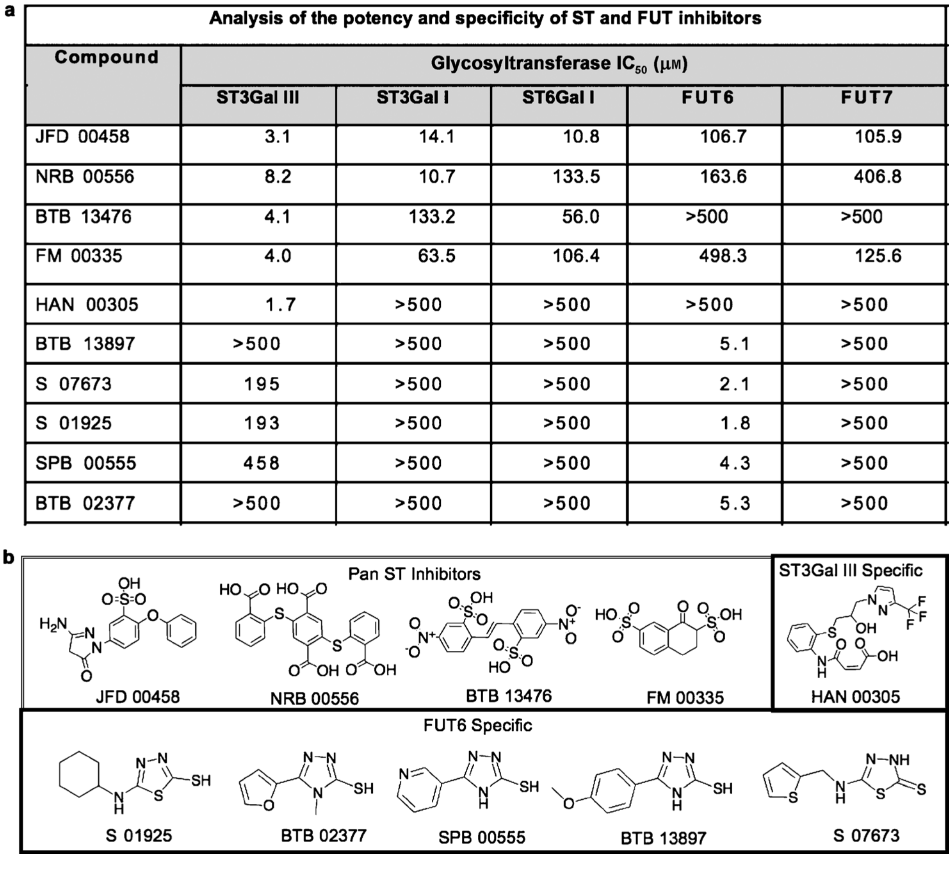


Scheme 1. Synthesis of CMP-^{FITC}NeuAc **3**. Reagents and conditions: a) CTP, CMP-NeuAc synthetase, 85%; b) FITC, MeOH, H_2O, $NaHCO_3$, 58%. CTP = cytidine triphosphate.



Scheme 2. Synthesis of GDP-^{FLOUR}Fuc **7**. Reagents and conditions: a) *B. Fragilis* FKP enzyme, ATP, GTP, pyrophosphatase, Ref. [6]; b) H_2, Pd/C 20 mm NH_4OH, 95–99%; c) N-Cbz-aminocaproic acid NHS ester, DMF, H_2O, $NaHCO_3$, 90%; d) 5-carboxyfluorescein NHS ester, DMF, H_2O, $NaHCO_3$, 81%. GTP = guanosine triphosphate, ATP = adenosine triphosphate, Cbz = benzyloxycarbonyl, NHS = *N*-hydroxysuccinimide.



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Analysis of the potency and specificity of ST and FUT inhibitors

Compound	Glycosyltransferase IC_{50} (μm)				
	ST3Gal III	ST3Gal I	ST6Gal I	FUT6	FUT7
JFD 00458	3.1	14.1	10.8	106.7	105.9
NRB 00556	8.2	10.7	133.5	163.6	406.8
BTB 13476	4.1	133.2	56.0	>500	>500
FM 00335	4.0	63.5	106.4	498.3	125.6
HAN 00305	1.7	>500	>500	>500	>500
BTB 13897	>500	>500	>500	5.1	>500
S 07673	195	>500	>500	2.1	>500
S 01925	193	>500	>500	1.8	>500
SPB 00555	458	>500	>500	4.3	>500
BTB 02377	>500	>500	>500	5.3	>500

Figure 2. Novel inhibitors of STs and FTs identified from HTS. a) Potency and selectivity of validated inhibitors was assessed by titration analysis using the FP assay. Results are the average of at least two independent experiments carried out in triplicate with standard deviations less than 10%. b) Structures of validated ST and FT hits include a related set of pan ST inhibitors, an ST3Gal-III-specific inhibitor, and a set of FUT6-specific inhibitors with a related pharmacophore.

