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Fucosyltransferase-specific inhibition *via* next generation of fucose mimetics†‡

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The ability to custom-modify cell surface glycans holds great promise for treatment of a variety of diseases. We propose a glycomimetic of L-fucose that markedly inhibits the creation of sLex by FTVI and FTVII, but has no effect on creation of Lex by FTIX. Our findings thus indicate that selective suppression of sLex display can be achieved, and STD-NMR studies surprisingly reveal that the mimetic does not compete with GDP-fucose at the enzymatic binding site.

The capacity to custom-modify cellular glycosylation without genetic manipulation of target cells holds great implications for the pharmacology of biologic agents and for cell-based therapeutics. To achieve glycoengineering of cell surface glycans, non-toxic inhibitors are needed that possess exquisite specificity for the target Golgi glycosyltransferase(s). Critically, such inhibitors should be selective solely for the target and should have no effect(s) on other (non-target) glycosyltransferases, thereby yielding construction only of desired glycan product(s). Fucosyltransferases (FTs) are key enzymes involved in the biosynthesis of important fucosylated glycoconjugates on the cell surface.[1] These enzymes catalyze the transfer of L-fucose (as a nucleotide-activated donor substrate, GDP-fucose (GDP-Fuc), Fig. 1A) to structurally diverse acceptors. Among the family of FTs, there are α-1,3-FTs that specifically modify terminal lactosaminyl glycans, the last step in biosynthesis of Lewis X antigens (*i.e.* "Lewis X" (Le[X]; CD15) and "sialyl Lewis X" (sLe[X]; CD15s), Fig. 1A). The α-1,3-FTs are responsible for installing terminal L-fucose residues on type 2 lactosamines located at the termini of glycan chains as "neutral type 2 lactosamines" (*i.e.* Gal-β(1,4)-GlcNAc-α-1-R, 'LacNAc', the precursor of Le[X]) or as "sialylated type 2 lactosamines" (*i.e.* NeuAc-α(2,3)-Gal-β(1,4)-GlcNAc-α-1-R, 'sLacNAc', the precursor of sLe[X]).[1a] In humans there are six α-1,3-FTs.[2] The principal α-1,3-FTs that mediate sLe[X] creation are fucosyltransferase VI (FTVI) and fucosyltransferase VII (FTVII), with fucosyltransferase IX (FTIX) dominating Le[X] synthesis.[2] Notably, FTVII makes only sLe[X], FTIX makes only Le[X] and FTVI makes both sLe[X] and Le[X]. The tetrasaccharide sLe[X] is the canonical binding determinant for the selectins (CD62E, CD62L, CD62P), a family of Ca^{++}-dependent lectins that direct critical cell–cell adhesive

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‡ Abbreviations: FTs: fucosyltransferases; GDP-Fuc: guanosine diphosphate fucose; LeX: Lewis X; sLeX: sialyl Lewis X; LacNAc: Gal-β(1,4)-GlcNAc-α-1-R; sLacNAc: NeuAc-α(2,3)-Gal-β(1,4)-GlcNAc-α-1-R; RPMI-8402: human lymphoblastic leukemia cells; MSCs: human mesenchymal stem cells; UT: untreated; Pre: preincubation; HUVEC: human umbilical vein endothelial cells; TNF: tumor necrosis factor; STD: saturation transfer difference; GFP: green fluorescent protein; DLS: dynamic light scattering.

† Electronic supplementary information (ESI) available: Cell assays, saturation transfer difference NMR. Protocol for FTVI purification. DLS. MD simulations. See DOI: 10.1039/d0cc04847j

