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Acceptor specificities and selective inhibition of recombinant human Gal- and GlcNAc-transferases that synthesize core structures 1, 2, 3 and 4 of O-glycans⋆

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Abstract

Background—Modifications of proteins by O-glycosylation determine many of the properties and functions of proteins. We wish to understand the mechanisms of O-glycosylation and develop inhibitors that could affect glycoprotein functions and alter cellular behavior.

Methods—We expressed recombinant soluble human Gal- and GlcNAc-transferases that synthesize the O-glycan cores 1 to 4 and are critical for the overall structures of O-glycans. We determined the properties and substrate specificities of these enzymes using synthetic acceptor substrate analogs. Compounds that were inactive as substrates were tested as inhibitors.

Results—Enzymes significantly differed in their recognition of the sugar moieties and aglycone groups of substrates. Core 1 synthase was active with glycopeptide substrates but GlcNAc-transferases preferred substrates with hydrophobic aglycone groups. Chemical modifications of the acceptors shed light on enzyme–substrate interactions. Core 1 synthase was weakly inhibited by its substrate analog benzyl 2-butanamido-2-deoxy-α-D-galactoside while two of the three GlcNAc-transferases were selectively and potently inhibited by bis-imidazolium salts which are not substrate analogs.

⋆Dedicated to Professor Hans Paulsen on the occasion of his 90th birthday.

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Conclusions—This work delineates the distinct specificities and properties of the enzymes that synthesize the common O-glycan core structures 1 to 4. New inhibitors were found that could selectively inhibit the synthesis of cores 1, 2 and 3 but not core 4.

General significance—These studies help our understanding of the mechanisms of action of enzymes critical for O-glycosylation. The results may be useful for the re-engineering of O-glycosylation to determine the roles of O-glycans and the enzymes critical for O-glycosylation, and for biotechnology with potential therapeutic applications.

Keywords

O-Glycans; Specificity; Inhibitors; C1GalT; C3GnT; C2GnT

1. Introduction

The GalNAcα-Ser/Thr based mucin type O-glycans of glycoproteins and mucins have a number of important functions. They ensure resistance to proteases, and affect physical, chemical and antigenic properties of proteins [1]. Carbohydrate structures linked to O-glycans form blood group and tissue antigens and ligands for protein interactions which control cell adhesion, migration and cell death [2–4]. Alterations of O-glycosylation can therefore dramatically affect the functions of glycoproteins and cellular behavior. Our interest is to understand the detailed mechanisms of O-glycosylation and to develop technologies to change O-glycosylation for further studies of the role of O-glycans.

A large family of polypeptide GalNAc-transferases initiates O-glycan biosynthesis [5], followed by various glycosyltransferases that sequentially or competitively add sugar residues to GalNAcα-Ser/Thr to synthesize different core subtypes [6]. Polypeptide GalNAc-transferases have overlapping specificities towards their peptide or glycopeptide substrates [5,6]. The most common second step of O-glycosylation is the addition of Gal to GalNAc by core 1 β1,3-Gal-transferase (C1GalT, core 1 synthase, T-synthase) that synthesizes the core 1 structure Galβ1–3GalNAcα-Ser/Thr (Fig. 1) [6,7]. Core 1, also known as the T antigen, is often present in the unmodified form in cancer cells but is further modified in most cell types [8]. A deficiency of core 1 production is associated with several human diseases and disorders, and with certain cancer cells that express terminal GalNAc (Tn antigen) or sialylα2–6GalNAc (sialyl-Tn antigen) [9]. This may be due to a deficiency in the chaperone Cosmc that normally ensures the expression of active C1GalT [10–14].

In specific tissues such as the colon, GalNAc in GalNAcα-Ser/Thr can also be converted to core 3 (Fig. 1), GlcNAcβ1–3GalNAc-, by core 3 β1,3-GlcNAc-transferase (C3GnT) [15,16]. C3GnT activity is low or undetectable in many cancer cells [17,18]. Metastatic colon and prostate cancer cells transfected with the gene encoding C3GnT were less capable of migration and invasion through extracellular matrix components, and suppressed tumor formation and metastasis in mice [19,20]. C3GnT gene knockout mice further showed that core 3 glycans have a protective function in normal gastro-intestinal epithelia [21].

C1GalT and C3GnT utilize GalNAcα-Ser/Thr-peptides as natural acceptor substrates but can be assayed using synthetic GalNAc-derivatives [14–16,22–24]. However, the detailed specificities of human C1GalT and C3GnT have not yet been determined.

Core 1 can be branched to form core 2, GlcNAcβ1–6(Galβ1–3) GalNAc-, by a family of core 2 β1,6-GlcNAc-transferases (C2GnT) (Fig. 1). While C2GnT1 is highly specific and only acts on core 1 [25–27], the related enzyme, core 2/4 β1,6-GlcNAc-transferase (C2GnT2), has a broader specificity and can also synthesize core 4, GlcNAcβ1–6(GlcNAcβ1–3)GalNAc-, from core 3 [15,28,29]. The levels of expression or activities of C2GnT1 have been shown to be abnormal and variable in cancer cells [8,18]. Core 2 O-glycans act as a scaffold structure for sialyl-Lewis x (SLe^x) which plays a critical role in cell adhesion and cell migration [30,31]. Moreover, MUC1 mucin, which contains core 2 O-glycans, functions as a molecular shield against immune cell attacks, facilitating bladder tumor metastasis [32]. Transfection experiments showed that the expression of C2GnT2 in colon cancer cells HCT116 is associated with reduced cell growth, increased apoptotic cell death and reduced tumor formation in nude mice [33].

Knowledge of the substrate recognition of glycosyltransferases is the basis for the rational design of biologically applicable glycosylation inhibitors that allow studies of the biological and pathological functions of glycans. We previously described a potent UV-activated inhibitor for C2GnT1, core 1 *p*-nitrophenyl (pnp) [34]. Recently, bis-imidazolium salts were examined in glycosyltransferase assays [35]. These bivalent imidazolium salt compounds contain two positively charged imidazolium groups linked by an aliphatic chain, and also had been reported to be potent and specific inhibitors of *Plasmodium* replication [36]. The structures of these bis-imidazolium inhibitors are not related to glycosyltransferase substrates and represent a new class of glycosyltransferase inhibitors. We have now studied the inhibition of the enzymes involved in the synthesis of O-glycan core 1 to 4 structures in more detail.

