A high-throughput glycosyltransferase inhibition assay to identify molecules targeting fucosylation in cancer cell-surface modification

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Abstract

In cancers, increased fucosylation (attachment of fucose sugar residues) on cell-surface glycans—resulting from the abnormal upregulation in the expression of specific fucosyltransferase enzymes (FUTs)—is one of the most important types of glycan modifications associated with malignancy. Fucosylated glycans on cell surfaces are involved in a multitude of cellular interactions and signal regulation in normal biological processes. For example, sialyl Lewis^X is a fucosylated cell-surface glycan that is abnormally abundant in some cancers where it has been implicated in facilitating metastasis, allowing circulating tumor cells to bind to the epithelial tissue within blood vessels and invade into secondary sites by taking advantage of glycan-mediated interactions.

To identify inhibitors of FUT enzymes as potential cancer therapeutics, we have developed a novel high-throughput assay that makes use of a fluorogenically labeled oligosaccharide as a probe of fucosylation. This probe, which consists of a 4-methylumbelliferyl glycoside, is recognized and hydrolyzed by specific glycoside hydrolase enzymes to release fluorescent 4-methylumbelliferone, yet when the probe is fucosylated prior to treatment with the glycoside hydrolases, hydrolysis does not occur and no fluorescent signal is produced. We have demonstrated that this assay can be used to measure the inhibition of FUT enzymes by small molecules, since blocking fucosylation will allow glycosidase-catalyzed hydrolysis of the labeled oligosaccharide to produce a fluorescent signal.

Employing this assay, we have screened a focused library of small molecules for inhibitors of a human FUT enzyme involved in the synthesis of sialyl Lewis^X, and demonstrate that our approach can be used to identify potent FUT inhibitors from compound libraries in microtitre plate-format.

Introduction

Carbohydrate structures (or glycans) that are present on cell-surfaces constitute a very important mode of intercellular communication. A great deal of information can be encoded on the oligosaccharides presented on cells by virtue of the many combinations through which different sugar units can be linked, and proper communication of this information is important within an organism for normal biological processes and healthy development, whereas miscommunication of glycan signals can have deleterious effects. This is acutely evident in cancers, which can hijack these sugar-encoded signals in order to proliferate at the expense of the host, exploiting receptors that are vulnerable to this kind of disinformation [1]. Given that cancer is characterized by aberrant cellular processes that lead to pathological proliferation of malignant tissue, it should not be surprising that abnormal glycosylation—i.e. the attachment of glycans with specific structures—on cell surfaces is a hallmark of essentially all types of cancer. Alterations in patterns of glycosylation have long been recognized clinically as biomarkers for cancer diagnostics, due to their importance in oncogenesis—yet, despite this, the glycosyltransferases involved in biochemical pathways that result in altered glycosylation in cancer have not yet been well explored as therapeutic targets.

In cancers, increased fucosylation (attachment of fucose sugar residues) on cell-surface glycans, resulting from the abnormal upregulation in the expression of specific fucosyltransferase enzymes (FUTs), is one of the most important types of glycan modification associated with malignancy [2]. Fucosylated glycans on cell surfaces are involved in a multitude of cellular interactions and signal regulation in normal biological processes. For example, sialyl Lewis^X is a fucosylated cell-surface glycan that is normally involved in cell-cell interactions including those involved the binding of human sperm to egg [3], and in the homing of leukocytes to sites in the vascular system in inflammatory and immune responses [4]. These interactions are mediated by the binding of sialyl Lewis^X to protein receptors known as selectins. In several types of cancer, including colorectal, prostate, and pancreatic cancers, fucosylation in malignant tissue resulting in increased formation of sialyl Lewis^X on cell surfaces has been implicated in facilitating

metastasis, allowing circulating tumor cells to bind to the epithelial tissue within blood vessels and invade into secondary sites by taking advantage of glycan-mediated interactions [5-7]. The role that upregulated FUT enzymes play towards the progression of cancers to metastatic stages, where the risk of death in patients is most grave, makes them very important targets for potential anticancer drugs.

The search for small molecule inhibitors of enzyme targets in drug development depends upon high-throughput screens and activity assays that are facile and sensitive, something that is lacking for glycosyltransferases in general. In the present study, we have developed a strategy to detect inhibition of specific glycosyltransferase activities and applied it towards the screening of compounds for FUT inhibitors. This involves utilizing synthetic, fluorogenically labeled oligosaccharides, and specific glycoside hydrolase (glycosidase) enzymes whose activities result in a fluorescence signal when the specific labeled glycan structures that they recognize are hydrolyzed. We explore the strategy of using this high-throughput screening approach in a microtitre-plate format in identifying drug leads that target fucosylation in cancer.

Prior work has demonstrated the use of synthetic fluorogenic oligosaccharide substrates for highly sensitive fluorescence-based assays of glycosidase activity, wherein the enzymatic hydrolysis of a terminal sugar unit at the non-reducing end of an oligosaccharide that was labeled at the reducing end with a 4-methylumbelliferyl group could be detected through the use of coupled enzymes. The coupled enzymes act specifically to digest the initial enzyme product further, one sugar unit at a time, to release monosaccharides and fluorescent 4-methylumbelliferone. For example, this method was used to assay for glycosidase activities that cleave the terminal sugar (and antigenic determinant) of oligosaccharides that make up blood group antigens [8, 9].

With few adjustments, we have adapted this strategy to assay glycosyltransferase activity rather than glycosidase activity. More specifically, we have developed a highly sensitive fluorescence-based test of glycosyltransferase inhibition. This can be achieved by incubating glycosyltransferase and glycosidase reactions sequentially. If the 4-methylumbelliferyl oligosaccharide that is recognized by the glycosidase

coupled enzymes is first incubated in a glycosyltransferase reaction, then the resulting glycosylated product will no longer be recognized in a subsequent reaction by the specific glycosidases, blocking hydrolysis that would result in a fluorescence signal. Yet if the glycosyltransferase is inhibited, glycosylation of the substrate will be prevented, allowing its hydrolysis by glycosidase coupled enzymes resulting in fluorescence detection of released 4-methylumbelliferone.

The work we describe here addresses a significant problem for identifying inhibitors of glycosyltransferases in the lack of simple, robust, and broadly applicable methods to perform high-throughput screening assays. Some generalized approaches previously described by others rely upon modified nucleotide sugars. For example fluorescently labeled nucleotide sugars can be used in fluorescence polarization-based binding assays to detect inhibitor compounds that bind competitively with the donor substrate [10, 11], or they can be used to detect activity by labeling glycans through the transfer of the modified sugar [12, 13]. The main difficulty with these methods is the necessity of the glycosyltransferase assayed to recognize a modified donor substrate. Other strategies involve detecting the released nucleoside diphosphate or nucleoside monophosphate from a glycosyltransferase reaction [14]. Assay kits based on this concept are commercially available [15, 16], and are highly sensitive to released nucleotides through luminescence detection, however a key drawback is that the signal is not linked to the transfer of a sugar unit to an acceptor molecule and it can be sensitive to donor nucleotide-sugar hydrolysis or to contaminating nucleoside mono- and diphosphates. Consequently, screening glycosyltransferase activity by detection of nucleotide release is also not suited for assays using whole cells or cell extract. In contrast, the assay method that we have developed is free of several of the drawbacks inherent in other approaches, having the advantage that it uses the natural sugar donor substrate—allowing for the assay of glycosyltransferases that may not accommodate modified nucleotide-sugars—and instead of detecting nucleotide release, it measures glycosyltransferase activity by the resulting glycosylation of a specific probe. These features may also prove useful for cell-based screens,

allowing for the assay of cell lysate in high-throughput to identify drug leads that may target glycosyltransferase activity at the gene expression level.

Here, we describe the application of our approach to detect the activity and inhibition of human fucosyltransferase VI (FUT6), misregulation of which is implicated in cancer and inflammatory diseases [17]. Establishing this as a high-throughput *in vitro* screening method, we tested a focused compound library for inhibition of FUT6, carrying out assays in 384-well microtitre plate format, and demonstrated its utility in the identification of inhibitory molecules and accurate determination of their IC_{50} values.

