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Specificity of β1,4-galactosyltransferase inhibition by 2-naphthyl 2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside

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Abstract Inhibitors of Galactosyltransferase (GalT) have the potential of reducing the amounts of adhesive carbohydrates on secreted and cell surface-bound glycoproteins. We recently found a potent inhibitor of β4GalT, 2-naphthyl 2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside (compound **612**). In this work, we have tested compound **612** for the specificity of its inhibition and examined its effect on GalT, and on GlcNAc- and GalNAc-transferases in homogenates of different cell lines, as well as on recombinant glycosyltransferases. Compound **612** was found to be a specific inhibitor of β4GalT. The specificity of recombinant human β3GalT5 that also acts on GlcNAc-R substrates, revealed similarities to bovine milk β4GalT. However, **612** was a poor substrate and not an inhibitor for β3GalT5. To further determine the specific structures responsible for the inhibitory property of **612**, we synthesized (2-naphthyl)-2-butanamido-2-deoxy-β-D-glucopyranosylamine (compound **629**) containing nitrogen in the glycosidic linkage, and compared it to other naphthyl and quinolinyl derivatives of GlcNAc as substrates and inhibitors. Compound **629** was a substrate for both β4GalT and β3GalT5. This suggests that properties of **612** other than the presence of the naphthyl ring alone were responsible for its inhibitory action. The results suggest a usefulness of **612** in specifically blocking the synthesis of type 2 chains and thus epitopes attached to type 2 chains. In addition, **612** potently inhibits β4GalT in cell homogenates and thus allows assaying β3GalT activity in the presence of β4GalT.

Keywords Galactosyltransferase · Inhibition · Substrate specificity · Glycosyltransferases · Chemical synthesis

Abbreviations

Bn	benzyl
FBS	fetal bovine serum
GalT	galactosyltransferase
Me	methyl
PBS	phosphate buffered saline
Ph	phenyl
Pnp	p-nitrophenyl
HMBC	Heteronuclear Multiple Bond Correlation Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy

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Introduction

The synthesis of glycoconjugates by glycosyltransferases plays a critical role in mediating biological recognition processes [1, 2]. Galactosyltransferases (GalT) add a Gal residue to glycoprotein-bound glycans, which can be further extended to form sialylated, fucosylated and other

complex carbohydrate epitopes. These epitopes play important roles in cell-cell and cell-lectin interactions, in homing of lymphocytes, the immune system, inflammation, bacterial adhesion, fertilization and other biological processes. Gal residues are recognized by galectins that control cell-cell or cell-matrix interactions, apoptosis and signal transduction [3–6]. In cancer, these interactions can regulate the invasive and metastatic behaviour of cells [7–10].

Members of the β3GalT and β4GalT families [11–13] are involved in the extension of glycan chains. β4GalT synthesize the common type 2 chain backbone, Galβ1-4GlcNAcβ1-3, which can be extended and branched, and serves as a scaffold for subsequent modifications and the attachment of blood group and Lewis x and y antigens. β3GalT catalyze the biosynthesis of type 1 chains, Galβ1-3GlcNAcβ1-3, that can carry Lewis a and b antigens. The abundance of these epitopes is often altered in cancer and is related to the metastatic potential of cancer cells [7, 10, 14, 15].

β3GalT5 is the key enzyme involved in the synthesis of type 1 chains [16–20]. The activity and transcript levels of β3GalT5 were shown to be decreased in tissue samples of human colon adenocarcinoma, relative to the surrounding normal mucosa [15–17]. In contrast, the activity of β4GalT has been found to be increased in cancer [21–23]. The activity or mRNA levels of β4GalT also increase in endothelial and cartilage derived cells after treatment of cells with tumor necrosis factor (TNF)α [24, 25]. These alterations are expected to cause a shift towards type 2 chains in inflamed and tumor tissues. We have therefore developed inhibitors for β4GalT that may serve to decrease the abundance of type 2 chains, and reduce the levels of sialyl-Lewis x determinants involved in selectin binding, cancer invasiveness and metastasis [26–28].