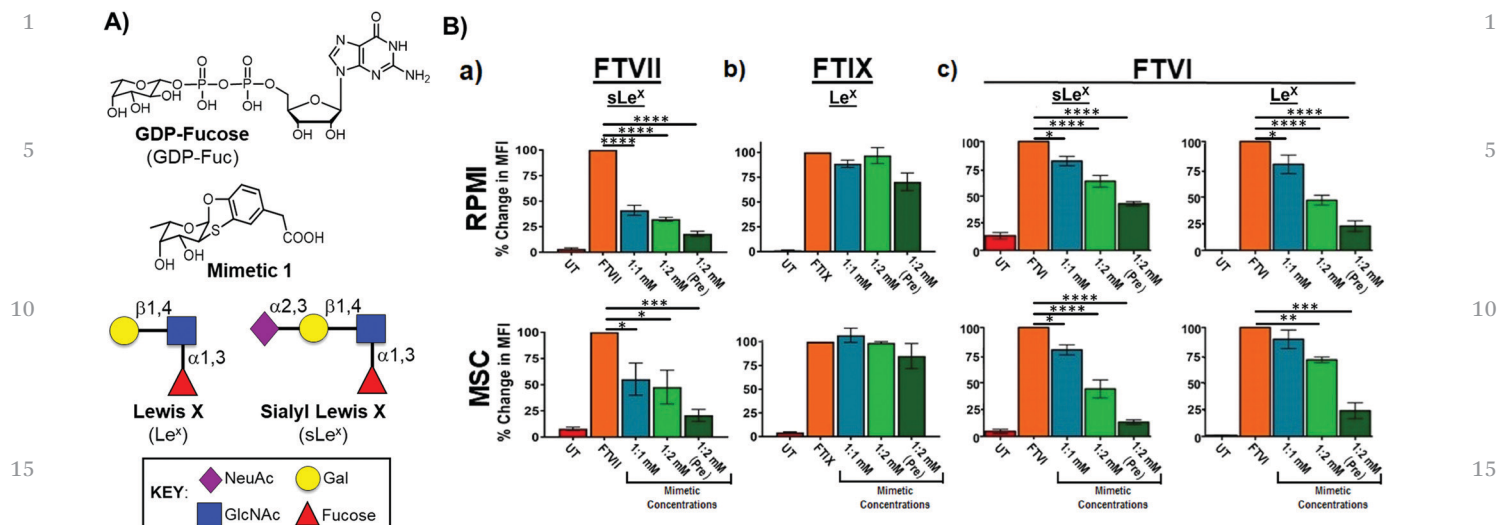§ BR and RS are co-last and co-corresponding authors.

Fig. 1   (A) Structures of GDP-Fuc and mimetic **1**. Schematic representation of Le[X] and sLe[X]. (B) Mimetic **1** inhibition experiments: (a–c) RPMI-8402 cell line (top row) and MSCs (bottom row) were exofucosylated using FTVII (a), FTIX (b), or FTVI (c) with 1.0 mM GDP-Fuc (orange), 1.0 mM GDP-Fuc to 1.0 mM **1** (blue), 1.0 mM GDP-Fuc to 2.0 mM **1** (light green), or cell suspension was preincubated (Pre) with FTs and mimetic **1** (2.0 mM) for 45 min followed by addition of GDP-Fuc (1.0 mM) for 1 h (dark green). Percentage change in mean fluorescence intensity (MFI) from baseline. Comparison between treatments was carried out by means of a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

interactions. Thus, type 2 lactosaminyl glycan fucosylation mediated by α-1,3-FTs plays a crucial role in a variety of biologic events including leukocyte trafficking, human fertilization, embryo development, metastasis, and immune cell differentiation.[3] Indeed, upregulated α-1,3-FTs activity, resulting in the overexpression of Le[X] and sLe[X] determinants, is etiologic in several human diseases including cancer and autoimmune conditions (*i.e.*, rheumatoid arthritis, Crohn's disease, diabetes).[4] Viewed from this perspective alone, α-1,3-FTs are rational targets for drug design[5] in that downregulation of their activity could provide therapeutic relief for a multitude of life-threatening and debilitating diseases.

Although there have been intensive efforts focused on inhibition of FTs,[5] progress in this area has been hindered by the complexity of the transition state of the catalytic reaction and the lack of X-ray crystallographic structural information regarding this process.[6] To date, data are limited to the binding of the nucleotide sugar donor (GDP-Fuc) to phylogenetically distant FTs such as the *Helicobacter pylori* α-1,3-FT complexed with the natural donor,[5e,f,k] and no crystal structure of α-1,3-FTs with acceptor substrate (especially involving natural acceptors displayed on glycoconjugates in native states) has been reported. This absence of information is likely due to the difficulties in expressing these enzymes in sufficient amounts for crystallization purposes or to the low binding affinity of acceptor glycans to the FT catalytic domain. Accordingly, the development of donor nucleotide sugar analogues/mimetics has thus garnered significant attention for utility in FT inhibition. In this regard, a per-acetylated 2F-fucose analog has been developed[5g] as a global metabolic inhibitor of FTs. It is metabolically converted into the corresponding donor analog inside the cell, which in turn acts as inhibitor either of FTs and of the *de novo* synthesis of GDP-Fuc.