Results and Discussion

Validation of a glycosidase-dependent fucosyltransferase activity assay

The high-throughput assay of glycosyltransferase activity and inhibition that we present is dependent upon glycosidase activities that can recognize and hydrolyze a specific oligosaccharide core structure and distinguish it from a further enzyme-modified (e.g. fucosylated) structure which will not be hydrolyzed. In initial work, we validated this strategy using the human fucosyltransferase VI (FUT6) enzyme which catalyzes the transfer of the fucosyl moiety from the nucleotide sugar substrate, guanosine 5′-diphospho-β-L-fucose (GDP-fucose), to *N*-acetyllactosamine (LacNAc) or 3′-sialyl-*N*-acetyllactosamine (3′-SLN) to form the $Lewis^X$ trisaccharide or sialyl $Lewis^X$ tetrasaccharide respectively (**Fig 1a**). We sought to develop a fluorogenically labeled acceptor substrate as a probe for FUT6-catalyzed fucosylation. For simplicity, we chose an oligosaccharide substrate consisting of the 4-methylumbelliferyl β-glycoside of the LacNAc disaccharide (MU-LacNAc), which we synthesized chemoenzymatically (**Supplementary Fig. S1**), yet the same strategy could be employed using a fluorogenically labeled β-glycoside of the 3′-SLN trisaccharide. Upon treatment with specific glycosidase enzymes—*Streptococcus pneumoniae* β-galactosidase, BgaA [18], and *Streptomyces plicatus* β-*N*-acetylhexosaminidase, SpHex [19, 20]—hydrolysis of MU-LacNAc releases fluorescent 4-methylumbelliferone. We anticipated that FUT6-catalyzed fucosylation of MU-LacNAc would result in the formation of the 4-methylumbelliferyl

β-glycoside of Lewis^X (MU-Le^X) and that this product would be resistant to hydrolysis catalyzed by BgaA and SpHex (**Fig 1b**). It follows then that conversion to MU-Le^X would diminish the amount of MU-LacNAc available for subsequent glycosidase-catalyzed reactions and consequently result in less fluorescent 4-methylumbelliferone being released by hydrolysis (unless the MU-Le^X-producing fucosylation reaction is otherwise inhibited).

To test whether MU-LacNAc is indeed fucosylated by FUT6, we performed assays in buffered solution (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) with varying amounts of FUT6 using MU-LacNAc (10 μM) as the acceptor substrate in the transfer of a fucosyl residue from donor GDP-fucose (100 μM). Reactions were carried out for one hour at 37 °C and stopped with the addition of a solution containing EDTA (100 mM) to inactivate the metal ion-dependent FUT6 [21], then analyzed by HPLC-MS (**Fig 2a**). We observed that MU-LacNAc was consumed in FUT6-catalyzed reactions to produce MU-Le^X as identified by MS (m/z expected $[M-H]^-$: 686.2302; m/z observed $[M-H]^-$: 686.2298) and MS/MS (**Supplimentary Fig S2**). We further verified that MU-LacNAc is specifically hydrolyzed by glycosidases BgaA and SpHex to release 4-methylumbelliferone whereas MU-Le^X is not. This was done by performing similar MU-LacNAc fucosylation reaction assays in which glycosidase enzymes BgaA and SpHex were included in the stop solution containing EDTA to simultaneously stop the FUT6 reaction and hydrolyze any remaining MU-LacNAc. HPLC-MS analysis confirmed that MU-LacNAc was hydrolyzed in these reactions, releasing 4-methylumbelliferone, while MU-Le^X did not act as a substrate for the glycosidases (**Fig 2b**). Quantitation against authentic standards showed that the amount of remaining MU-LacNAc measured following fucosylation was consistent with the amount of released 4-methylumbelliferone measured after the subsequent incubation with BgaA and SpHex (**Supplementary Table S1**).

Given these results we were confident that, following glycosidase treatment, measurement of the 4-methylumbelliferone fluorescence signal could be used to determine the amount of MU-LacNAc that remained unconverted by FUT6 in a fucosylation assay. The amount of MU-Le^X produced could therefore be inferred by lowered relative intensity of fluorescence in these end-point measurements. Indeed, we

found that the fluorescence intensity from FUT6 assays—involving sequential fucosyltransferase and glycosidase treatment—is inversely correlated to the amount of FUT6 present in the assay, consistent with our HPLC-MS results (**Fig 2b**, **Supplementary Table S1**). No fluorescence detectable above background was observed in assays where BgaA and SpHex were omitted.

Optimizing the fucosyltransferase assay for high-throughput screening

Having carried out our assay with different amounts of FUT6, we sought to continue testing a variety of conditions for further optimization. Optimal conditions for the fucosyltransferase assay should produce a measurable difference in fluorescence signal in response to FUT6 inhibition while remaining within the dynamic range.

To optimize the concentration of fucosyl donor substrate and the incubation time for the fucosyltransferase reaction, we performed our assay with fixed acceptor MU-LacNAc concentration (10 µM) and fixed FUT6 concentration (15 µg/mL, ~40 µU/mL) while varying the concentration of GDP-fucose between 10 and 200 µM. Assays were stopped at different time points, after incubation at 37 °C, with the addition of a solution containing EDTA and the glycosidases, BgaA and SpHex. Fluorescence was measured for each time point and varying GDP-fucose concentrations (**Fig. 3**). We found that by using only one equivalent of GDP-fucose at 10 µM, incomplete conversion of MU-LacNAc to MU-Le^X occurred over the course of a four-hour incubation, with the reaction proceeding to less than 70% during that time. Assays containing 40 µM or more of GDP-fucose went to at least 98% completion over four hours. At the one-hour time point, FUT6 assays using 10, 40, 100, and 200 µM of GDP-fucose had gone to roughly 50%, 70%, 80%, and 90% completion, respectively. Given those results, to perform assays optimized in substrate concentrations and incubation time, we continued subsequent tests using 100 µM GDP-fucose in FUT6 assays incubated for one hour at 37 °C prior to the addition of stop solution containing BgaA and SpHex.

We also tested the effect of varying the concentration of the glycosidases, BgaA and SpHex, in the reaction to release 4-methylumbelliferone from MU-LacNAc. To buffered solutions containing 10 μM of MU-LacNac, equal volumes of stop solution containing varying concentrations of BgaA and SpHex were added and the mixtures were then incubated at 37 °C for ten minutes. We observed that using BgaA and SpHex each at a concentration of 0.1 mg/mL in the stop solution was sufficient to produce the maximum fluorescence signal after ten-minute incubation (**Fig. 4**). We continued subsequent tests using the glycosyidases at these concentrations for ten-minute incubations at 37 °C. However, since these end-point measurements of glycosidase reactions are not time-sensitive—given that they occur after the fucosyltransferase reaction has been stopped (with the addition of EDTA)—it is possible to use less BgaA and SpHex with longer incubation times to achieve quantitative hydrolysis of MU-LacNAc. Since our assay is intended to measure FUT6 inhibition using the fluorescence signal of 4-methylumbelliferone released from the hydrolysis of unmodified MU-LacNAc, FUT6 inhibitors that also inhibit the hydrolytic glycosidases may result in false negatives. However, it is possible to control for this contingency by performing assays omitting FUT6.

To evaluate our assay for high-throughput screening, we performed multiple replicate experiments in the presence or absence of FUT6 (15 μg/mL, ~40 μU/mL) in order to calculate Z′ (or Z-factor) (**Fig 5**), which is a statistical measure of the robustness of a high-throughput assay [22] (where a an ideal value is 1). Current practice suggests that for a screen to be considered viable, the Z′ value must be greater than 0.5. For our FUT6 assay, we determined a Z′ value of 0.8, indicating a highly robust assay suitable for high-throughput screening.

Synthesis and high-throughput assay of a focused library of derivatized FUT6 inhibitors

Next, we focused efforts on evaluating our assay system in detecting the inhibition of FUT6. Since blocking fucosylation would allow the glycosidase-catalyzed hydrolysis of more MU-LacNAc to occur (**Fig 1b**), inhibition of FUT6 should correspond to an increase in fluorescence signal compared to an assay

performed in the absence of an inhibitor. Using a small molecule, compound **1** (**Fig. 6**), which had previously been identified by Rillahan *et al.* as an inhibitor of FUT6 [12], we indeed observed that inhibition of FUT6 in our assay system resulted in increasing fluorescence signal with increasing inhibitor concentration (keeping the concentration of FUT6 and other components fixed) (**Fig. 7**). HPLC-MS analysis was also performed for data points and found to be consistent with the fluorescence readings (**Supplementary Fig. S3**). From the fluorescence measurements, we determined an IC_{50} of 130 μM for compound **1** towards FUT6 under our assay conditions. We attribute the difference in the IC_{50} that we observed from that reported for the same compound by Rillahan *et al.* [12] (5.3 μM) to differences in the assay system. Whereas in previous reports, FUT6 was assayed using a fluorescently labeled derivative of the GDP-fucose donor and a glycoprotein acceptor substrate, our setup uses the natural GDP-fucose and MU-LacNAc as donor and acceptor substrates, respectively.