GDP (Figure 1d and Figure S1 in the Supporting Information). A powerful aspect of this assay is that it is catalytic in nature, as evidenced by the lack of FP signal when the acceptor substrate is withheld (Figure S2 in the Supporting Information), and therefore the assay allows for the identification of both donor- and acceptor-substrate-site inhibitors (Figure S3 in the Supporting Information) and requires minimal amounts of difficult-to-express enzymes.

To assess the performance of the assay in a real HTS setting, the Maybridge Hitfinder collection of 16000 compounds was screened at 10 μm against each of the five GT targets. The results of these screens were successful in two respects. First, on a plate-to-plate basis, *Z'* was consistently above 0.8 for each enzyme. This parameter is a measure of assay robustness, with $Z' > 0.5$ being the industry standard for HTS. Second, inhibitors were identified for all targets, which demonstrates that small-molecule inhibitors for this family of enzymes can indeed be discovered (Table S1 in the Supporting Information). This finding is especially encouraging given the size of the library screened and the low substrate concentrations used for screening. It was striking, however, that the hit rates varied considerably amongst enzymes (Table S1 in the Supporting Information), which is likely due to library bias rather than assay design, because each enzyme assay was set up similarly; the substrate concentrations were well below the Michaelis constant K_M, and reactions were allowed to proceed to approximately 75% transfer of the donor substrate.

To validate hits from the screening campaign, a simple secondary assay was employed. Reactions were carried out identically as in the primary screen, but instead of reading FP, the reactions were quenched and the reaction mixtures loaded onto an SDS-PAGE gel, and in-gel fluorescence was measured (Figure S4 in the Supporting Information). This method, along with analysis of the total fluorescence of any putative hit, allows for rapid removal of false positives occurring from fluorescent compound interference or other HTS artifacts, which, we should note, were remarkably low