2. Material and methods

2.1. Materials

Materials were purchased from Sigma unless otherwise stated. Gal- and GlcNAc-analogs, core 1 and core 3 disaccharide-containing compounds were synthesized as previously reported [26,27,37–40]. Synthetic glycopeptides [41] and many other sugar derivatives were synthesized and kindly provided by Hans Paulsen (University Hamburg, Germany). The intactness of glycopeptides was confirmed by MALDI-TOF mass spectrometry in the negative or positive ion modes as previously described [27].

2.2. Enzymes

Active, soluble human recombinant core 1 β1,3-Gal-transferase (C1GalT) was prepared in insect Hi-5 cells by co-expression with human Cosmc as previously described [11] and the crude cell extracts were used as the enzyme source. His-tagged soluble human recombinant core 2 β1,6-GlcNAc-transferase (C2GnT1) was produced in insect cells as described and used as the crude cell extract [42]. Soluble human recombinant core 3 β3GlcNAc-transferase (C3GnT) and core 2/4 β6GlcNAc-transferase (C2GnT2) containing His-tags

were also produced in Sf9 insect cells [43; http://glycoenzymes.ccrc.uga.edu/]. C3GnT and C2GnT2 activities were barely detectable before purification. Therefore, both enzyme proteins were purified by Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography. Briefly, the insect cell supernatants were dialyzed against dialysis buffer (50 mM NaH_2PO_4, 500 mM NaCl; pH 8.0) for 18 h at 4 °C with a buffer change after the first 6 h. Ni^{2+}-NTA resin (Thermo Scientific) was first equilibrated with dialysis buffer. The dialyzed insect cell supernatant was then incubated with the equilibrated resin at room temperature for 3 h with gentle agitation. The mixture was transferred into an empty column and the resin was allowed to settle. The resin was washed with 10 column volumes of dialysis buffer containing 20 mM imidazole, which was gradually increased to 50 mM. Enzyme was eluted with 5 column volumes of dialysis buffer containing 250 to 500 mM imidazole. The eluted fractions were concentrated with polyethylene glycol at 4 °C, and then dialyzed against HEPES buffer (20 mM HEPES, 1 mM $MgCl_2$, 20 mM NaCl, 1 mM DTT) and 1 mL protease inhibitor (Sigma Protease inhibitor cocktail for general use) for 3 h at 4 °C. Aliquots of purified enzyme solutions were adjusted to 20% glycerol and stored at −80 °C. The protein concentrations of the enzyme stock solutions were determined by the Bio-Rad (Bradford) protein assay method using bovine serum albumin as the standard.

Western blot analysis was performed with mouse monoclonal anti-His antibody against the His-tag as the primary antibody (Cell Biolabs, Inc.) and horseradish peroxidase-conjugated goat anti-mouse IgG as the secondary antibody (Santa Cruz Biotechnology). Labeling was visualized with Western blot detection system (iNtRON Biotechnology).

2.3. Glycosyltransferase assays

All glycosyltransferase assays were carried out in at least duplicate determinations with less than 10% difference between assays [14,27,44]. The standard assay mixtures for human recombinant C1GalT contained in a total volume of 40 μL: 5 μL of insect cell supernatant containing C1GalT (0.036 mg protein), 0.125 M MES, pH 7.0, 12.5 mM $MnCl_2$, 10 mM AMP, 0.4 mM UDP-[^{3}H]Gal (2000–3000 cpm $nmol^{-1}$) and 0.5 mM GalNAcα-Bn. Control assays contained no acceptor substrate or no inhibitor.

Affinity purified human recombinant C3GnT was assayed in mixtures containing 10 μL C3GnT solution (0.003 mg protein), 0.125 M MES buffer, pH 7.0, 10 mM AMP, 0.125 M GlcNAc, 12.5 mM $MnCl_2$ 1.05 mM UDP-[^{3}H]GlcNAc (5800 cpm/nmol) and acceptors as indicated in the Tables, or water in negative controls.

Purified human recombinant C2GnT2 was assayed similarly as C3GnT but without $MnCl_2$. 15 μL purified C2GnT2 (0.002 mg protein) was used in each assay and Galβ1–3GalNAcα-pnp (Core 1-pnp) or GlcNAcβ1–3GalNAcα-pnp (Core 3-pnp) were employed as standard acceptor substrates. C2GnT1 produced in insect cells was assayed as described [27].

For inhibition studies, inhibitors were present at the same concentration as the acceptor in the assays, or as indicated in figure legends and tables. Assay mixtures were incubated for 1 h at 37 °C and reactions were quenched with 600 μL of cold water. Reaction products were isolated using 0.4 mL AG1 × 8 ion exchange resin (100–200 mesh). Radioactivity of the

eluate was determined by scintillation counting. The Origin Pro 8.0 program was used to determine kinetic parameters K_M, V_{max} and IC_{50}.

3. Results

3.1. Characterization of Core1 β1,3-Gal-transferase (C1GalT)

Human recombinant C1GalT was highly active with GalNAcα-Bn as acceptor substrate. The reaction was linear with respect to protein concentration up to 0.8 mg/mL and incubation time up to 1.5 h. The optimal reaction pH was 7.0. The conserved presence of the DxD motif in the C1GalT sequence suggested the involvement of divalent metal ions as cofactors in Gal-transferase catalysis. Of several divalent metal ions studied, Mn^{2+} at 12.5 mM concentration was shown to be an efficient cofactor (taken as 100% activity), while 15% activity was observed in the presence of 12.5 mM Co^{2+}. None of the other metal ions tested, including Mg^{2+}, Ca^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+}, was effective in stimulating C1GalT activity (Supplementary Fig. 1), and no activity was observed in the presence of 12.5 mM ethylenediamine tetraacetic acid (EDTA). Half-maximal activity was reached at 0.25 mM $MnCl_2$ concentration (Supplementary Fig. 2A). C1GalT was specific for UDP-Gal as the donor substrate (Fig. 2). The apparent K_M for UDP-Gal was 1.0 mM, and V_{max} was 0.16 μmol/h/mg with 5 mM GalNAcα-Bn as acceptor substrate. The apparent K_M for GalNAcα-Bn was 0.8 mM and V_{max} was 0.14 μmol/h/mg (Table 1, Supplementary Fig. 3).