We also synthesized a series of derivatives of **1** (compounds **2** – **13**), generating a focused library of small molecules to screen as inhibitors. These were prepared with a general two-step synthetic strategy wherein an aryl hydrazide was coupled to an alkyl-substituted isothiocyanate followed by cyclization of a 1,2,4 triazole ring in basic aqueous solution (**Fig 6**). We screened the resulting collection of substituted triazoles for FUT6 inhibition in our high-throughput assays in varied concentrations to determine the IC_{50} values for each (**Fig 7a** and **b** and **Table 1**) and we were pleased to find that several of these showed improved inhibition compared to the parent compound **1**. The molecules that show inhibitory activity have in common a 1,2,4-triazole-3-thione functional group, which can tautomerize to the 1,2,4-triazole-3-thiol in solution (**Supplementary Fig. S4**). Inclusion of compounds **12** and **13** in our assays demonstrated the importance of the thione/thiol in this panel of molecules towards enzyme inhibition. Compound **13**, which was synthesized from **12** and differs from it by the replacement of the thione/thiol with a methylthio group (**Fig 6**)**,** shows no detectable inhibition of FUT6 when tested up to 1.6 mM, whereas **12** has an IC_{50} in the range of the compounds in the series (230 μM) (**Fig 7b** and **Table 1**). Rillahan *et al.* observed time-dependent, non-competitive, irreversible inhibition of FUT6 by compounds that include this thione- or

thiol-bearing pharmacophore [12], which—along with our results showing the requirement of the group for inhibition—is consistent with it potentially acting as a reactive functional group that can covalently modify the enzyme.

Conclusions

Our work has established a platform for high-throughput screening of glycosyltransferase activity and inhibition using a glycosidase coupled enzyme approach with fluorogenically labeled oligosaccharides that is more commonly employed towards detecting enzymatic hydrolysis of specific carbohydrate structures. The methodology that we have demonstrated with our assay of FUT6-catalyzed fucosylation—and inhibition thererof—using a simple fluorogenic glycoside substrate, can be easily adapted to target other glycosyltransferase enzymes, making it a versatile tool in their study.

Altered glycosylation patterns are not only markers of many pathologies including cancer, but they often play direct roles in disease progression by influencing cellular processes and cell-cell interactions. The glycosyltransferase enzymes involved are therefore important, yet underexplored targets for therapeutics. A major hurdle towards developing glycosyltransferases as druggable targets is the challenge of finding simple and sensitive biochemical assays to probe their activity. We envisage that the strategy we have described here for screening inhibitors of FUT6 can be used for a variety of cell surface-modifying glycosyltransferases, by using fluorogenically labeled oligosaccharides with distinct structures that are recognized as acceptor substrates for glycosylation, along with glycosidase enzymes that hydrolyze them.

Due to the high sensitivity of the fluorescence-based assay, we have been able to perform measurements with less-than-nanomole quantities of substrate and enzyme. This is of crucial importance when screening vast collections of small molecules in compound libraries, as often tens to hundreds of thousands of molecules will be tested to identify only a handful of promising leads. Anticipating that a fraction of these will be cell-permeable and active as drugs—inhibiting their target enzymes within the

Golgi where glycan assembly occurs—increasing screening-capacity with highly sensitive assays is imperative.

With the chemical biology tools and methodologies that we have developed towards this screening assay, the groundwork has been laid for future large-scale efforts towards identifying inhibitors of glycosyltransferases, like FUT6, involved in cancer and other diseases using a combination of library screening and compound diversification through combinatorial chemistry leading towards effective drug design.

Methods

Organic Synthesis of 1,2,4-triazoles

Representative procedure for the synthesis of 1-Aroylthiosemicarbazide: N-ethyl-2-(furan-2-carbonyl)hydrazinecarbothioamide. The procedure was adapted from methods described by Kane *et al*. for the synthesis of 1,2,4-triazoles [23]. In a 25 mL round-bottom flask, furan-2-carbohydrazide (139 mg, 1.1 mmol) was added to a stirred solution of isothiocyanatoethane (87 mg, 1 mmol) in anhydrous THF (6.5 mL). A condenser was connected to the round-bottom flask and the mixture was kept at 80 °C for 18 hours. After the reaction was complete, solvent was removed under vacuum and the solid residue was recrystallized in a MeOH-EtOH mixture to yield the pure product (142 mg, 67%).

*Representative procedure for the synthesis of 5-Aryl-2,4-dihydro-3H-1,2,4-triazole-3-thiones: 4-methyl-5-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (compound **6**).* The method followed that of Kane *et al.* [23]. **To** a 25 mL round bottom flask, *N*-methyl-2-phenylhydrazinecarbothioamide (193 mg, 0.92 mmol) and 10.14 mL of 1M $NaHCO_3$ (aq) were added and heated at 85 °C for 24 hours. The mixture was quenched with 12N HCl (744 μL) to pH 2, then extracted with EtOAc (3x15 mL). The organic portions were combined and dried over Na_2SO_4. Solvent was removed under vacuum and the solid was recrystallized with 99% EtOH to obtain product as a white solid (86 mg, 49%).

*5-(4-methoxyphenyl)-4-methyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (compound **12**).* The procedure was adapted from methods described by Samanta *et al*. for the synthesis of 1,2,4-triazoles [24]. A solution of 2-(4-methoxybenzoyl)-*N*-methylhydrazine-1-carbothioamide (160 mg, 1.0 mmol) in 2.5 mL of 2M NaOH (aq) were placed in a microwave vial equipped with a stir bar. The reaction vial was placed in the microwave reactor for 3 min at 150 °C (900 rpm, 30 s pre-stir, high absorption level). After the reaction was done, the mixture was quenched with 12N HCl (416 μL) and extracted with EtOAc (3x15 mL). The organic portions were combined and dried over Na_2SO_4. Solvent was removed under vacuum and the solid was recrystallized with 99% EtOH to obtain a white solid (140 mg, 63%).

*3-(4-methoxyphenyl)-4-methyl-5-(methylthio)-4H-1,2,4-triazole (compound **13**).* The procedure was adapted from Kane *et al.* [23]. In a 10 mL round-bottom flask, **12** (100 mg, 0.45 mmol) were dissolved in 1 mL of H_2O. NaOH (40 mg, 1 mmol) were added to the reaction vessel and the mixture was kept stirring for 5 min. Then a solution of MeI (50 μL, 0.73 mmol) in 99% EtOH (230 uL) was added slowly and the reaction was incubated overnight at room temperature. After the reaction was complete, the mixture was diluted with water, and the formed precipitate was filtered and then recrystallized from EtOAc to obtain a white solid (73 mg, 69%).

The identities of the synthesized compounds were confirmed by NMR spectrometric analysis (supporting information).

Enzymatic synthesis of MU-LacNAc

MU-LacNAc was synthesized from 4-methylumbelliferyl-β-*N*-acetylglucosamine (Carbosynth, EM03177) with the addition of a galactose residue from UDP-galactose (Carbosynth, MU06699), catalyzed by the galactosyltransferase from *H. pylori*, HpGalT (HP0826) [25]. Briefly, in a 2 mL solution buffered with 100 mM Tris-HCl (pH 8) and 10 mM $MgCl_2$, to which 4-methylumbelliferyl-β-*N*-acetylglucosamine (3.8 mg, 10 μmol) was added from a 500 mM solution in DMSO, and UDP-galactose (10 mg, 17.7 μmol) was added from dry powder, HpGalT (HP0826) was added from a semi-pure protein

extract to a total of 0.3 mg/mL of protein. The mixture was incubated for about 13 hours at 22 °C. The protein was then removed using a Vivaspin 6 MWCO 10000 centrifugal filter (GE, 28932296) and the mixture was applied to a HyperSep C18 cartridge (ThermoFisher, 60108-301), from which pure MU-LacNAc was eluted using a stepwise gradient of 0 to 50% methanol. Fractions containing the desired product were pooled and the solvent removed *in vacuo*, yielding MU-LacNAc (5.2 mg, 91%).