As an alternative approach, we have pursued the stereoselective synthesis of molecule **1** (Fig. 1A),[7a] a conformationally constrained mimetic of L-fucose. It contains a fucose-like pyranose ring fused with an oxathine six-member ring bearing a phenyl acetic residue. Importantly, fucose mimetic **1** is recognized by fucose-binding lectins with an affinity comparable to that the natural ligand, L-fucose.[7] Then, according to molecular dynamics (MD) simulations performed on GDP-fucose and mimetic **1**, while the fucopyranosyl ring of both compounds exists in the $^1C_4$ conformation, mimetic **1** is more rigid, exhibiting a single conformation in solution (Fig. S1, ESI†). On this basis, we decided to extend the study of the glycan mimicry properties of mimetic **1** by exploring its ability to interfere with the catalytic activity of FTs in competition against GDP-Fuc as a donor. Exofucosylation is a technique whereby a pertinent FT together with GDP-Fuc is placed in a cell suspension to stereoselectively install fucose on pertinent acceptor cell surface glycan(s). For the case of α-1,3-FTs, this approach provides the ability to pinpoint the effect(s) of mimetic addition on creation of fucosylated glycans Le[X] and sLe[X], while keeping the rest of the cell's biological functions and its viability intact. Specifically, this study focused on three α-1,3-FTs that can create sLe[X] and Le[X] determinants: FTVII, FTIX, and FTVI.[2,8] To assess enzymatic activity, two cell types were utilized, the human lymphoblastic leukemia cell line RPMI-8402 and human mesenchymal stem cells (MSCs). Each were selected since these cell types do not natively express either sLe[X] or Le[X] determinants (CD15s and CD15, respectively), but express both the sLacNAc and LacNAc acceptors. Accordingly, mimetic **1** was tested as an inhibitor of FT-mediated exofucosylation on these cells and the extent of fucosylation was monitored using antibodies that detect the creation of the relevant fucosylated epitopes (*i.e.* sLe[X] or Le[X]).

Cells were treated directly with reaction buffer containing the relevant FTs together with 1.0 mM GDP-Fuc; cells untreated with FTs (UT) were used as reference reaction negative control (Fig. 1B, Fig. S2 and S3, ESI†). FTVI is capable of adding L-fucose to both sialylated and neutral LacNAc acceptors (sLac-NAc and LacNAc), whereas FTVII and FTIX hold strict specificity for sialylated (sLacNAc) and neutral type II LacNAc acceptors, respectively.[2] In the first set of explorative experiments, we sought to determine if mimetic 1 could inhibit production of sLe^X on RPMI-8402 cells (Fig. 1B(a)). We performed several reactions that contained either no enzyme (UT), FTVII with GDP-Fuc (1.0 mM) and then FTVII with two different concentrations of the mimetic 1 (1.0 mM and 2.0 mM respectively). Moreover, a pre-incubation protocol was also analyzed, wherein the cell suspension was pre-treated with FTVII and a 2.0 mM solution of mimetic 1 followed by addition of 1.0 mM solution of GDP-Fuc (Pre). Results revealed that mimetic 1 was able to inhibit FTVII-driven sLe^X expression on RPMI-8402 cells (Fig. 1B(a)), with the degree of inhibition correlating with the increasing concentration of 1. Notably, pre-incubation of FTVII with mimetic 1 provided a significant inhibition of sLe^X production (Fig. 1B(a)). To further test the specificity range of mimetic 1 effect(s), we performed exofucosylation with FTIX (Fig. 1B(b)). Unlike with FTVII, our results using RPMI-8402 cells indicate that mimetic 1 does not inhibit FTIX-mediated exofucosylation (i.e. has minimal effect on the creation of Le^X, Fig. 1B(b)). To assess whether these findings are cell/glycocalyx specific, we employed a second cell type, MSCs, as they also display surface sLacNAc and LacNAc acceptors. Fig. 1B(a and b) is representative of exofucosylation reactions performed on MSCs using FTVII and FTIX, under the same conditions used for RPMI-8402 cells. Again, FTVII inhibition was clear and proceeded stepwise with the addition of additional mimetic, and the level of inhibition was stronger in the pre-incubation reactions. In contrast, and similar to results using RPMI-8402 cells, the mimetic did not inhibit FTIX production of Le^X. To determine if the selective inhibition of sLe^X creation, but not Le^X creation, was due to the ability of mimetic to only interfere with FT-sLacNAc interactions, or if it was inherent to the inability of mimetic 1 to inhibit FTIX, we examined mimetic effects on FTVI activity (Fig. 1B(c)). We found that unlike FTIX, FTVI production of Le^X was clearly inhibited by mimetic 1 (Fig. 1B(c)) yet like FTVII, the ability of FTVI to produce sLe^X was also markedly diminished in both cell lines. Pre-incubation with the mimetic prior to addition of GDP-Fuc inhibited more completely exofucosylation reactions mediated by FTVI and FTVII, but had no effect on FTIX, providing further evidence that mimetic 1 does no inhibit FTIX. These differences between isoforms at the level of their inhibition can be a reflection of their discrete sequence identity ($\sim$40%), implying that their active sites or regions around are likely different. We then performed two titration experiments using FTVI and FTVII respectively, GDP-Fuc (1.0 mM) and different concentrations of mimetic 1 (Fig. S4, ESI†) to determine the relative IC$_{50}$ value. Bell-shaped like concentration curves were obtained either in the FTVI and in the FTVII reactions, which suggested the