A series of synthetic compounds was employed to study the acceptor substrate specificity of C1GalT (Table 2). The activity of C1GalT with 0.5 mM of GalNAcα-Bn as substrate was 0.042 μmol/h/mg (taken as 100% activity). GalNAc derivatives were variably active as substrates when the aglycone groups were benzyl (Bn), *p*-nitrophenyl (pnp), perillyl or peptide. The diphosphate aglycone group prevented activity. GalNAcα-peptide substrates exhibited up to 5.64-fold the activity observed with GalNAcα-Bn. The most active substrate was the acetyl and amide-protected glycopeptide Ac-GHA-(GalNAcα) TSLPVTG-NH_2, derived from the tandem repeat sequence of MUC4 mucin. Free glycopeptides that had a sequence of 10 amino acids derived from mucins MUC2 and MUC3 were also very active (Table 2) and the activity levels varied according to the position of GalNAc in the glycopeptide. However, a MUC1 mucin-derived glycopeptide having 21 amino acids with GalNAc attached to Ser-6 was only 39% active compared to GalNAcα-Bn. No activity was detected with the short glycopeptide A-(GalNAcα)T. Free GalNAc showed only 6% activity, indicating that the presence of a hydrophobic or peptide aglycone group was beneficial. In contrast to the GalNAcα-anomer, the β-anomer, GalNAcβ-pnp, was not a substrate for C1GalT. In addition, removal or substitution of the hydroxyl group at the 3- or 4-position of GalNAc yielded analogs that were inactive as substrates. However, the hydroxyl group at the 6-position of GalNAc was not required in an active substrate. Thus, deletion or modifications of the 6-hydroxyl, or substitutions with a variety of substituents including fluoro groups or saccharide moieties yielded substrates with activities of 49 to 191%, compared to GalNAcα-Bn. However, no activity was detected with the glycopeptide TE-(GlcNAcβ1–6GalNAcα-)TTSHSTPG, although the corresponding glycopeptide containing a single GalNAcα-residue was very active with 3.7 times the activity observed with GalNAcα-Bn (Table 2). These results show that the activity is determined not only by

the sugar moiety but also by its position within the peptide, its substitution, by the amino acid composition and sequence as well as the length of the peptide.

No C1GalT activity was detected when GalNAc was replaced by derivatives of Gal, galactosamine (GalN) or GlcNAc which suggested a requirement for the *N*-acyl group as well as the *galacto* configuration in the substrate. 2-*N*-Propionyl-GalNα-Bn showed 60% activity compared to GalNAcα-Bn, while the bulky 2-*N*-butyryl substituent prevented enzyme activity.

3.2. Characterization of core 3 β1,3-GlcNAc-transferase (C3GnT)

Purified human recombinant C3GnT showed a major protein band at 56 kDa in the elution fractions seen by SDS-PAGE (Supplementary Fig. 4A). Western blotting revealed a single protein band at 56 kDa for both the crude enzyme preparation and the purified enzyme (Supplementary Fig. 4B) which had an activity of 0.19 μmol/h/mg with 0.5 mM GalNAcα-Bn as acceptor substrate. The conserved DxD motif in the C3GnT sequence suggested the requirement for divalent metal ion as a cofactor for GlcNAc transfer. Mn^{2+} was the most effective cofactor, while Co^{2+} gave 18% activity compared to Mn^{2+}. None of the other metal ions tested including Mg^{2+}, Ca^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+} was effective in stimulating C3GnT activity (Supplementary Fig. 1) and there was no activity in the presence of EDTA. This pattern was similar to that for C1GalT. However, the effect of Mn^{2+} on C3GnT differed from its effect on C1GalT, since it required a higher concentration of $MnCl_2$ to saturate the reaction (Supplementary Fig. 2B).

C3GnT was most active with UDP-GlcNAc as the donor substrate (Fig. 2), but also showed 0.8% activity with UDP-Glc. This may be an *in vitro* phenomenon since the Glcβ1–3GalNAc- structure has never been reported in human mucin O-glycans. The apparent K_M for UDP-GlcNAc was 2.8 mM, and V_{max} was 1.30 μmol/h/mg (Supplementary Fig. 5A, Table 1).

The acceptor substrate specificity of C3GnT was investigated with a series of synthetic compounds and compared to that of C1GalT (Table 2). The aglycone groups of GalNAc derivatives had a large effect on activity. The apparent K_M value for GalNAcα-Bn was 3.0 mM with a V_{max} of 0.95 μmol/h/mg while the apparent K_M for GalNAcα-perillyl was 0.3 mM with a V_{max} of 0.4 μmol/h/mg (Table 1, Supplementary Fig. 5B). Similar to C1GalT, the β-anomer GalNAcβ-pnp was not recognized as a substrate for C3GnT. In addition, removal or substitution of the hydroxyl group at the 3- or 4-position of GalNAc yielded inactive substrates. Modifications at position 2 of GalNAc led to similar results for both C1GalT and C3GnT. However, in contrast to C1GalT, the 6-hydroxyl of GalNAc was found to be absolutely essential for C3GnT activity. The reactivity towards glycopeptide substrates containing GalNAcα-Thr was generally lower for C3GnT, compared to C1GalT (Table 2). In addition, these two enzymes had very different reactivity ratios using GalNAcα-glycopeptide substrates. Some of the glycopeptides that had very high activity with C1GalT were poorly active with C3GnT. The glycopeptide Ac-A-(GA)**T**G-NH_2 was the best substrate with an apparent K_M of 0.7 mM and a V_{max} of 1.1 μmol/h/mg. The MUC4-derived glycopeptide Ac-GHA-(GalNAcα)TSLPVTG-NH_2, was only 22% active compared to GalNAcα-Bn, and the MUC2-derived glycopeptides were poorly active while no activity

was observed with MUC3-derived glycopeptides. It is interesting that the glycopeptide A-(GalNAcα)T that was inactive with C1GalT showed 15% activity with C3GnT. Thus, the recognition of these GalNAc-glycopeptides was not only a function of the glycopeptide structure and composition but was specific for each enzyme.

3.3. Characterization of core 2 β1,6-GlcNAc-transferase (C2GnT1) and core 2/4 β1,6-GlcNAc-transferase (C2GnT2)

We previously examined the specificities of recombinant human C2GnT1 [27] and the enzyme from human leukemia cells [26] that synthesizes the core 2 structure. In the present work we expanded on the specificity of the enzyme towards additional substrates having hydrophobic aglycone groups, as well as glycopeptides carrying the core 1 structure (Table 3). We compared this with the specificity of human recombinant C2GnT2 that synthesizes both the core 2 and core 4 structures. After purification, C2GnT2 showed a major protein band at 53 kDa by SDS-PAGE and Western blot analysis (Supplementary Fig. 6A, B) with a specific activity of 0.22 μmol/h/mg.

Although both C2GnT1 and 2 have a SPDE sequence resembling a DxD motif which was shown for C2GnT1 to contain the catalytic base Glu320 [45,46], a metal ion cofactor was not required for catalysis. Interestingly, the presence of 12.5 mM Mn^{2+} in the assay led to an 83–85% reduction of C2GnT2 activity using core 1 or core 3 substrates. Similarly, C2GnT1 activity was reduced by 61% in the presence of 12.5 mM Mn^{2+} (Supplementary Fig. 7A, B).