Enzymatic synthesis of GDP-fucose

GDP-fucose was prepared enzymatically following a previously described procedure [26]. Briefly, the reaction was performed in a 10 mL reaction buffer containing Tris-HCl (100 mM, pH 7.5), $MgCl_2$ (20 mM), fucose (100 mg, 0.6 mmol), adenosine triphosphate (0.72 mmol), guanosine triphosphate (0.72 mmol), bifunctional fucokinase/GDP-fucose pyrophosphorylase, FKP (2 mg/mL) [27, 28], and inorganic pyrophosphatase (34 μg/mL), was incubated at 37 °C with agitation at 200 rpm for 4 hours. The formation of GDP-fucose was monitored by TLC (*n*-butanol/water/acetic acid = 2:1:1 (v/v/v), R_f = 0.21). The reaction was quenched by addition of the same volume of ethanol, followed by incubation at 4 °C for 30 minutes. Insoluble precipitates and the denatured proteins were removed by centrifugation at 4 °C, 10,000 x *g* for 10 minutes. The supernatant was concentrated under vacuum, redissolved in 10 mL deionized water, and chromatographed on a 60 mL Q FF anion-exchange column (GE Life Sciences, 17051001) at 4 mL/min with a gradient of 0% B for 15 minutes; 25% B for 15 minutes; 25-100% B for 45 minutes; and 100% B for 15 minutes, where buffer A is deionized water and buffer B is NH_4HCO_3 solution (500 mM). Product-containing fractions were identified by TLC analysis and were pooled together, lyophilized, and applied to a TOYOPEARL® HW-40F gel chromatography column (1.5 cm x 75 cm) with distilled water (10 mL/h) to obtain purified GDP-fucose.

Recombinant proteins

We obtained recombinantly expressed human FUT6—purified from recombinant *Spodoptera frugiperda* cells—from Dr. Donald L. Jarvis (University of Wyoming). BgaA, SpHex, HpGalT (HP0826), and FKP were expressed and purified following procedures similar to those previously reported [18, 20, 25, 28].

N-terminally His_6-tagged BgaA was expressed in *E. coli.* BL21(DE3) that was transformed with the plasmid pET28-BgaA, and cultured in LB media. The cells were grown to $OD_{600} \approx 0.6$ at 37 °C at which point 0.5 mM isopropyl β-D-1-thiogalactorpyranoside (IPTG) was added, followed by overnight incubation at 16 °C. Cells were harvested by centrifugation and lysed by sonication. The cell extract was purified using a 1 mL HisPur Ni-NTA column (ThermoFisher, 88225), and the protein was stored in buffer (20 mM Tris, pH 7.5, 100 mM NaCl).

N-terminally His_7-tagged SpHex was overexpressed in *E. coli.* BL21(DE3) that had been transformed with the plasmid pET3-SpHex. An expression culture was grown in LB at 37 °C prior to induction with 0.4 mM IPTG at a cell density of $OD_{600} \approx 0.5$. Following 3-hour incubation at 25 °C, cells were harvested and lysed and SpHex was affinity purified using a 1 mL HisPur Ni-NTA column. Purified SpHex was stored at 4 °C in buffer (20 mM Tris, pH 6.0, 300 mM NaCl, 10 mM β-mercaptoethanol).

HpGalT (HP0826) was expressed in *E. coli* AD202 transformed with pHP21 encoding the galactosyltransferase. Expression cultures were grown in 2X YT medium at 37 °C until a cell density of $OD_{600} \approx 0.4$ was reached, whereupon cells were induced with 0.5 mM IPTG and further cultured at 25 °C for an additional 24 hours. Cells were then harvested and lysed by sonication and the cell extract was applied to a 1 mL column of Macro-Prep High Q resin (Bio-Rad, 1580040) in 100 mM HEPES buffer, pH 8. The semi-pure protein was collected in the flow-through.

FKP was expressed from *E. coli* BL21(DE3) transformed with the plasmid pET28a-FKP [28], cultured in 500 mL of LB containing ampicillin (100 μg/mL) at 37 °C until OD_{600} value reached 0.6-0.8. Protein expression was then induced by addition of 0.5 mM IPTG followed by incubation at 25 ˚C for 16 to 20 hours. The cell pellet was resuspended in buffer containing 25 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.1% Triton X-100, and then cells were lysed by sonication. Protein purification was performed by nickel-affinity chromatography—the supernatant of the cell lysate was chromatographed on a 5-mL HisTrap chelating HP column (GE Life Sciences, 17040901) with a linear gradient from 0 to 500 mM imidazole. Fractions with significant amounts of His_6-tagged protein were pooled and concentrated by centrifugation with a centrifugal filter device (Amicon Ultra, Merck Millipore), and stored at -20 °C.

HPLC-MS analysis

Analysis by HPLC-MS was performed using an Agilent 1290 Infinity II UHPLC in conjunction with an Agilent 6560 Ion Mobility Q-TOF mass spectrometer. Samples obtained from enzymatic assays were diluted 1:1 with distilled water and 10 μL of these dilutions were injected for chromatography using a Phenomenex 150 × 2.00 mm Synergi 4 μM Hydro-RP column. Samples were eluted from the column with the following gradient (buffer A = water + 0.1% formic acid; buffer B = acetonitrile + 0.1% formic acid): 1-3% buffer B at a flow rate of 0.3 mL/min over 3.0 minutes; 3-80% buffer B at a flow rate of 0.3 mL/min over 4.0 minutes; 80% buffer B at a flow rate of 0.4 mL/min for 1.5 minutes; 1% buffer B at a flow rate of 0.4 mL/min for 1.0 minute; then 1% buffer B for 2.5 minutes at a flow rate of 0.3 mL/min. Detection of analytes by mass spectrometry was carried out by QTOF in negative mode with a source voltage of 2000 V and a scan range of m/z values between 75 and 1750.

High-throughput FUT6 inhibition assay

Volumes of 2.5 – 320 nL from inhibitor stock solutions (100 mM in DMSO) were transferred to wells of a black 384-well assay plate (Nunc 262260) and diluted with DMSO to a final volume of 400 nL per well using an Echo 550 (Labcyte Inc., San Jose, USA) acoustic liquid handler. A 10 µL solution of FUT6 enzyme (30 µg/mL, ~80 µU/mL) in 2× assay buffer (100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$) was added to each well of the assay plate using a Biomek FXP liquid handling robot (Beckman Coulter, Indianapolis, USA), and the plate was incubated at 37 °C for 30 minutes. To each well, 10 µL of substrate solution (0.02 mM MU-LacNAc and 0.2 mM GDP-Fuc) was added, and the reaction mixtures were further incubated at 37 °C for 1 hour. To each well, 20 µL of stop buffer (50 mM Tris-HCl, pH 7.5, 100 mM EDTA, 0.1 mg/mL BgaA, and 0.1 mg/mL SpHex) was added to quench the fucosyltransferase reaction, and the fluorescence of 4-methylumbelliferone was developed at 37 °C for 10 minutes. The signal was measured with excitation at 372 nm, reading emission at 445 nm using a CLARIOstar® monochromator microplate reader (BMG Labtech, Germany), and the data were recorded using MARS data analysis software 3.20 R2. The fluorescence intensity was normalized against a control reaction that had no FUT6 enzyme present. The resulting relative activity was fit into equation IC50 - 4 Parameter Logistic using GraFit 7.0.3 (Erithacus Software Limited). The experiments were performed in triplicates for each inhibitor concentration.

Associated content

Supporting Information

The Supporting Information is available free of charge via the internet at http://pubs.acs.org. Included in the SI are tables and figures detailing MS and fluorescence data, figures illustrating chemical reactions, and NMR data.

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Table 1. IC_{50} values obtained from assay of compounds for inhibition of FUT6, determined in triplicates. NI: not inhibited up to 1.6 mM.