GlcNAc-naphthyl derivatives have previously been shown to be inhibitors of bovine milk β4GalT and did not act as acceptor substrates [26]. In particular, 2-naphthyl 2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside (compound **612**) has been used in airway cell cultures to decrease cell surface galactosylation and receptor sites for *Pseudomonas aeruginosa* [27]. However, it has not been determined if these inhibitors are specific for β4GalT. In the present work, we determined the nature and specificity of **612,** and examined the inhibition of a number of recombinant glycosyltransferases and enzymes from cell homogenates. We focused on β4GalT1 and β3GalT5, two enzymes that are important for the extension of glycoprotein-bound glycans. We found that **612** is a specific inhibitor for β4GalT and thus has the potential to reduce the synthesis of type 2 glycan chains. Replacement of the sulfur atom with nitrogen in the glycosidic linkage of **612** abrogated the inhibitory properties of **612**.

Materials and methods

Materials

Materials were purchased from Sigma unless stated otherwise. GlcNAc-analogs, including compounds were synthesized or obtained as previously described [26, 29–33]. Insect cell supernatants containing soluble human GlcNAc-transferases I and II (GnT I or GnT II) were kindly donated by H. Schachter, Hospital for Sick Children, Toronto. GnT II substrate Manα1-6(GlcNAcβ1-2Manα1-3)Manβ-octyl was produced from Manα1-6(Manα1-3)Manβ-octyl (Man_3-octyl, from Toronto Research Chemicals) using GnT I [30]. The GnT I product was purified by anion exchange chromatography and reverse phase HPLC, and was free of GnT I substrate as judged by GnT I assays and mass spectrometry [31]. Recombinant soluble bovine polypeptide GalNAc-transferase T1 (ppGalNAcT1) was donated by A. Elhammer [34] and stored at −20°C. The enzyme that synthesizes O-glycan core 2, soluble human core 2 GlcNAc-transferase (C2GnT1), was produced in insect cells and stored at −80°C as described [35]. NMR and mass spectrometry was carried out in the Department of Chemistry, Queen's University, as described [26, 32].

Genes encoding soluble human β3GalT1 and 2 [12], cloned into baculovirus vectors were obtained from Henrik Clausen, University of Denmark, Copenhagen. Sf9 insect cells were grown by Don Jarvis, University of Wyoming, in complete TNM-FH medium containing 10% fetal bovine serum (FBS) [36]. Cells (2 million cells/5 ml medium) were infected with baculovirus (E2) and the culture supernatants were harvested at 24, 48 and 72 hpi. Soluble human β3GalT5 was expressed in insect cells in the absence of FBS and in the presence of BSA. The cell supernatant, as well as the monoclonal antibody MAb5B8, which is specific to β3GalT5, were kindly provided by Henrik Clausen. Enzymes were stored at −80°C or for several weeks at 4°C.

Purification of β3GalT5

β3GalT5 from Sf9 insect cell supernatants with a specific activity of 19.3 nmol/h/mg using 0.5 mM GlcNAcβ-Bn substrate was purified as follows. Enzyme was applied to Q-Sepharose and eluted with 25 mM Tris–HCl, pH 7.2, 10 mM NaCl, 1 mM $MnCl_2$. Subsequently, eluted enzyme was applied to S-Sepharose and eluted with 35 mM MES, pH 6.0, 1 mM $MnCl_2$, and a gradient of 0.05 to 1 M NaCl. Fractions were assayed for β3GalT5 activity and analyzed by SDS-PAGE. Fractions that contained the highest β3GalT5 activity were pooled and stored at −80°C. This purified enzyme solution had an activity of 6.5 μmol/h/mg using 0.5 mM GlcNAcβ-Bn as the acceptor substrate.

ELISA assay

The confirmation of β3GalT5 expression was made with an ELISA assay using antibody MAb5B8. Briefly, purified β3GalT5 was diluted 1:5, 1:10 and 1:50 in coating buffer (0.05 M carbonate-bicarbonate, pH 9.7). 100 μl of β3GalT5 preparation were transferred into each well of 24 well plates and incubated at 4°C overnight. After incubation, supernatants were removed and wells washed 3 times with 200 μl PBS containing 10% Tween, followed by blocking at 4°C overnight with 200 μl PBS containing 1% BSA. Wells were then washed 3 times before transfer into each well of 100 μl primary antibody MAb 5B8 (1:4 dilution in PBS containing 10% Tween and 1% BSA). After incubation for 1 h at room temperature (rt), wells were washed and 100 μl of secondary antibody (donkey anti-mouse IgG-HPR at 1:1000 dilution in PBS containing 10% Tween and 1% BSA) were applied and incubated for 1 h at rt. Each well was washed carefully before adding 100 μl of chromogenic TMB substrate solution (3,3′, 5,5′-tetramethylbenzidine). The reaction was allowed to proceed for 30 min; the reaction was then stopped with 1% SDS. The absorbance at 370 nm was read with a microplate reader.