Both, C2GnT1 and C2GnT2 were found to be specific for UDP-GlcNAc as the sugar donor substrate (Fig. 2). For C2GnT1, the apparent K_M for UDP-GlcNAc with 5 mM Galβ1–3GalNAcα-pnp as acceptor was 3.2 and V_{max} was 0.1 μmol/h/mg (Table 1, Supplementary Fig. 8A). For C2GnT2, the apparent K_M for UDP-GlcNAc with 5 mM Galβ1–3GalNAcα-pnp as acceptor was 2.2 mM and V_{max} was 1.7 μmol/h/mg. With 5 mM GlcNAcβ1–3GalNAcα-pnp as the acceptor substrate, the apparent K_M for UDP-GlcNAc was 2.1 mM and V_{max} was 0.8 μmol/h/mg (Table 1, Supplementary Fig. 9A).

A series of Galβ1–3GalNAc- and GlcNAcβ1–3GalNAc derivatives was used to study the substrate specificity of C2GnT1 and C2GnT2 (Table 3). Both enzymes preferred the core 1 over the core 3 substrate, and the hydrophobic aglycone groups had significant but different effects on the activities. C2GnT1 had an apparent K_M value of 1.1 mM for the core 1 substrate Galβ1–3GalNAcα-pnp and V_{max} was 0.08 μmol/h/mg (Table 1, Supplementary Fig. 8B). For C2GnT2, the apparent K_M value for Galβ1–3GalNAcα-pnp was 2.1 mM with a V_{max} of 1.5 μmol/h/mg. The apparent K_M for the corresponding core 3 substrate GlcNAcβ1–3GalNAcα-pnp was much higher at 5.8 mM with a V_{max} was 1.0 μmol/h/mg (Tables 1, Supplementary Fig. 9B).

As was the case for C2GnT1 [26], the 4- and 6-hydroxyl groups of both Gal and GalNAc residues were absolutely essential for C2GnT2 activity. Galβ1–3GlcNAcα-Bn was not active indicating that the axial position of the 4-hydroxyl was critical for C2GnT2. Substitution of the 2-hydroxyl of Gal with Fuc also prevented activity. The 3-hydroxyl of Gal was not essential for C2GnT1 or C2GnT2. The 3-O-methyl substitution of Gal also had little effect on C2GnT1 and resulted in 41% C2GnT2 activity. However, the extension of the

Gal moiety by a GlcNAcβ1–3 residue resulted in inactive substrate, probably by steric hindrance (Table 3).

Both, C2GnT1 and C2GnT2 activities were dramatically reduced when the aglycone group was a peptide derived from MUC1 MUC2, MUC 3or MUC4. Generally, C2GnT2 showed a much lower activity towards glycopeptide substrates containing the core 1 structure, and no detectable activity towards glycopeptides containing the core 3 or core 6 (GlcNAcβ1–6GalNAc-) structure (Table 3). As reported previously, one of the core 1-containing glycopeptides tested, (Galβ1–3GalNAcα-)TAGV, had high activity with C2GnT1. However, C2GnT2 obviously recognized this glycopeptide differently since no C2GnT2 activity was detected (Table 3). MUC1 glycopeptides having 21 amino acids were generally poor substrates for C2GnT1 and showed no activity of C2GnT2. In contrast, acetyl- and amide-protected shorter glycopeptides were more active with C2GnT1 (7 to 49% of the activity with Gal-1–3GalNAc-pnp) and with C2GnT2 (<1 to 8% activity).

Both, C2GnT1 and C2GnT2 were surprisingly active with glycopeptides having the β-linked core 1 structure (Galβ1–3GalNAcβ-Thr) which showed 43 to 72% activity for C2GnT1 and 15 to 32% activity for C2GnT2 (Table 3).

3.4. Inhibition of Gal- and GlcNAc-transferases

Compounds that were inactive as substrates were screened as inhibitors for Gal- and GlcNAc-transferase activities. Substrate analogs, as well as a series of neutral imidazoles and monovalent imidazolium salts [36], and a series of bis-imidazolium salts containing an aliphatic linker-chain were tested. Although some of the bis-imidazolium salts inhibited β3GalT5 and β4GalT1 [35], none of these compounds produced an inhibitory effect on C1GalT activity (Table 4). Naphthyl derivatives of GlcNAc were previously shown to be potent inhibitors of β4GalT1 [37,38] but did not inhibit C1GalT or any of the other three enzymes. In this large series of compounds only benzyl 2-butanamido-2-deoxy-α-D-galactoside (2-*N*-butyryl-GalNα-Bn) weakly inhibited C1GalT activity with an IC_{50} of 2.31 mM (Supplementary Fig. 10) but did not inhibit the other enzymes tested (Table 4).

None of the inactive GalNAc derivatives had any inhibitory effect on C3GnT. However, bis-imidazolium salts containing an aliphatic linker-chain of 20 or 22 carbons significantly inhibited C3GnT activity (Table 4). The IC_{50} of an inhibitor with a 20-carbon chain, 1,20-bis-(3-methyl-1*H*-imidazolium-1-yl)eicosane dichloride, was 0.14 mM, while the IC_{50} of the respective dimesylate salt was 0.13 mM. The IC_{50} of the inhibitor with a 22-carbon chain, 1,22-bis-(3-methyl-1*H*-imidazolium-1-yl)docosane dimesylate, was 0.26 mM (Table 4, Supplementary Fig. 11). Imidazolium salts with linker-chains of 18 carbons or less did not inhibit C3GnT.

Although none of the sugar derivatives inhibited C2GnT1, the bis-imidazolium salts with aliphatic linker-chains of 16, 18, 20 or 22 carbons in length were potent inhibitors (Table 4, Supplementary Fig. 12). The lowest IC_{50} value (0.062 mM) was seen with 1,20-bis-(3-methyl-1*H*-imidazolium-1-yl)eicosane dichloride. Surprisingly, none of these compounds inhibited the related enzyme C2GnT2.