Compound	IC_{50} (μM)
1	130 ± 40
2	90 ± 20
3	60 ± 20
4	330 ± 40
5	330 ± 50
6	250 ± 60
7	210 ± 40
8	110 ± 10
9	58 ± 7
10	110 ± 10
11	200 ± 10
12	230 ± 40
13	**NI***

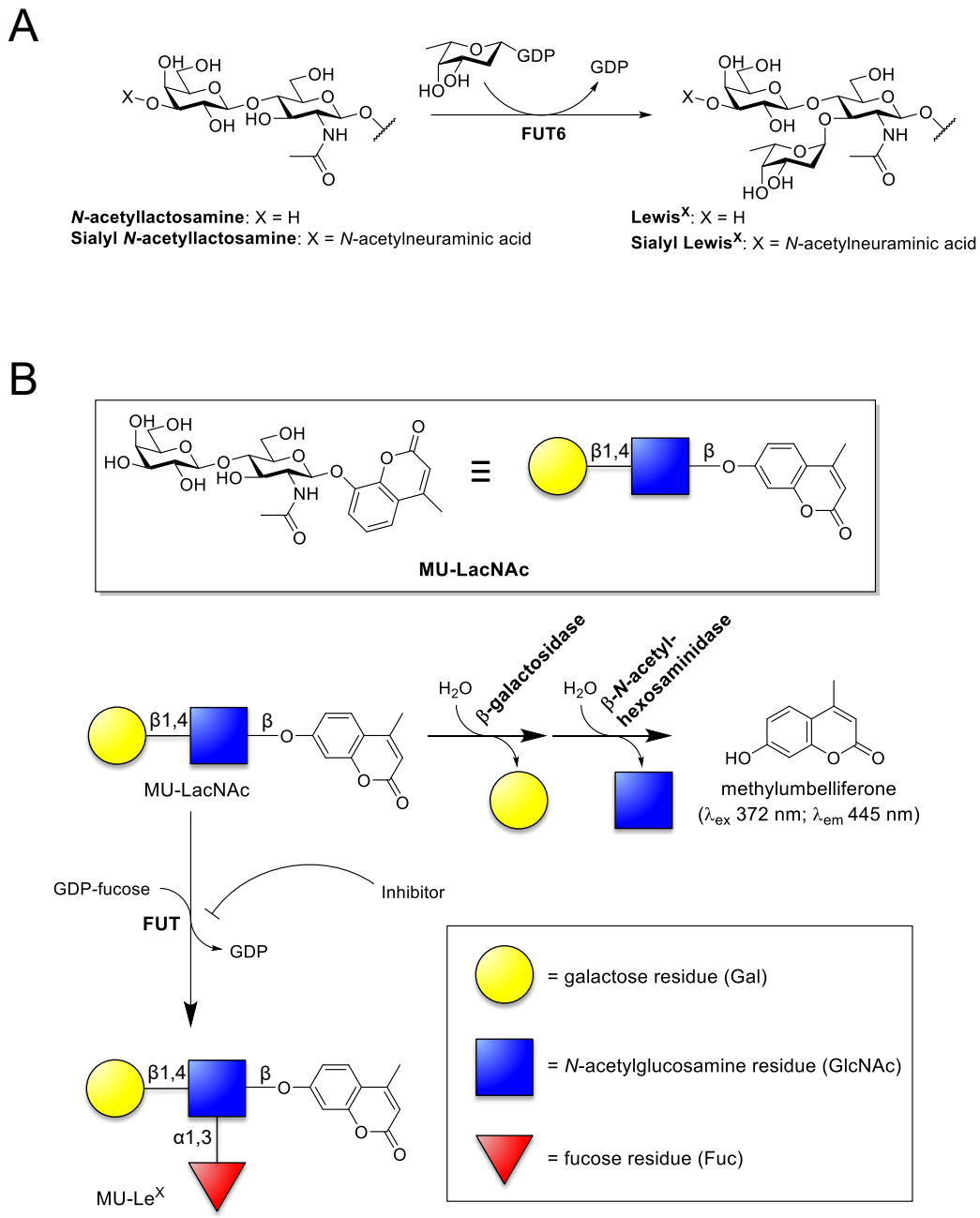

Figure 1. (A) FUT6-catalyzed addition of a fucose residue to *N*-acetyllactosamine (LacNAc) or 3′-sialyl *N*-acetyllactosamine (3′-SLN). (B) A fluorescence-based assay to test for fucosyltransferase (FUT) activity or inhibition by the sequential incubation of FUT enzyme with MU-LacNAc and GDP-fucose in the presence or absence of an inhibitor, followed by glycoside hydrolase enzymes for the specific hydrolysis of MU-LacNAc.

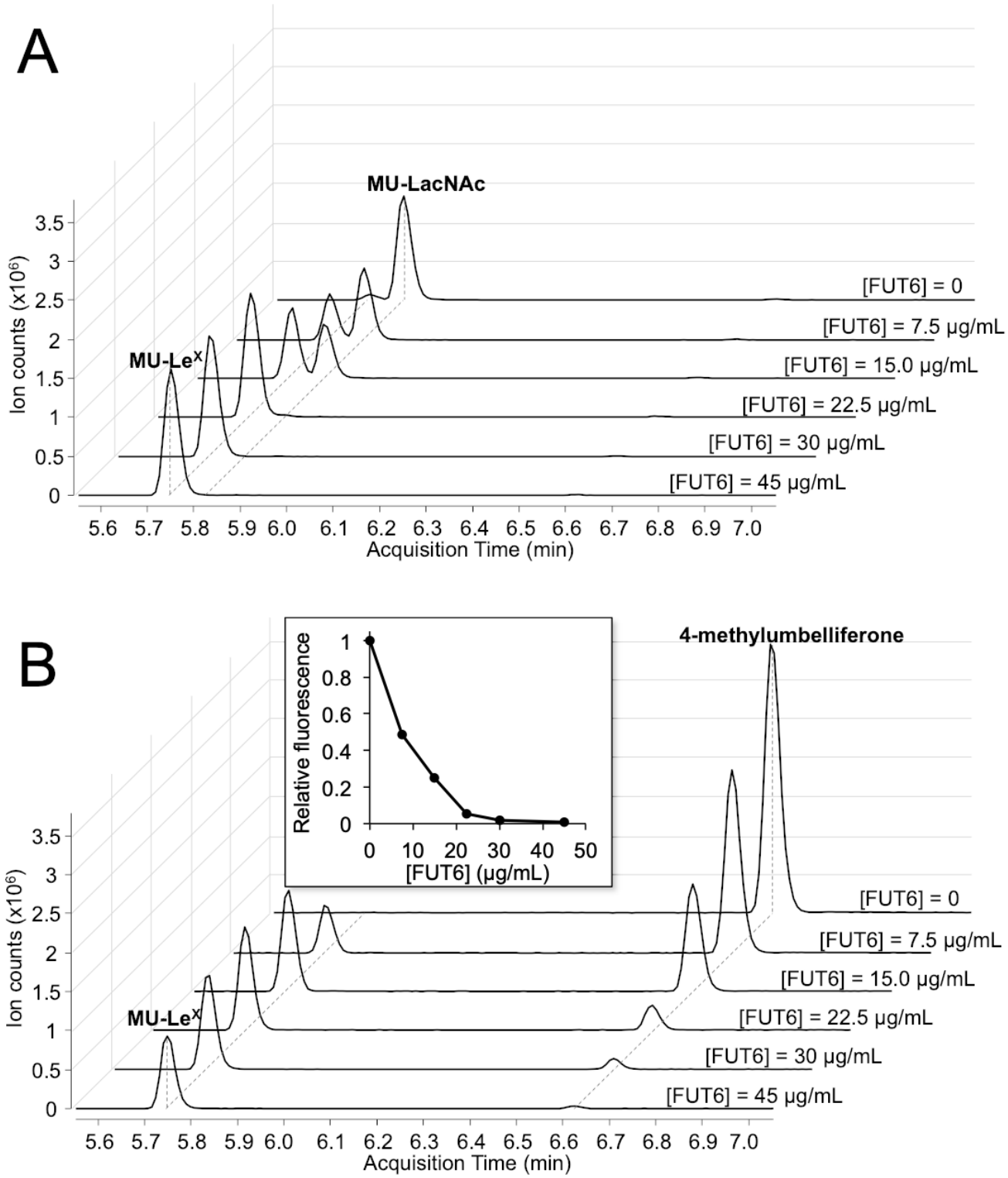

Figure 2. FUT6-catalyzed fucosylation of MU-LacNAc. HPLC-MS chromatograms showing extracted ion counts (EIC) scanning for m/z values for MU-LacNAc ($[M-H]^-$: 540.1722, $[M+formate]^-$: 586.1770), MU-Le^X ($[M-H]^-$: 686.2302; $[M+formate]^-$: 732.2357) and 4-methylumbelliferone ($[M-H]^-$: 175.0400). **(a)** HPLC-MS analysis of one-hour fucosyltransferase reactions (37 °C) using MU-LacNAc (10 µM) as an acceptor and GDP-fucose (100 µM) as a donor with varying concentrations of FUT6. **(b)** HPLC-MS analysis of reactions following treatment with BgaA and SpHex glycosidases. Inset: relative fluorescence measurements of the same.