Glycosyltransferase assays

Glycosyltransferases were assayed as previously described [26, 28, 29, 31–33, 35]. Enzyme products were isolated by the AG1x8 or C18 Sep-Pak method, and analyzed by reverse phase HPLC using a C18 column and acetonitrile/water mixtures as the mobile phase. Assays were carried out at least in duplicate determinations. GalT were assayed in a total volume of 40 μl containing 0.5 mM acceptor substrate (or as indicated in the tables), recombinant enzyme preparation or cell homogenate (0.1 to 0.2 mg protein), 0.5 mM UDP-[^{3}H]Gal (1600–1800 cpm/nmol), 0.125 M MES buffer (pH 7), 12.5 mM $MnCl_2$, 10 mM AMP, and 5 mM γ-galactonolactone. In assays of cell homogenates, 0.125% Triton X-100 was present. The standard GalT assays contained GlcNAcβ-Bn as the acceptor substrate, and control assays lacked the acceptor. Reaction mixtures were incubated for 1 h at 37°C, and products isolated using 0.2 ml columns of AG1×8 (100–200 mesh). Radioactivity of the eluate was determined by scintillation counting. For further analysis of products, mixtures were separated by reverse phase HPLC [26]. Kinetic parameters were determined using the program OriginPro [26, 28].

Core 1 β3GalT was assayed similarly using 0.5 or 1 mM GalNAcα-Bn as the acceptor. Polypeptide GalNAc-transferase activities were assayed using 1 mM UDP-[^{3}H]GalNAc as the donor and 1 mM **362** (TTTVTPTPTG) or 2.5 mM AQPTPPP as the acceptor. GlcNAc-transferases were assayed using 1 mM UDP-[^{3}H]GlcNAc as the donor and 2 mM Galβ1-3GalNAcα-Bn acceptor (for core 2 β6-GlcNAc-transferase), 2 mM GlcNAcβ1-3GalNAcα-pnp (for core 4 β6-GlcNAc-transferase), 2 mM GalNAcα−Bn (for core 3 β3-GlcNAc-transferase), 0.5 mM Man3octyl (for GnT I) or 1.7 mM Manα1-6(GlcNAcβ1-2Manα1-3)Manβ-octyl (for GnT II). Mn^{2+} was omitted in β6-GlcNAc-transferase assays. GlcNAc-transferase V (Gn-TV) was assayed as described [33], followed by HPLC separation of substrates and products. For inhibition studies, compound **612** was present at 0.5 mM concentration with 10% methanol in the assay, or as indicated in the tables.

Large scale preparation of GalT products

For the large scale preparation of non-radioactive GalT enzyme products for structural analysis, GlcNAcβ-benzyl was used as acceptor substrate and non-radioactive UDP-Gal, and the assay was scaled up 100-fold. Products were purified by ion exchange chromatography (using 10 ml AG1x8, 100–200 mesh), followed by HPLC, using a C18 column and 7% acetonitrile/93% water as the eluant [26]. The enzyme products, Galβ1-3/4GlcNAcβ-benzyl were exchanged with 99.96% D_2O and analyzed by 600 MHz ^{1}H-NMR spectroscopy, using a Bruker spectrometer at Queen's University, Department of Chemistry.

Synthesis of (2-naphthyl)-2-butanamido-2-deoxy-β-D-glucopyranosylamine (**629**)