4. Discussion

All four human enzymes studied here are inverting glycosyltransferases that transfer a sugar residue from an α-linkage in UDP-α-Gal or UDP-α-GlcNAc to form β-linkages in the products, which are the major mucin type O-glycan core structures 1 to 4 (Fig. 1, Table 5). These core structures are found in the highly O-glycosylated tandem repeat regions of colonic or lung mucins [6,47]. These enzymes therefore efficiently accept the peptide and glycopeptide moieties of their mucin substrates. However, *in vitro*, only core 1 synthase has a pronounced preference for mucin-derived glycopeptides and may thus bind preferably to GalNAcα-Thr. The enzymes that assemble core 2, 3 and 4 generally were poorly active using mucin-derived glycopeptides. Although the sugar moieties have a stabilizing effect on peptide conformations, large mucin molecules having rigid 'bottle brush' conformations appear to be better substrates for these enzymes, and are efficiently glycosylated *in vivo*. Mucin-derived glycopeptides have been used *in vitro* to synthesize the Tn and T antigens [27,48]. However, the relatively short size and flexible nature of glycopeptides used in this study did not allow efficient synthesis of core 3 and 4 O-glycans. The *in vitro* synthesis of core 3-glycopeptides by recombinant C3GnT has been achieved using MUC1-derived glycopeptide derivatives linked to beads [48]. These solid phase-linked glycopeptides are expected to be more stable in their conformations and may thus be better substrates.

Polypeptide GalNAc-transferases act as the first enzymes in the O-glycan core synthesis pathways to synthesize the Tn antigen and bind the peptide moieties in the substrate binding site [49–51]. The crystal structures of human T2 and T10 [49,50] as well as mouse T1 [51] reveal a lectin binding domain, which suggests that the enzymes can bind a glycopeptide as the acceptor substrate. Of the four enzymes that extend the Tn antigen, only the crystal structure of mouse C2GnT1 is known, with and without the acceptor or UDP [45,46]. It appears that the peptide moiety of glycopeptides is not bound to the enzyme but extrudes into the solution. Thus, most glycopeptides are poor acceptor substrates for C2GnT1, independent of the α- or β-configurations of core 1, while hydrophobic groups may assist substrate binding. It is remarkable that core 1-TAGV is an excellent substrate and may uniquely bind to hydrophobic amino acids near the acceptor binding site of C2GnT1 but not to the similar enzyme C2GnT2.

Human C1GalT has a distinct substrate specificity recognizing the GalNAc-ring in α-configuration as well as the aglycone group, with a preference for peptide. GlcNAcβ1–6GalNAcα-Bn is a good substrate for C1GalT but the MUC3 mucin-derived glycopeptide with this disaccharide structure is not. This suggests that glycopeptides can have unfavorable conformations or cause poor accessibility of the acceptor to the catalytic site. GalNAc in β-configuration also does not bind to the enzyme and future protein structure of C1GalT may reveal a steric hindrance of β-linked GalNAc.

C1GalT does not require the presence of the 6-hydroxyl of GalNAc for substrate binding but it does require the 2-*N*-acyl group. While 2-*N*-propyl GalNα-Bn shows reduced activity, 2-*N*-butyryl-GalNα-Bn is inactive but binds and inhibits the enzyme, probably by steric hindrance of catalysis. This is the first report of a substrate analog inhibitor of C1GalT.

C3GnT also requires GalNAc substrate in the α-configuration as well as all of the substituents of the GalNAc sugar ring. While the N-propyl group allows significant activity, the N-butyryl group prevents binding of the compound to the enzyme. The aglycone groups of the GalNAc-substrates are also important. Glycopeptides showed variable activity and it appears that the length and amino acid composition of peptides determine the efficiency of GlcNAc transfer.

Human core 1 and core 3 synthases are related to Gal-transferase families. Both of them are within the GT31 family of glycosyltransferases with a GT-A fold. They have DxD motifs (Table 4) which may provide the catalytic base to activate the acceptor sugar by de-protonation. This motif may also coordinate the Mn^{2+} ion and thus interact with the phosphate of UDP-Gal/UDP-GlcNAc [52]. The $MnCl_2$ requirement of human C1GalT (Supplementary Fig. 2A) resembles that of the rat liver enzyme which has an optimum at 10 mM $MnCl_2$ [14]. This *in vitro* concentration is much higher than the Mn^{2+} concentration in the Golgi which is less than 1 mM. Thus Mn^{2+} regulates glycoprotein glycosylation reactions but also glycoprotein trafficking and turnover [53]. C2GnT1 and C2GnT2 do not require divalent metal ions for activity. The inhibition of these enzymes by the unphysiologically high concentration of 12.5 mM Mn^{2+} *in vitro* may be a result of altered protein structure.

Human C2GnT1 and C2GnT2 are members of the β1,6-GlcNAc-transferase family and have 57% sequence identity and 73% similarity. Both enzymes are in the GT14 family (Table 5). The structure of mouse C2GnT1 suggests that Arg378 and Lys401 residues are in proximity to the β-phosphate of UDP-GlcNAc, thus replacing the function of the Mn^{2+} ion in catalysis [45]. The interactions may serve to stabilize the UDP leaving group after GlcNAc transfer. The human counterpart of C2GnT1 also has Arg378 and Lys401 residues close to the DxD motif near the C-terminus that may replace the function of a divalent metal ion.

A conserved SPDE motif resembling the DxD motif is present in mouse and human C2GnT1 as well as in human C2GnT2. The Glu320 of this sequence in mouse C2GnT1 is near the O-4 and O-6 of GalNAc of bound substrate while Arg254 forms a hydrogen-bond with O-4 of Gal [45,46]. Glu243 also binds to O-4 as well as the O-6 of the Gal residue. This explains the absolute requirement of C2GnT1 for the Galβ1–3GalNAc sequence where the 4- and 6-hydroxyls of both sugar residues have to be unmodified. The acceptor binding site of C2GnT2, in addition to core 1, also accommodates the GlcNAc residue of core 3. This could be due to the replacement of Glu253 with Thr in C2GnT2 that may allow the binding of the equatorial O-4 of GlcNAc. Tyr358 is in the vicinity of O-2 of Gal in mouse C2GnT1, but this residue appears to be replaced by the smaller Gly368 residue in C2GnT2, explaining why the bulkier GlcNAc is accepted instead of Gal at that site. However, the core 3 substrate GlcNAcβ1–3GalNAcα-pnp showed an almost 3-fold higher K_M value compared to the core 1 substrate, suggesting that core 3 does not bind as well as core 1 to C2GnT2.

A third enzyme of the C2GnT family, C2GnT3, which is primarily expressed in the thymus was shown to be specific for UDP-GlcNAc as the sugar donor substrate and also has the SPDE motif [54]. The enzyme has the acceptor specificity of C2GnT1 and acts on core 1 but not on core 3, although a very low distal I-antigen GlcNAc-transferase (IGnT) activity was

detected with the i-antigenic GlcNAcβ1–3Galβ-methyl substrate. Interestingly, Hashimoto et al. [55] reported that mouse C2GnT2 acted on core 1, core 3 and GlcNAcβ1–3Gal-substrates and thus has the core 2-, core 4- and IGnT activities, similar to that of human C2GnT2 [29] and the crude M-enzyme (C2GnT2) in rat colon [15,26]. However, in our current study we did not observe the IGnT activity of human C2GnT2, and our observations correspond to the specificity of human C2GnT2 cloned by Schwientek et al. [28]. Thus, depending on the assay conditions, the enzyme might have a very low activity towards the GlcNAcβ1–3Gal-substrate.