formation of colloidal aggregates at higher concentrations under our experimental conditions ($>$2.0 mM).[9] The mimetic reached the highest inhibition at 2.0 mM against both FTVI and FTVII, with a slightly higher extent of inhibition on FTVII over FTVI.

As sLe^X is the primary ligand for E-selectin, which is induced on endothelial cells by inflammatory cytokine (e.g., TNFα), we sought to evaluate the biological impact of the mimetic's inhibition of sLe^X binding to E-selectin under hemodynamic shear. We therefore used a parallel plate flow chamber (Fig. S5, ESI†) seeded with monolayers of human umbilical vein endothelial cells (HUVEC) that were stimulated with TNFα. RPMI cells were then introduced under defined fluid shear conditions to determine inhibition. Low shear conditions revealed that the binding interactions of integrins on all the treatment were intact, but as expected, the use of 1 in all the reactions dampened the ability of cells to adhere to HUVEC under physiologically relevant shear conditions (Fig. S5, ESI†).

To gain further insights on the structural features of the interaction of mimetic 1 with FTs, we performed saturation transfer difference (STD)-NMR experiments.[10] As recently reported, the production of relevant amounts of some glycosyl-transferases is a challenge.[6] Therefore, FTVI was selected as we required high quantitative enzyme amounts for pertinent NMR experiments. We produced FTVI from HEK293 cells (ESI†) as a fusion protein containing an N-terminal His-tagged green fluorescent protein. The expression of FTVII was poor and impeded us to perform STD-NMR experiments. STD NMR build-up curves allowed for binding epitope mapping of the donor substrate (Fig. S6, ESI†). The binding mode of GDP-Fuc is characterized by the recognition of the nucleoside moiety by FTVI, which is key to the donor substrate interaction. The fucopyranose ring shows clear contacts with the protein but the lower intensities indicate that fucose is farther from the surface of the binding pocket, most likely oriented towards a shallower acceptor binding site. This is reminiscent of the binding epitope of GDP-Fuc to other fucosyltransferases.[11] Then, we carried out analogue STD-NMR with the mimetic. In particular, a 1.0 mM solution of mimetic 1 was used, where no colloidal aggregation occurred (Fig. S7, ESI†). Strong STD signals were observed upon addition of mimetic 1 to a solution containing FTVI (Fig. 2a), unambiguously indicating that mimetic 1 interacts with FTVI under fast exchange conditions, in agreement with an affinity in the low mM range. The contacts that mimetic 1 creates with FTVI in the bound state were elucidated by binding epitope mapping (Fig. 2b) from mathematical fitting of experimental STD build-up curves.[12] From these data we delineated the specific pattern of interactions that mimetic 1 makes with FTVI in the bound state, revealing that on one side of the inhibitor, the aromatic protons Ha, Hb and fucose protons H1 and H6 make the closest contacts with the protein surface, followed by proton H5. On the other hand, the methylene group showed the lowest saturation transfer from FTVI in the bound state, suggesting this side of mimetic 1 is more solvent-exposed in the bound state, in agreement with the higher polarity of the carboxylate group of
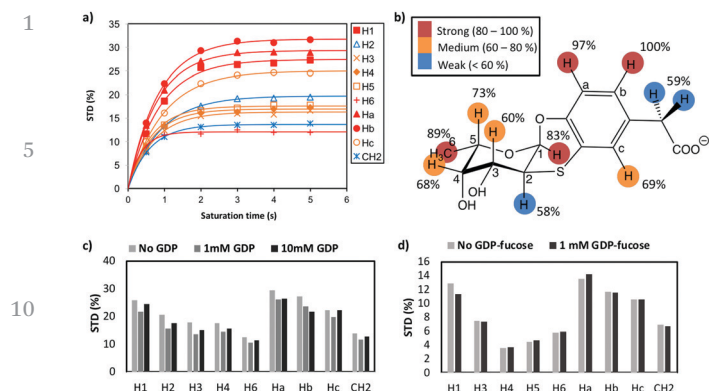
Fig. 2 STD NMR study of the binding of mimetic **1** to FTVI. (a) Experimental STD NMR build-up curves (symbols); the initial slopes of each curve were determined by mathematical fitting (solid lines) to obtain the epitope mapping (ESI†). (b) Binding epitope mapping for the interaction of mimetic **1** with FTVI. Results of the STD NMR competition experiments of (c) **1** and GDP for binding to FTVI; (d) **1** and GDP-Fuc for binding to FTVI.