To a solution of 2-butanamido-2-deoxy-D-glucopyranose [37] (500 mg, 2.0 mmol) and 2-naphthylamine (200 mg, 1.4 mmol) in methanol–water (9:1 v/v, 10 ml) were added ~3 drops of 0.5 N HCl and the reaction mixture was stirred at rt for 6 h, then heated at 60°C for 10 h. The mixture was neutralized with $NaHCO_3$, washed with ethyl acetate and filtered. The filtrate was concentrated, and the residue was washed with ethyl acetate–hexane (1:3 v/v) to give the title compound as a white solid (340 mg, 45%): mp 196–197°C; $[\alpha]_D^{25} -18.8°$ (c 0.2, CH_3OH); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.79 (t, J=7.4 Hz, 3 H), 1.43–1.58 (m, 2 H), 2.01–2.16 (m, 2 H), 3.16–3.25 (m, 1 H), 3.25–3.30 (m, 1 H), 3.40–3.57 (m, 2 H), 3.64–3.77 (m, 2 H), 4.47–4.56 (m, 2 H), 4.97 (app d, J=5.6 Hz, 1 H), 5.04 (app d, J=5.2 Hz, 1 H), 6.27 (d, J=6.8 Hz, 1 H, H-1), 6.84–6.93 (m, 2 H), 7.16 (app t, J=7.0 Hz, 1 H), 7.32 (app t, J=7.0 Hz, 1 H), 7.54–7.71 (m, 3 H), 7.98 (d, J=8.0 Hz, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.4, 18.7, 37.4, 55.2, 60.9, 70.8, 74.4, 77.4, 85.7 (C-1), 105.4, 117.5, 121.8, 125.8, 126.1, 127.2, 127.5, 128.5, 134.7, 144.6, 174.4; HRMS (EI) Calculated for $C_{20}H_{27}N_2O_5$: 375.1919 $[M+H]^+$. Found: 375.1909.

Cell cultures and treatments

All of the cell growth media contained 100 U/ml Penicillin and 0.1 mg/ml Streptomycin. Human colonic cancer cells HT29 and mouse lymphocytic cells MOPC and P388 were grown in DMEM medium (GIBCO) containing 10% FBS. Human airway epithelial cancer cells NCI-H292 were propagated in RPMI 1640 medium (GIBCO) containing 10% FBS. Human colon cancer cells Caco-2 were grown in DMEM medium (GIBCO) containing 10% FBS. Prostate cancer cells PC-3, DU145, LNCap and VCap, as well as normal prostate cells (ATCC) were grown as recommended by ATCC. Cell homogenates were prepared by hand homogenizing in 0.25 M sucrose of cells washed with PBS. The protein content was determined by the BioRad (Bradford) method.

Results

Inhibition of glycosyltransferases in cell homogenates by **612**

Mammalian cells express a combination of different members of the β3GalT and β4GalT families which are Golgi membrane-bound. We used cell homogenates to test whether these membrane-bound enzymes would be inhibited by **612.** Utilizing GlcNAcβ-Bn as the acceptor substrate, GalT activities were high in all homogenates tested, including those from lung cancer cells H292, intestinal cancer cells Caco-2, mouse lymphocytic cells MOPC and P388, and prostate cell lines RWPE-1, PC-3, DU145, LNCap and VCap. The addition of 0.5 mM compound **612** inhibited GalT activities by 68% in P388 cells, and by more than 93% in all other cell homogenates.

In order to determine the specificity of inhibition in cell homogenates, we tested a number of cell-derived glycosyltransferases in the presence and absence of **612** (Table 1). In the absence of the inhibitor, the activity of polypeptide GalNAc-transferase (ppGalNAcT), using 2.5 mM AQPTPPP as the acceptor substrate, was high in H292 cell homogenates (115.3 nmol/h/mg). The presence of 1 mM **612** in the assay reduced ppGalNAcT activity in lung and other cell homogenates by <4% (Table 1). No inhibition by compound **612** of core 1 β3Gal-transferase (C1GalT) was observed in cell homogenates from lung and lymphocytic cells. While homogenates from P388 cells did not show detectable activity of core 2 β6GlcNAc-transferase (C2GnT), MOPC cell homogenates showed low activities, which were not inhibited by **612**. HPLC assays showed that C2GnT activity in lung cells (2.8 nmol/h/mg) was inhibited by less than 14%. There was no detectable activity of core 3 β3GlcNAc-transferase (C3GnT), and core 4 β6-GlcNAc-

Table 1 Inhibition by compound **612** of glycosyltransferase activities in cell homogenates