It is interesting that imidazolium salts potently inhibit C3GnT and C2GnT1 but not C1GalT or C2GnT2. These inhibitors are not substrate analogs and also inhibit β1,3- and β1,4-Gal-transferases [35] in a mixed non-competitive/uncompetitive fashion. This selective mode of inhibition does not appear to involve binding of the positively charged salts to UDP-sugars, since only selected transferases are inhibited. It will have to be shown if there are hydrophobic and negatively charged amino acid residues on the surface of enzyme proteins in an appropriate configuration that allows the binding of imidazolium salts with spacers of specific lengths (20 to 22 carbons for C3GnT and 16 to 22 carbons for C2GnT1). This binding then may cause an unfavorable protein conformation or block access to the substrate binding site. The glycosyltransferases studied here are among those affected by cancer and other diseases [8], and inhibitors have therapeutic potential. Future knowledge of the structures of these glycosyltransferases will help our understanding of substrate recognition and aid in further development of inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ac-	acetyl
C1GalT	core 1 β1,3-Gal-transferase
C3GnT	core 3 β1,3-GlcNAc-transferase
C2GnT1	core 2 β1,6-GlcNAc-transferase
C2GnT2	core 2/4 β1,6-GlcNAc-transferase
Gn	GlcNAc
onp	*o*-nitrophenyl

pnp *p*-nitrophenyl

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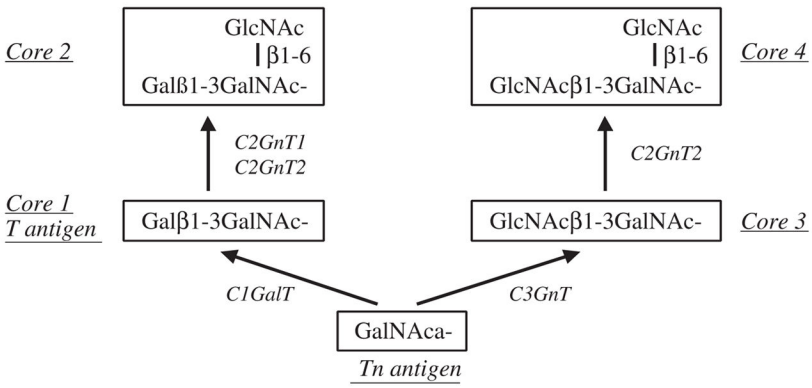

Fig. 1.

Synthesis of the common O-glycan core structures 1 to 4. C1GalT and C3GnT both act on GalNAc-substrates to synthesize core 1 and core 3, respectively. Core 1 is branched by either C2GnT1 or C2GnT2 to form core 2. Core 3 is branched only by C2GnT2 to form core 4. Core 1–4 structures can be extended and terminated in many different ways.

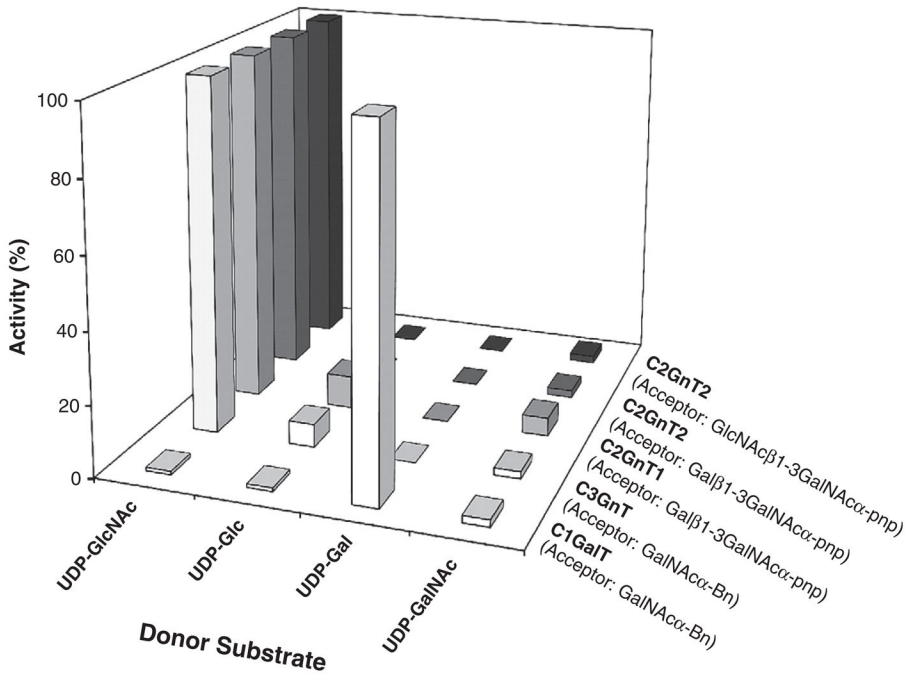

Fig. 2.

Donor specificities of C2GnT2, C2GnT1, C3GnT, and C1GalT. Glycosyl transfer to acceptor substrate was measured as a function of nucleotide sugar donor. Enzymes were assayed as described in the Methods section, except in the presence of different nucleotide sugars replacing the standard donor substrates; 0.40 mM UDP-Gal, 2266 cpm/nmol; 0.345 mM UDP-Glc, 5549 cpm/nmol; 0.5 mM UDP-GlcNAc, 5876 cpm/nmol; or 0.6 mM UDP-GalNAc, 1708 cpm/nmol. The activity with the nucleotide sugar donor specific for each enzyme was set to 100%. C1GalT and C3GnT activities were assayed using GalNAcα-Bn as acceptor substrate; C2GnT1 activity was assayed using Galβ1–3GalNAcα-pnp as acceptor substrate; C2GnT2 activity was assayed using Galβ1–3GalNAcα-pnp and GlcNAcβ1–3GalNAcα-pnp as acceptor substrates.

Table 1

Kinetic parameters of glycosyltransferases for acceptor and donor substrates. Assays were carried out as described in the Material and methods section, using the indicated acceptor substrates to determine the K_M and V_{max} values (Origin Pro 8.0). Bn, benzyl; pnp, *p*-nitrophenyl.