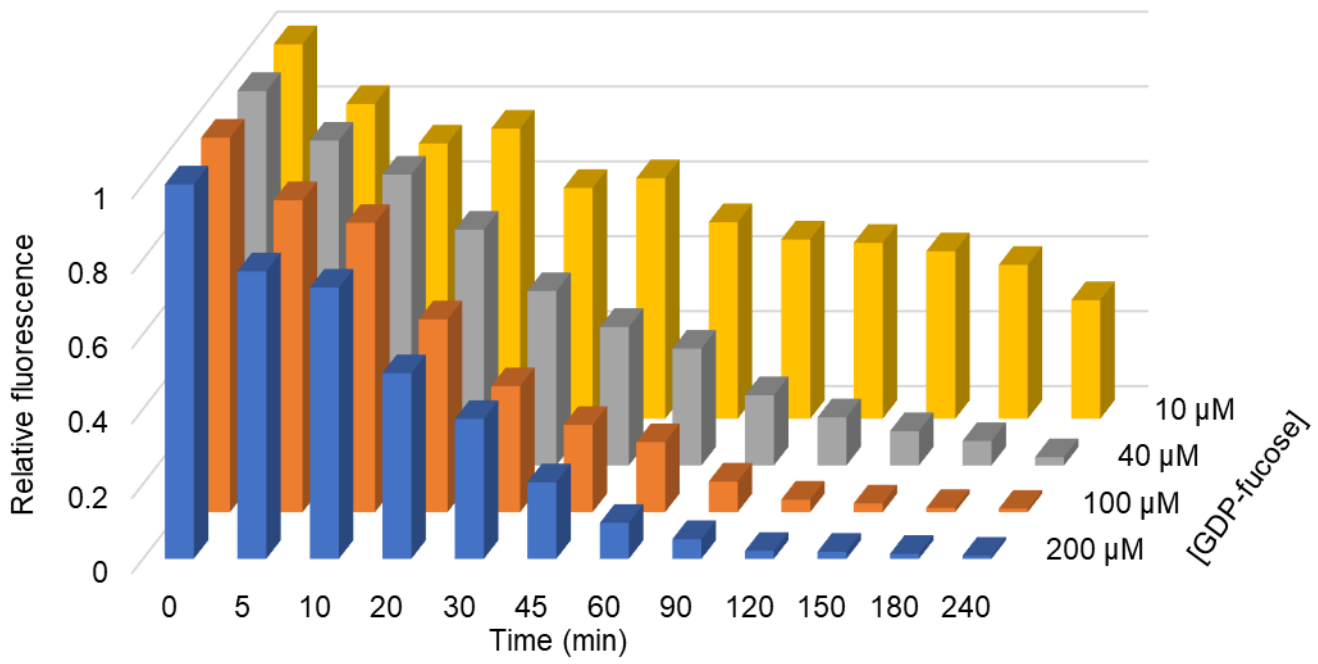

Figure 3. Optimization of donor substrate concentration and FUT6 incubation time. FUT6-catalyzed fucosylation reactions were incubated at 37 °C with different concentrations of GDP-fucose (10, 40, 100, and 200 µM) for different time periods up to four hours. Stop solution was added and after hydrolysis was complete, fluorescence was measured.

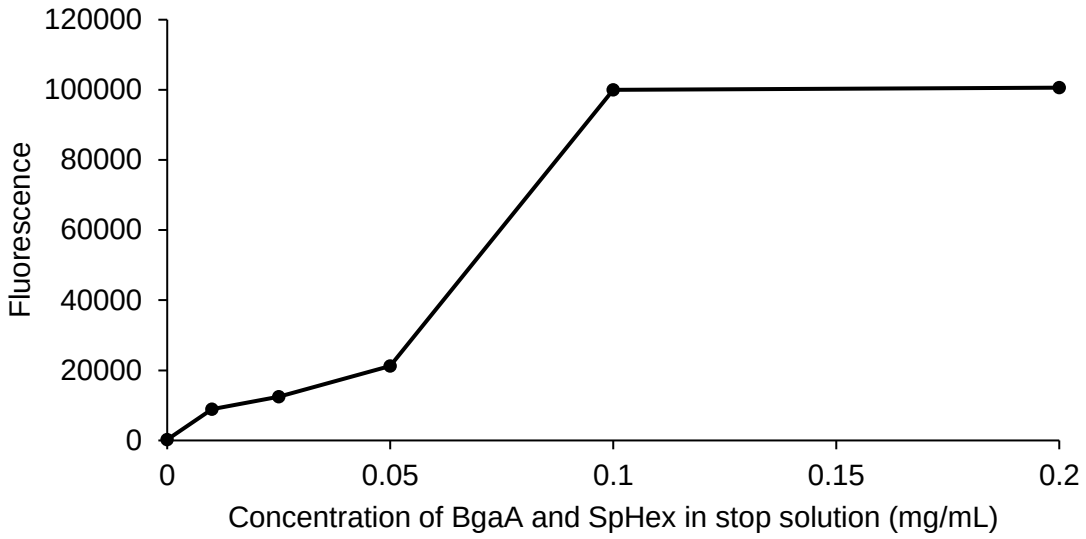

Figure 4. Optimization of BgaA and SpHex concentrations in stop solution. After addition of an equal volume of stop solution containing different amounts of the glycosidases to a buffered solution containing 10 µM MU-LacNAc, mixtures were incubated at 37 °C for ten minutes before fluorescence measurements.

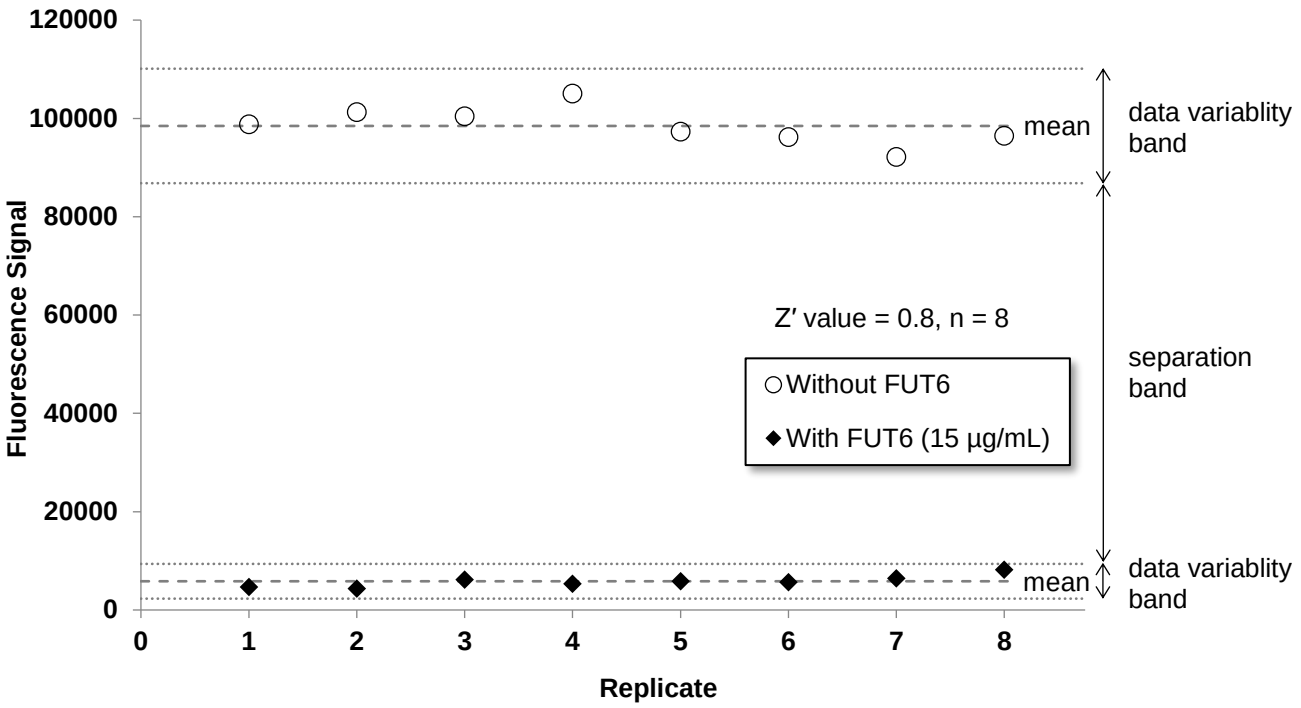

Figure 5. Replicates of the assay performed with FUT6 (15 µg/mL, ~40 µU/mL) and without, plotted to show range of data variability and separation between positive and negative controls used to calculate Z′.