Enzyme	Cell type	Substrate	Inhibitor	Activity without inhibitor (nmol/h/mg)	Inhibition (%)
β3/4GalT	H292	0.5 mM GlcNAcβ-Bn	0.5 mM **612**	34.9	95
β3/4GalT	CaCo-2	0.5 mM GlcNAcβ-Bn	0.5 mM **612**	38.4	98
β3/4GalT	MOPC	1.0 mM GlcNAcβ-Bn	1.0 mM **612**	2.3	93
β3/4GalT	P388	1.0 mM GlcNAcβ-Bn	1.0 mM **612**	167.0	68
ppGalNAcT	H292	2.5 mM AQPTPPP	1.0 mM **612**	115.3	< 4
ppGalNAcT	MOPC	1.0 mM AQPTPPP	1.0 mM **612**	0.7	< 1
C1GalT	H292	0.5 mM GalNAcα-Bn	0.5 mM **612**	1.4	< 1
C1GalT	P388	1.0 mM GalNAcα-Bn	1.0 mM **612**	15.8	< 1
C2GnT	H292	2.0 mM Galβ1-3GalNAcα-Bn	0.5 mM **612**	2.8	< 14
C3GnT	H292	2.0 mM GalNAcα-Bn	0.5 mM **612**	<1	nd
C4GnT	H292	2.0 mM GlcNAcβ1-3GalNAcα-pnp	0.5 mM **612**	<1	nd
GnT I	H292	0.5 mM Man_3Oct	0.5 mM **612**	1.4	<1
GnT II	H292	1.7 mM $GnMan_3Oct$	0.5 mM **612**	7.7	<1
GnT V	PC-3	0.5 mM GnMan(Gn4Man)4ManOct	0.5 mM **612**	0.6	<1

Glycosyltransferase activities in cell homogenates were assayed as described in Materials and Methods. Compound **612** (2-naphthyl-2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside) was added to the complete assay at a final concentration indicated in the table. The methanol concentration in assays was 10%. Homogenates were prepared from human airway cells H292, human colonic cancer cells Caco-2, and mouse lymphocytic cells MOPC and P388. ppGalNAcT, polypeptide GalNAc-transferase; C1GalT, core 1 β3Gal-transferase; C2GnT, core 2 β6GlcNAc-transferase; C3GnT, core 3 β3GlcNAc-transferase; C4GnT, core 4 β6-GlcNAc-transferase; GnT I, GlcNAc-transferase I; GnT II, GlcNAc-transferase II. Man_3Oct, Manα1-6(Manα1-3)Manβ-octyl; $GnMan_3Oct$, Manα1-6(GlcNAcβ1-2Manα1-3)Manβ-octyl; GnMan(Gn4Man)4Man-Oct, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[4-deoxy-Manα1-3]4-O-methylManβ-octyl; Bn, benzyl; nd, not determined; pnp, *p*-nitrophenyl

Table 2 Activities and inhibition of purified and recombinant glycosyltransferases

Enzyme	Substrate	Activity (%)	Inhibitor	Inhibition (%)
Bovine milk β4GalT	0.5 mM GlcNAcβ-Bn	100		
	0.5 mM **612**	<1		
	0.5 mM **633**	<1		
	0.5 mM **629**	23		
	0.5 mM GlcNAcβ-Bn		0.5 mM **612**	95
	0.5 mM GlcNAcβ-Bn		0.5 mM **633**	0
	0.5 mM GlcNAcβ-Bn		0.5 mM **629**	29
Bovine ppGalNAcT	0.5 mM AQPTPPP		0.5 mM **612**	<7
Human C2GnT1	0.5 mM Galβ1-3GalNAcα -Bn		0.5 mM **612**	<7
Human GnTI	0.5 mM Man_3Oct		0.5 mM **612**	<7
Human GnTII	1.7 mM $GnMan_3Oct$		0.5 mM **612**	<7

Purified and recombinant glycosyltransferases were assayed using the substrates and inhibitors indicated, as described in the Materials and Methods section. ppGalNAcT, polypeptide GalNAc-transferase; C2GnT1, core 2 β6GlcNAc-transferase 1, GnT I, GlcNAc-transferase I; GnT II, GlcNAc-transferase II; Man_3Oct, Manα1-6(Manα1-3)Manβ-octyl; $GnMan_3Oct$, Manα1-6(GlcNAcβ1-2Manα1-3)Manβ-octyl; Bn, benzyl

transferase (C4GnT) activity was very low in H292 cell homogenates (0.03 nmol/h/mg). However, GnT I and II were active in H292 cell homogenates but were not affected by the presence of 0.5 mM **612**. Gn-TV activity in prostate cancer PC-3 cells was not inhibited by **612**. This suggested that **612** blocks the synthesis of Gal extensions of complex O- and N-glycans but does not affect the synthesis of underlying structures.