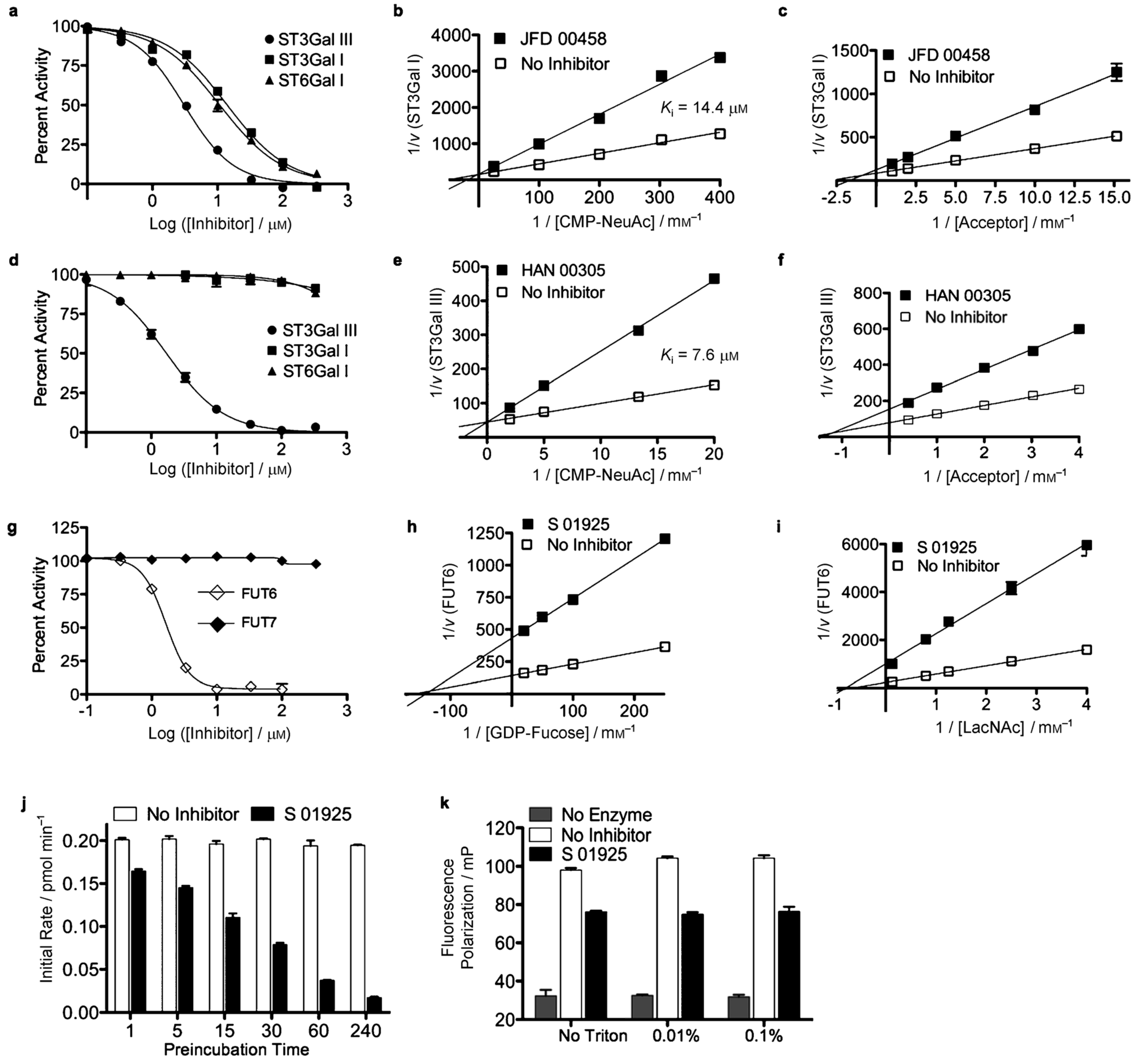

Figure 3. Mechanism of inhibition of ST and FUT inhibitors. a) Inhibition by the pan ST inhibitor JFD00458 was assessed for all three STs using the FP assay. Percent activity is relative to DMSO only (100%) and no enzyme (0%). Kinetic analysis of JFD00458 inhibition of ST3Gal I (v = reaction rate) was assessed with b) CMP-NeuAc as the varied donor substrate and c) Galβ1-3GalNAc as the varied acceptor substrate. d) The ST3Gal-III-specific inhibitor HAN00305 was assessed for potency against all three STs. To assess the mechanism of inhibition, kinetics were performed with e) CMP-NeuAc as the varied donor substrate, and f) Galβ1-4GlcNAc as the varied acceptor substrate. g) A representative inhibitor of FUT6, S01925, exhibits selective inhibition as assessed using the FP assay. Kinetic analysis of the mode of inhibition of FUT6 by S01925 shows that it is noncompetitive with respect to h) the donor substrate GDP-Fucose and i) the acceptor substrate Galβ1-4GlcNAc. j) Inhibition of FUT6 increases with time of preincubation of S01925 and k) is not reversible by detergents that disrupt nonspecific aggregators. All experiments are representative of three independent experiments. K_i = inhibition constant.

(ca. 6% of the analyzed hits; Figure S4 in the Supporting Information).

Of the confirmed hits, structural analysis and specificity profiles revealed several interesting inhibitor classes. As shown in Figure 2, a group of negatively charged pan ST inhibitors showed either weak (e.g. JFD 00458) or negligible activity against the FTs. One inhibitor (HAN 00305) exhibited a more than 250-fold higher potency for ST3Gal III than for all the other STs and FTs, and in contrast, a group of structurally related inhibitors of FUT6 showed negligible inhibition of the other GTs.

Representative inhibitors of each of these groups were selected for kinetics analysis using the natural nucleotide sugar to better understand the mechanism of inhibition and to confirm that the inhibitors were not interfering solely with the binding of the fluorescent substituents on the donor substrates used in the screen. As expected, owing to the presence of a negatively charged sulfonic acid moiety, the pan ST inhibitor JFD 00458 was competitive with the common donor substrate CMP-NeuAc (Figure 3 a–c). However, the extremely selective ST3Gal III inhibitor HAN 00305 was also found to be competitive with the donor substrate site (Figure 3 d–f), thus highlighting the fact that although similarities may exist at the CMP-NeuAc binding site of the STs, there are also significant differences that allow for selective inhibition. A group of inhibitors with a common pharmacophore were highly selective for FUT6, as exemplified by S 01925 (Figure 3 g), which showed noncompetitive inhibition with respect to both donor and acceptor substrates (Figure 3 h, i). Further studies showed that the degree of inhibition was time-dependent, suggesting that S 01925 was an irreversible inhibitor (Figure 3 j). Although promiscuous aggregators would also exhibit these characteristics, these molecules are selective, and moreover, they are completely detergent-insensitive (Figure 3 k).[6]

The successful identification of inhibitors of STs and FUTs from a pilot screening campaign highlights the ability of this simple assay to identify inhibitors of high potency and selectivity. Although the results presented herein were for a set of five exemplary STs and FUTs, based on literature precedence,[3] the assay should be applicable to most members of the mammalian ST and FUT families, as well as to certain pathogenically important bacterial enzymes such as the FUTs of *H. pylori*[7] and the Cst-II ST of *C. jejuni*.[8] We suggest that this approach may also work with other families of GTs that can transfer donor substrates with unnatural fluorescent substituents. Metabolic engineering studies utilizing GlcNAc and GalNAc analogues with unnatural N-acyl groups suggest the potential for designing suitable donor substrates for these GT families.[9]

Owing to the catalytic nature of the assay and the sensitivity of FP measurements, the above assay has minimal reagent requirements, thus making the transition to large-scale screens feasible. Ultimately it will be important to obtain inhibitors that are cell-permeable since GTs are Golgi-resident enzymes. Thus, cell-based secondary assays will be an important addition to large-scale campaigns to identify inhibitors with potential for in vivo applications.

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