1. Next, STD NMR competition experiments were performed between mimetic **1** and both the nucleotide GDP and the GDP-Fuc (Fig. 2c, d, Fig. S8 and S9, ESI†). NMR signals of mimetic **1** (1.0 mM) were monitored upon addition of GDP. Binding of GDP was confirmed by the observation of STD-NMR signals, but neither 1 mM nor 10 mM of GDP were able to displace mimetic **1** from its complex with FTVI, as no reduction in its STD intensities was observed (Fig. 2c and Fig. S8, ESI†). We then carried out further competition experiments between mimetic **1** and the whole donor substrate, GDP-Fuc (Fig. 2d and Fig. S8, ESI†). Again, NMR signals of mimetic **1** (1.0 mM) were monitored upon addition of GDP-Fuc (Fig. S9, ESI†). Binding of **1** was confirmed by STD NMR signals, however, as with results using GDP, no displacement of mimetic **1** was observed (Fig. S9, ESI†). To confirm the apparent absence of competition, we repeated the experiments with a sample containing GDP-Fuc and FTVI. NMR signals of donor GDP-Fuc were then monitored upon addition of equimolar concentrations of **1** (Fig. S10, ESI†). The absence of reduction of STD signals of GDP-Fuc after addition of mimetic **1** confirmed that the inhibitor does not compete with donor substrate. Indeed, unexpectedly, the competition experiments indicated mimetic **1** binds to a region of FTVI different from the donor substrate binding site. Competition experiments with acceptor were no feasible as STD NMR experiments on samples with sLacNAc acceptor substrate and FTVI did not show signals, indicating its binding is out of the appropriate kinetics range of affinities for STD NMR (Fig. S11, ESI†).

The possibility of modulating cell surface expression of E-selectin ligands by using selective and non-toxic enzymatic inhibitors could have significant therapeutic applications, such as controlling inflammatory conditions and in reducing the spread of cancer. In the course of identifying new potential bioactive scaffolds, we report here on the ability of the fucose mimetic **1** to selectively interfere with creation of sLe[X]. Notably, it does not affect the catalytic activity of FTIX, the α-1,3-FT that

principally mediates Le[X] synthesis, yet markedly interferes with creation of sLe[X] by FTVI and FTVII, and favors FTVII inhibition over that of FTVI. Structural information about the interactions of the mimetic with FTVI obtained by STD-NMR revealed mimetic **1** binding epitopes, and defined the different particular positions of the fucose and the aromatic moiety as the driving forces of the enzyme inhibition. Surprisingly, our findings indicate that the mimetic **1** and the GDP-fucose do not compete for the same enzymatic binding site. Therefore, further binding studies of **1** with FTs, and in particular FTVII, aimed at a deeper understanding of the binding mode of this molecule (*i.e.*, structural, biochemical, pharmacological and biophysical characterizations) and the type of inhibition, are ongoing. Moreover, mimetic **1** has a modular structure, which can be further modified following a rational structure-based design to improve affinity and selectivity. Therefore, further refinements of the structure of such mimetic will pave the way for developing next-generation inhibitors for these enzymes.

Conflicts of interest

There are no conflicts to declare.

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