To confirm the absence of inhibitory effects of **612** on specific glycosyltransferases, recombinant enzymes were tested with and without **612** (Table 2). Soluble bovine polypeptide GalNAcT1, human GnT I and GnT II, and human C2GnT1 activities showed inhibition of <7% by 0.5 mM **612**. However, bovine milk β4GalT1 was strongly inhibited (>95%). It is interesting that the inhibitor is effective in GalT assays in aqueous solution and in the presence of 10% methanol, although the compound shows no apparent solubility in water.

In contrast to the strong inhibition seen by compound **612**, compound **633** was neither a GalT substrate nor an inhibitor of purified β4GalT or GalT in H292 lung cell homogenates. Peracetylation of sugar derivatives has been shown to facilitate entry of disaccharides into cells [8, 9], with subsequent de-acetylation and metabolism inside cells. We therefore tested both, compounds **612** and the peracetylated form of **612** (compound **633**) (Table 2, 3), for their effect in lung cell cultures [27]. Preliminary data (not shown) suggested that 1 mM **612** in the cell medium of lung cells H292 reduced cell surface galactosylation but **633** had no effect. Therefore, **612** appeared to be taken up by cells. The peracetylated compound **633** may have also penetrated into cells but was probably not converted to its active, deacetylated form of the GalT inhibitor (**612**).

Inhibition of GalT by GlcNAc-naphthyl derivatives

We have previously shown that several derivatives of GlcNAcβ-2-naphthyl, in spite of containing GlcNAc as the acceptor site, were not substrates for β4GalT but these compounds inhibited the enzyme [26]. Using human lung cell H292 homogenate, naphthyl derivatives **581** (GlcNAcβ-O-2-naphthyl) and **589 (**GlcNAcβ-S-2-naphthyl) were ineffective as substrates for GalT and inhibited the activity by 5% (Table 3).

Several hydrophobic amino acids are present in the acceptor binding site of β4GalT [38, 39]; thus the hydrophobic properties of the naphthyl ring structure may enable these substrate analogs to tightly bind to GalT, forming potent inhibitors. The introduction of a nitrogen atom into the

Table 3 Inhibition of GalT in H292 cells by GlcNAc-naphthyl derivatives

Substrate	Activity %	Inhibitor	Inhibition %
0.5 mM GlcNAcβ-Bn	100		
0.5 mM **612**	<1		
0.5 mM **633**	<1		
0.5 mM **629**	22		
0.5 mM **581**	<1		
0.5 mM **589**	<1		
0.5 mM GlcNAcβ-Bn		0.5 mM **612**	95
0.5 mM GlcNAcβ-Bn		0.5 mM **633**	<5
0.5 mM GlcNAcβ-Bn		0.5 mM **629**	7
0.5 mM GlcNAcβ-Bn		0.5 mM **581**	< 5
0.5 mM GlcNAcβ-Bn		0.5 mM **589**	< 5

GalT was assayed in H292 cell homogenates using the substrates and inhibitors indicated, as described in the Materials and Methods section

Table 4 ^{1}H and ^{13}C NMR parameters of enzyme products and substrate

^{1}H-chemical shift (ppm)	GlcNAcβ-Bn	Galβ1-4GlcNAcβ-Bn	Galβ1-3GlcNAcβ-Bn
GlcNAc			
H-1	4.53	4.47 (8.5 Hz)	4.45
H-2	3.61	3.69	3.70
H-3	3.39	3.58	3.59
H-4	3.40	3.67	3.41
H-5	3.36	3.52	3.34
H-6	3.68,3.86	3.77, 4.95	3.34, 3.65
Galβ1-4			
H-1		4.42 (7.8 Hz)	
H-2		3.48	
H-3		3.61	
H-4		3.86	
H-5		3.67	
H-6		nd	
Galβ1-3			
H-1			4.25
H-2			3.36
H-3			3.48
H-4			3.76
H-5			3.55
H-6			3.61
^{13}C-chemical shift (ppm)	GlcNAcβ-Bn	Galβ1-4GlcNAcβ-Bn	Galβ1-3GlcNAcβ-Bn
GlcNAc			
C-1	99.7	99.6	99.5
C-2	55.4	54.9	54.5
C-3	75.8	72.3 (71.4)	82.3
C-4	73.6	78.2	68.6
C-5	69.8	74.7	75.4
C-6	60.7	60.0	60.8
Galβ1-4			
C-1		102.6	
C-2		70.8	
C-3		72.3 (71.4)	
C-4		68.5	
C-5		75.1	
C-6		60.9	
Galβ1-3			
C-1			103.3
C-2			70.6
C-3			72.5
C-4			68.5
C-5			75.3
C-6			61.0