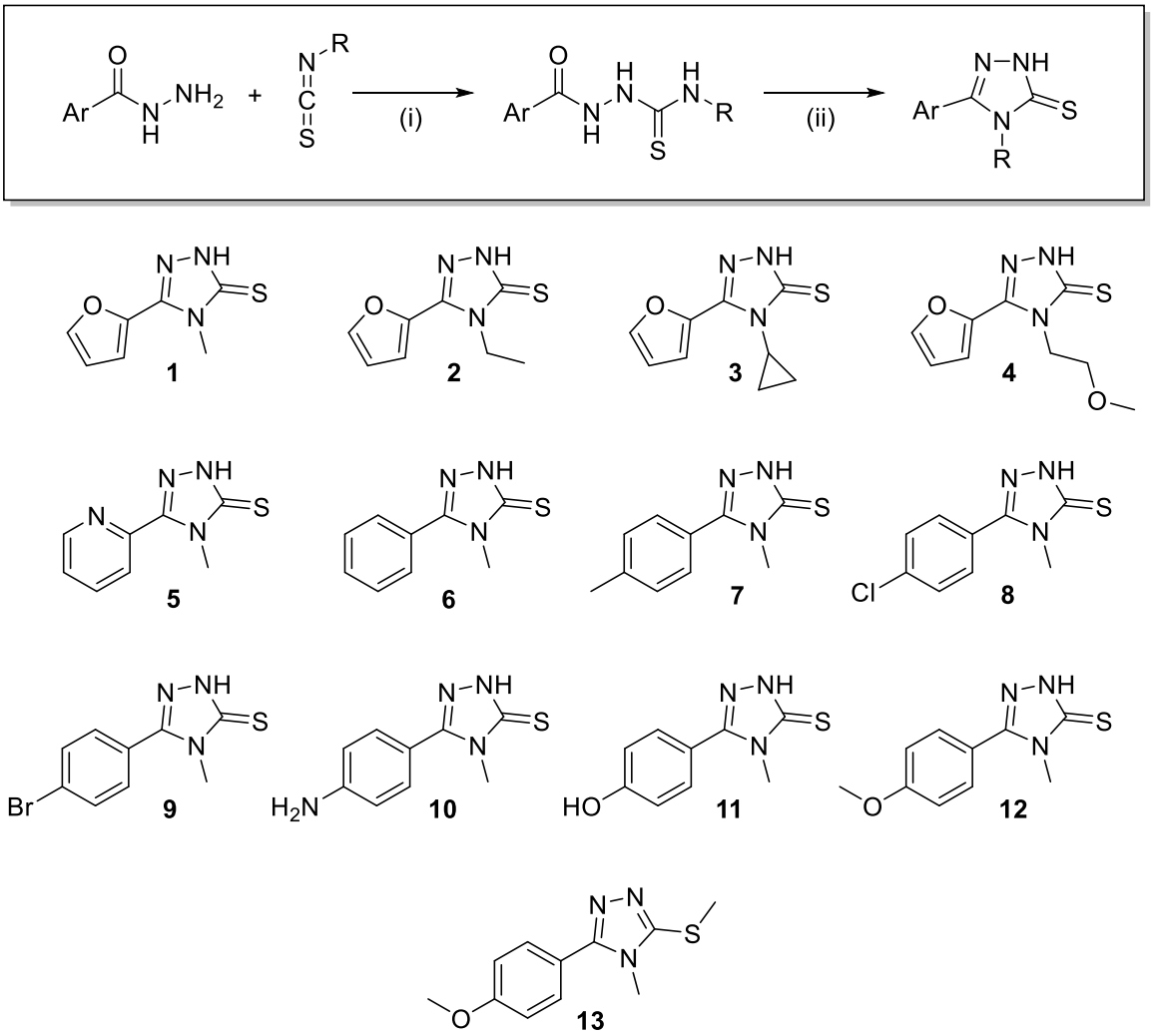

Figure 6. Synthesis of a focused library of 1,2,4-triazole compounds tested as inhibitors of FUT6. Inset: general scheme for a two-step synthesis of compounds **1** to **12**—(i) 18-hour reflux at 80 °C in THF; (ii) 24-hour reflux at 85 °C in 1M $NaHCO_3$ (aq) or 3-minute microwave reaction at 150 °C in 2M NaOH (aq), followed by quenching with 12N HCl.

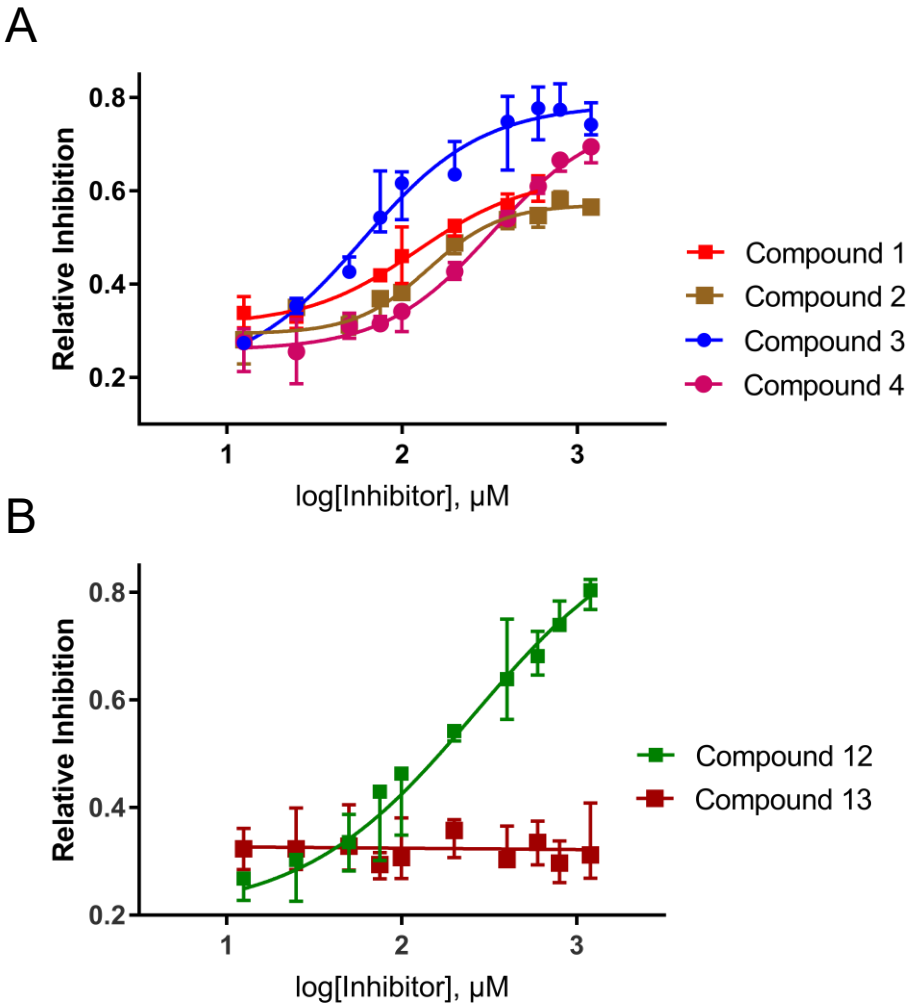

Figure 7. Plot of inhibition as determined by fluorescence signal against the log of concentration of select compounds. **(a)** IC_{50} curves of compound **1** and derivatives with varied 4-*N*-alkyl substituents (compounds **2**, **3**, and **4** with intermediate, low, and high IC_{50} values, respectively). **(b)** IC_{50} curve of inhibitor compound **12** in comparison to non-inhibitor compound **13**.