Enzyme	Substrate	K_M (mM)	V_{max} (μmol/h/mg)
C1GalT	GalNAcα-Bn	0.8	0.14
	UDP-Gal (using GalNAcα-Bn)	1.0	0.16
C3GnT	GalNAcα-Bn	3.0	0.95
	UDP-GlcNAc (using GalNAcα-Bn)	2.8	1.30
	GalNAcα-perillyl	0.3	0.40
	Ac-A-(GalNAcα)TG-NH_2	0.7	1.10
C2GnT1	Galβ1–3GalNAcα-pnp	1.1	0.08
	UDP-GlcNAc (using Galβ1–3GalNAcα-pnp)	3.2	0.10
C2GnT2	Galβ1–3GalNAcα-pnp	2.1	1.50
	UDP-GlcNAc (using Galβ1–3GalNAcα-pnp)	2.2	1.70
	GlcNAcβ1–3GalNAcα-pnp	5.8	1.00
	UDP-GlcNAc (using GlcNAcβ1–3GalNAcα-pnp)	2.1	0.80

Table 2

Acceptor substrate specificity of human C1GalT and C3GnT. C1GalT and C3GnT were assayed as described in Material and methods with 0.5 mM GalNAcα-Bn as the standard acceptor substrate (set to 100% activity), or with other GalNAc derivatives as substrates. Bn, benzyl; GalN, D-galactosamine; nd, not done; pnp, *p*-nitrophenyl; onp, *o*-nitrophenyl; bold **T** or **S** in glycopeptides indicate the attachment sites for an O-glycan.

Compound name (0.5 mM in assays)	C1GalT activity (%)	C3GnT activity (%)
GalNAcα-Bn	100[a]	100[b]
Section I: modifications of the aglycone		
GalNAcα-phenyl	181	37
GalNAcα-pnp	204	88
GalNAcα-perillyl	102	153
GalNAcβ-pnp	<1	<1
GalNAcα-O-PO_3-PO_3-$(CH_2)_{11}$-O-Ph	<1	<1
GalNAc	6	<1
GalNAcβ1–4GlcNAcβ-Bn	<1	<1
Section II: modifications of the ring substituents		
2-*N*-Propionyl-GalNα-Bn	60	62
2-*N*-Butyryl-GalNα-Bn	<1	<1
2-Deoxy-Galα-Bn	<1	<1
3-Deoxy-GalNAcα-Bn	<1	<1
3-*O*-Ethyl-GalNAcα-Bn	<1	<1
3-*O*-Propyl-GalNAcα-Bn	<1	<1
4-Deoxy-GalNAcα-Bn	<1	<1
6-Deoxy-GalNAcα-Bn	68	<1
6-*O*-(4,5-Anhydro)pentyl-GalNAcα-Bn	191	<1
GlcNAcβ1–6-GalNAcα-Bn (Core 6-Bn)	74	<1
Galβ1–4GlcNAcβ1–6GalNAcα-onp	38	<1
Galβ1–3(6-deoxy)GalNAcα-Bn	<1	<1
Galβ1–6GalNAcα-Bn	30	<1
Galβ1–4Glcβ1–6GalNAcα-Bn	63	<1
4-F-4-Deoxy-GlcNAcβ1–6-GalNAcα-Bn	88	<1
Galβ1–4GlcNAcβ1–6Galα-(2-naphthyl)	<1	<1
GlcNAcβ1–6-Galβ-OCD3	<1	<1
Section III: glycopeptides		
A-(GalNAcα)**T**	<1	15
Ac-V-(GalNAcα)**T**P-NH_2	71	36
Ac-A-(GalNAcα)**T**G-NH_2	143	170
Ac-GHA-(GalNAcα)**T**SLPVTG-NH_2	564	22
TTTVTP-(GalNAcα)**T**PTG	259	5
TTTV-(GalNAcα)**T**PTPTG	270	10
TT-(GalNAcα)**T**VTPTPTG	99	8
T-(GalNAcα)**T**TVTPTPTG	231	nd

Compound name (0.5 mM in assays)	C1GalT activity (%)	C3GnT activity (%)
TET-(GalNAcα)**T**SHSTPG	210	nd
TE-(GalNAcα)**T**TSHSTPG	373	nd
TE-(GlcNAcβ1–6GalNAcα)**T**TSHSTPG	<1	nd
AHGVT-(GalNAcα)**S**APDTRPAPGSTAPPA	39	15

[a] 100% C1GalT activity corresponds to 0.042 μmol/h/mg.

[b] 100% C3GnT activity corresponds to 0.190 μmol/h/mg.

Table 3

Acceptor substrate specificity of human C2GnT1 and C2GnT2. C2GnT1 and C2GnT2 were assayed as described in Material and methods with 0.5 mM Galβ1–3GalNAcα-pnp as the standard acceptor substrate (set to 100% activity). Bn, benzyl; GalN, D-galactosamine; pnp, *p*-nitrophenyl; onp, *o*-nitrophenyl. Bold **S** and **T** in glycopeptides indicate the attachment of O-glycans.

Compound name (0.5 mM in assays)	C2GnT1 activity (%)	C2GnT2 activity (%)
Galβ1–3GalNAcα-pnp (Core1-pnp)	100[a]	100[b]
Section I: modifications of the aglycone		
Galβ1–3GalNAcα-Bn (Core1-Bn)	64	35
Galβ1–3GalNAcα-perillyl	135	86
Galβ1–3GalNAcα-onp	79	100
Section II: modifications of the sugar moiety		
GalNAcα-Bn	<1	<1
2-*N*-Butyryl-GalNα-Bn	<1	<1
GlcNAcβ1–3-Galβ-methyl	<1	<1
GlcNAcβ1–3GalNAcα-pnp (Core 3-pnp)	<1	32
GlcNAcβ1–3GalNAcα-allyl	<1	27
Galβ1–3GlcNAcα-Bn	<1	<1
Fucα1–2Galβ1–3GalNAcα-methyl	<1	<1
3-Deoxy-GalNAcα-Bn	<1	<1
Galβ1–3(6-deoxy)GalNAcα-Bn	<1	<1
GlcNAcβ1–6GalNAcα-Bn (Core 6-Bn)	<1	<1
Galβ1–3(4-deoxy)GalNAcα-Bn	<1	<1
Galβ1–3(6-O-methyl)GalNAcα-Bn	<1	<1
3-Deoxy-Galβ1–3GalNAcα-Bn	90	52
3-*O*-Methyl-Galβ1–3GalNAcα-Bn	104	41
Galβ1–3GlcNAcβ1–3Galβ1–3GalNAcα-Bn	<1	<1
4-Deoxy-Galβ1–3GalNAcα-Bn	<1	<1
4-*F*-4-Deoxy-Galβ1–3GalNAcα-Bn	<1	<1
6-Deoxy-Galβ1–3GalNAcαBn	<1	<1
GlcNAcβ1–6(GlcNAcβ1–3)GalNAcα-Bn (Core 4-Bn)	<1	<1
Section III: glycopeptides		
(Galβ1–3GalNAcα)**T**AGV	92	<1
T-(GlcNAcβ1–3GalNAcα)**T**TVTPTPTG	<1	<1
TETTSHS-(GlcNAcβ1–3GalNAcα)**T**PG	<1	<1
TET-(GlcNAcβ1–3GalNAcα)**T**SHSTPG	<1	<1
TE-(GlcNAcβ1–3GalNAcα)**T**TSHSTPG	<1	<1
TE-(GlcNAcβ1–6GalNAcα)**T**TSHSTPG	<1	<1
TT-(Galβ1–3GalNAcα)**T**VTPTPTG	13	<1
T-(Galβ1–3GalNAcα)**T**TVTPTPTG	11	<1
TT-(Galβ1–3GalNAcα)**T**VTP-(Galβ1–3GalNAcα)**T**PTG	12	<1
TETTSHS-(Galβ1–3GalNAcα)**T**PG	70	7