600 MHz NMR spectra were collected in 1D and 2D experiments. The substrate for GalT was GlcNAcβ-Bn. Bovine milk β4GalT was used to produce Galβ1-4GlcNAcβ-Bn as described in the Materials and Methods section. Human β3GalT5 was used to produce Galβ1-3GlcNAcβ-Bn. GlcNAc derivatives were purified by HPLC and exchanged with D_2O

naphthyl ring structure (quinolinyl derivatives) yielded substrates for β4GalT, probably by distorting the ring structure and/or by adding a charge to the compound. In order to examine if the naphthyl ring structure was indeed responsible for the inhibitory activity, we introduced a nitrogen atom into the anomeric linkage, leaving the naphthyl ring intact, in GlcNBuβ-NH-2-naphthyl (compound **629**). In contrast to **612**, compound **629** proved to be a substrate for bovine milk β4GalT (Table 2). This shows that it was not the naphthyl ring alone that was responsible for the inhibition of GalT by

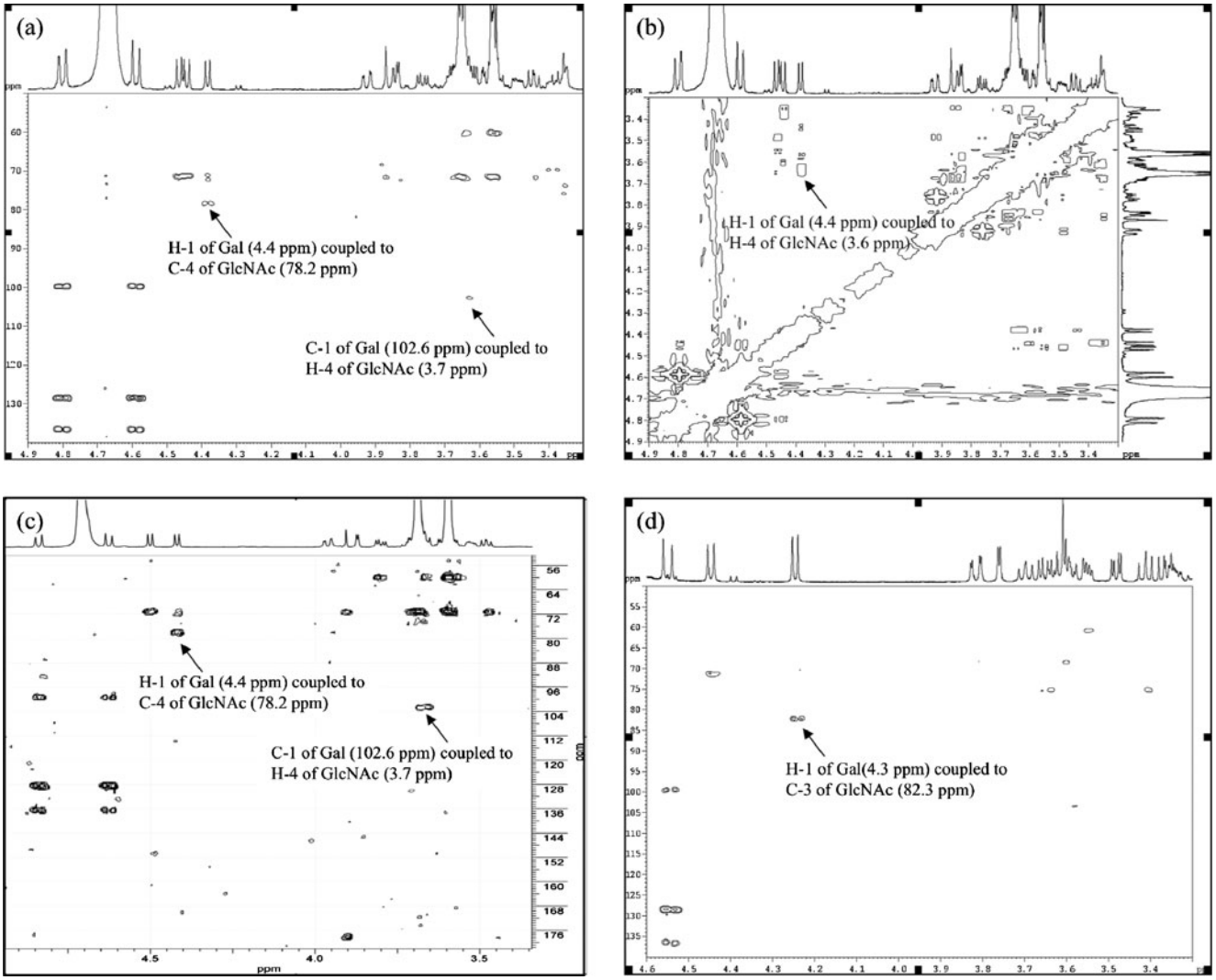

Fig. 1 Two-dimensional 600 MHz spectra of Gal-transferase enzyme products. **a** and **b** Galβ1-4GlcNAcβ-Bn was prepared from crude β4GalT1 (containing FBS) as described in the Materials and Methods section. **c** Galβ1-4GlcNAcβBn was prepared with purified bovine milk β4GalT. **d** Galβ1-3GlcNAcβBn was prepared with crude β3GalT5 (in the absence of FBS) using GlcNAcβ-Bn as the substrate. NMR experiments (HMBC) were performed at rt. **a**, **c**, **d**, HMBC; **b**, NOESY

compounds **612**, **581** and **589**. The overall charge and hydrophobicity of the compounds may also be important. When the nitrogen atom is present at various positions in the naphthyl ring (in quinolinyl derivatives), the compounds are good GalT substrates [26]. Thus, quinolinyl derivatives have apparent K_M values of 0.11 to 0.25 mM and V_{max} of 6.5–19.8 μmol/h/mg [26] with bovine milk β4GalT. Compound **629** was found to