References

1. Pinho, S. S. & Reis, C. A. (2015) Glycosylation in cancer: mechanisms and clinical implications, *Nat Rev Cancer*. **15**, 540-555.
2. Miyoshi, E., Moriwaki, K. & Nakagawa, T. (2008) Biological Function of Fucosylation in Cancer Biology, *J Biochem.* **143**, 725-729.
3. Pang, P.-C., Chiu, P. C. N., Lee, C.-L., Chang, L.-Y., Panico, M., Morris, H. R., Haslam, S. M., Khoo, K.-H., Clark, G. F., Yeung, W. S. B. & Dell, A. (2011) Human sperm binding is mediated by the sialyl-Lewis^X oligosaccharide on the zona pellucida, *Science.* **333**, 1761-1764.
4. Polley, M. J., Phillips, M. L., Wayner, E., Nudelman, E., Singhal, A. K., Hakomori, S. & Paulson, J. C. (1991) CD62 and endothelial cell-leukocyte adhesion molecule 1 (ELAM-1) recognize the same carbohydrate ligand, sialyl-Lewis x, *Proc Natl Acad Sci USA.* **88**, 6224-6228.
5. Nakamori, S., Kameyama, M., Imaoka, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Kabuto, T., Iwanaga, T., Matsushita, Y. & Irimura, T. (1993) Increased expression of sialyl Lewis^X antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study, *Cancer Res.* **53**, 3632-3637.
6. Barthel, S. R., Wiese, G. K., Cho, J., Opperman, M. J., Hays, D. L., Siddiqui, J., Pienta, K. J., Furie, B. & Dimitroff, C. J. (2009) Alpha 1,3 fucosyltransferases are master regulators of prostate cancer cell trafficking, *Proc Natl Acad Sci USA.* **106**, 19491-19496.
7. Mas, E., Pasqualini, E., Caillol, N., El Battari, A., Crotte, C., Lombardo, D. & Sadoulet, M.-O. (1998) Fucosyltransferase activities in human pancreatic tissue: comparative study between cancer tissues and established tumoral cell lines, *Glycobiology.* **8**, 605-613.
8. Kwan, D. H., Ernst, S., Kötzler, M. P. & Withers, S. G. (2015) Chemoenzymatic Synthesis of a Type 2 Blood Group A Tetrasaccharide and Development of High-throughput Assays Enables a Platform for Screening Blood Group Antigen-cleaving Enzymes, *Glycobiology.* **25**, 806-811.
9. Kwan, D. H., Constantinescu, I., Chapanian, R., Higgins, M. A., Kötzler, M. P., Samain, E., Boraston, A. B., Kizhakkedathu, J. N. & Withers, S. G. (2015) Toward Efficient Enzymes for the Generation of Universal Blood through Structure-Guided Directed Evolution, *J Am Chem Soc.* **137**, 5695-5705.
10. Soltero-Higgin, M., Carlson, E. E., Phillips, J. H. & Kiessling, L. L. (2004) Identification of Inhibitors for UDP-Galactopyranose Mutase, *J Am Chem Soc.* **126**, 10532-10533.
11. Gross, B. J., Kraybill, B. C. & Walker, S. (2005) Discovery of O-GlcNAc Transferase Inhibitors, *J Am Chem Soc.* **127**, 14588-14589.
12. Rillahan, C. D., Brown, S. J., Register, A. C., Rosen, H. & Paulson, J. C. (2011) High-Throughput Screening for Inhibitors of Sialyl- and Fucosyltransferases, *Angew Chem, Int Ed.* **50**, 12534-12537.
13. Gao, Z., Ovchinnikova, O. G., Huang, B.-S., Liu, F., Williams, D. E., Andersen, R. J., Lowary, T. L., Whitfield, C. & Withers, S. G. (2019) High-Throughput "FP-Tag" Assay for the Identification of Glycosyltransferase Inhibitors, *J Am Chem Soc.* **141**, 2201-2204.
14. Gosselin, S., Alhussaini, M., Streiff, M. B., Takabayashi, K. & Palcic, M. M. (1994) A Continuous Spectrophotometric Assay for Glycosyltransferases, *Anal Biochem.* **220**, 92-97.
15. Zegzouti, H., Engel, L., Hennek, J., Alves, J., Vidugiris, G. & Goueli, S. (2013) Detection of glycosyltransferase activities with homogenous bioluminescent UDP detection assay, *Glycobiology.* **23**, 1340-1341.
16. Zegzouti, H., Engel, L., Hennek, J., Alves, J., Vidugiris, G. & Goueli, S. (2014) Detection of glycosyltransferase activities with homogenous bioluminescent UDP and GDP detection assays, *Glycobiology.* **24**, 1156.
17. Kannagi, R. (2002) Regulatory roles of carbohydrate ligands for selectins in the homing of lymphocytes, *Curr Opin Struct Biol.* **12**, 599-608.
18. Singh, A. K., Pluvinage, B., Higgins, M. A., Dalia, A. B., Woodiga, S. A., Flynn, M., Lloyd, A. R., Weiser, J. N., Stubbs, K. A., Boraston, A. B. & King, S. J. (2014) Unravelling the Multiple Functions of the Architecturally Intricate Streptococcus pneumoniae β-galactosidase, BgaA, *PLoS Pathog.* **10**, e1004364.
19. Mark, B. L., Wasney, G. A., Salo, T. J. S., Khan, A. R., Cao, Z., Robbins, P. W., James, M. N. G. & Triggs-Raine, B. L. (1998) Structural and Functional Characterization of Streptomyces plicatus β-N-Acetylhexosaminidase by Comparative Molecular Modeling and Site-directed Mutagenesis, *J Biol Chem.* **273**, 19618-19624.

20. Mark, B. L., Vocadlo, D. J., Knapp, S., Triggs-Raine, B. L., Withers, S. G. & James, M. N. (2001) Crystallographic evidence for substrate-assisted catalysis in a bacterial beta-hexosaminidase, *J Biol Chem.* **276**, 10330-10337.
21. Kannagi, R. (2014) Fucosyltransferase 6. GDP-Fucose Lactosamine α3-Fucosyltransferase (FUT6) in *Handbook of Glycosyltransferases and Related Genes* (Taniguchi, N., Honke, K., Fukuda, M., Narimatsu, H., Yamaguchi, Y. & Angata, T., eds) pp. 559-571, Springer Japan, Tokyo.
22. Zhang, J.-H., Chung, T. D. Y. & Oldenburg, K. R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays, *J Biomol Screening.* **4**, 67-73.
23. Kane, J. M., Staeger, M. A., Dalton, C. R., Miller, F. P., Dudley, M. W., Ogden, A. M. L., Kehne, J. H., Ketteler, H. J. & McCloskey, T. C. (1994) 5-Aryl-3-(alkylthio)-4H-1,2,4-triazoles as selective antagonists of strychnine-induced convulsions and potential antispastic agents, *J Med Chem.* **37**, 125-132.
24. Samanta, S., Lim, T. L. & Lam, Y. (2013) Synthesis and in vitro Evaluation of West Nile Virus Protease Inhibitors Based on the 2-{6-[2-(5-Phenyl-4H-{1,2,4]triazol-3-ylsulfanyl)acetylamino]benzothiazol-2-ylsulfanyl}acetamide Scaffold, *ChemMedChem.* **8**, 994-1001.
25. Namdjou, D.-J., Chen, H.-M., Vinogradov, E., Brochu, D., Withers, S. G. & Wakarchuk, W. W. (2008) A β-1,4-Galactosyltransferase from Helicobacter pylori is an Efficient and Versatile Biocatalyst Displaying a Novel Activity for Thioglycoside Synthesis, *ChemBioChem.* **9**, 1632-1640.
26. Zhao, G., Guan, W., Cai, L. & Wang, P. G. (2010) Enzymatic route to preparative-scale synthesis of UDP-GlcNAc/GalNAc, their analogues and GDP-fucose, *Nat Protoc.* **5**, 636-646.
27. Yi, W., Liu, X., Li, Y., Li, J., Xia, C., Zhou, G., Zhang, W., Zhao, W., Chen, X. & Wang, P. G. (2009) Remodeling bacterial polysaccharides by metabolic pathway engineering, *Proc Natl Acad Sci USA.* **106**, 4207-4212.
28. Fang, J.-L., Tsai, T.-W., Liang, C.-Y., Li, J.-Y. & Yu, C.-C. (2018) Enzymatic Synthesis of Human Milk Fucosides α1,2-Fucosyl para-Lacto-N-Hexaose and its Isomeric Derivatives, *Adv Synth Catal.* **360**, 3213-3219.