Compound name (0.5 mM in assays)	C2GnT1 activity (%)	C2GnT2 activity (%)
TET-(Galβ1–3GalNAcα)**T**SHSTPG	27	<1
Ac-PTT-(Galβ1–3GalNAcα)**T**GIST-NH_2	36	<1
Ac-PT-(Galβ1–3GalNAcα)**T**TGIST-NH_2	49	6
Ac-P-(Galβ1–3GalNAcα)**T**TTGIST-NH_2	7	<1
Ac-GTT-(Galβ1–3GalNAcα)**T**PIST-NH_2	14	<1
Ac-GT-(Galβ1–3GalNAcα)**T**TPIST-NH_2	13	5
Ac-G-(Galβ1–3GalNAcα)**T**TTPIST-NH_2	12	8
Ac-PT-(Galβ1–3GalNAcβ)**T**TPIST-NH_2	43	15
Ac-P-(Galβ1–3GalNAcβ)**T**TTPIST-NH_2	72	32
AHGVT-(GalNAcα)**S**APDTRPAPGSTAPPA	<1	<1
AHGVT-(Galβ1–3GalNAcα)**S**APDTRPAPGSTAPNA	9	<1
AHGVT-(Galβ1–3GalNAcα)**S**APESRPAPGSTAPNA	9	<1
AHGVT-(Galβ1–3GalNAcα)**S**APDTRPAPGSTAPTA	9	<1
AHGVT-(Galβ1–3GalNAcα)**S**APETRPAPGSTAPTA	11	<1
AHGVT-(Galβ1–3GalNAcα)**S**APDTRPAPGSTAP- (Galβ1–3GalNAcα)**T**A	18	<1
AHGVT-(Galβ1–3GalNAcα)**S**APDTRPAPGS- (Galβ1–3GalNAcα)**S**APPA	9	<1

[a] 100% C2GnT1 activity corresponds to 0.034 μmol/h/mg.

[b] 100% C2GnT2 activity corresponds to 0.220 μmol/h/mg.

Table 4

Inhibition of human C1GalT, C3GnT, C2GnT1 and C2GnT2. Human recombinant enzymes were assayed as described in Material and methods with 0.5 mM GalNAcα-Bn (for C1GalT and C3GnT) or Galβ1–3GalNAcα-pnp (for C2GnT1 and C2GnT2) as the acceptor substrate. All inhibitor compounds were inactive as substrates. The inhibitor concentration in the assay was at a 1:1 ratio with the acceptor substrate (0.5 mM). Other bis-imidazolium compounds with similar structure but having aliphatic linker-chains containing 4, 6, 8, 10, 11, 12, 13 or 14 carbons in length were inactive as substrates and as inhibitors.

Inhibitor (0.5 mM in assays)	C1GalT		C3GnT		C2GnT1		C2GnT2
	Inhibition (%)	IC_{50} (mM)	Inhibition (%)	IC_{50} (mM)	Inhibition (%)	IC_{50} (mM)	Inhibition (%)
1,15-Bis-(3-methyl-1*H*-imidazolium-1-yl)pentadecane dichloride	<1		<1		<1		<1
1,16-Bis-(3-methyl-1*H*-imidazolium-1-yl)hexadecane dichloride	<1		<1		81	0.23	<1
1,18-Bis-(3-methyl-1*H*-imidazolium-1-yl)octadecane dichloride	<1		<1		100	0.02	<1
1,20-Bis-(3-methyl-1*H*-imidazolium-1-yl)eicosane dichloride	<1		95	0.14	100	0.06	<1
1,20-Bis-(3-methyl-1*H*-imidazolium-1-yl)eicosane dimesylate	<1		80	0.13	100		<1
1,22-Bis-(3-methyl-1*H*-imidazolium-1-yl)docosane dimesylate	<1		78	0.26	100	0.07	<1
1-Thio-*N*-butrylGlcNβ-(2-naphthyl)	<1		<1		<1		<1
2-*N*-Butyryl-GalNα-Bn	16	2.31	<1		<1		<1

Table 5

Glycosyltransferases used in this study. All of the enzymes used are human, inverting transferases having a predicted GT-A fold [52]. The GT family assignment is from the CAZy data bank. The DxD motif may contain one or more catalytically active acidic amino acids. Although N-glycosylation sites are present, their occupation by N-glycans and their function in enzyme stability has only been shown for C2GnT1 [42]. Multiple Cys residues function in forming protein dimers (C1GalT) [14] and/or disulfide bonds (C1GnT1) [45]. -T, -transferase.

Enzyme names	Accession #	EC#	GT family	Fold	DxD motif	N-Glycan sites	Cys
C1GalT, core 1 β1,3-Gal-T, Core 1 synthase, T-synthase, Core 1-T	NP_064541	2.4.1.122	GT31	GT-A	DAD	0	7
C3GnT, core 3 β1,3-GlcNAc-T, Core 3 synthase, β3GnT6	Q6ZMB0	2.4.1.147	GT31	GT-A	DDD	3	3
C2GnT1, core 2 β1,6-GlcNAc-T, Core 2-T	Q02742	2.4.1.102	GT14	GT-A	DE, DVDVD	2	2
C2GnT2, core 2/4 β1,6-GlcNAc-T, Core 4-T	AAD10824	2.4.1.148	GT14	GT-A	DE, DSD, DID	2	2