be a less effective substrate with an apparent K_M of 0.3 mM and a V_{max} of 2.7 nmol/h/mg for bovine milk β4GalT. As expected for a competing substrate, **629** decreased the transfer of Gal to the standard substrate GlcNAcβ-Bn by 29%, at 0.5 mM concentrations of both substrates, as shown by HPLC isolation of Galβ1-4GlcNAcβ-Bn product. Using H292 lung cell homogenates, **629** also served as a GalT substrate (22% of GlcNAcβ-Bn control), and showed a minor inhibition of Gal incorporation into GlcNAcβ-Bn (7%), at equimolar concentration of both **629** and GlcNAcβ-Bn substrates (Table 3).

Specificity and inhibition of β3GalT5

The inhibition of **612** was studied with an important member of the β3GalT family, β3GalT5, expressed in insect cells in the absence of FBS. A large scale enzyme product was prepared, purified by HPLC using a C18 column, and analyzed by NMR (Table 4). The enzyme product using GlcNAcβ-Bn substrate was identified as Galβ1-3GlcNAcβ-Bn, with a doublet of H-1 at 4.25 ppm due to Galβ. A major shift of the C-3 signal of the GlcNAc moiety from 75.8 ppm in the substrate to 82.3 ppm in the product, seen in the HMBC spectrum (Fig. 1), identified the 1–3 linkage.

β3GalT5 was purified 336-fold and on SDS-PAGE showed a major protein band at 34 kDa. The reaction was linear with respect to protein concentration up to 0.01 mg/ml and incubation time up to 1 h. The apparent K_M for UDP-Gal was 0.8 mM, and V_{max} was 87 μmol/h/mg using GlcNAcβ-Bn as the acceptor (Fig. 2a). The apparent K_M for GlcNAcβ-Bn was 4.9 mM and V_{max} was 159 μmol/h/mg. High activities were observed with GlcNAcβ1-3GalNAcα-pnp as the acceptor, with an apparent K_M of 1.7 mM and V_{max} of 172 μmol/h/mg. Similarly, GlcNAc1-3Gal-methyl acceptor showed an apparent K_M of 1.7 mM and V_{max} of 205 μmol/h/mg (Fig. 2b). This finding is consistent with those of Zhou *et al.* [18].

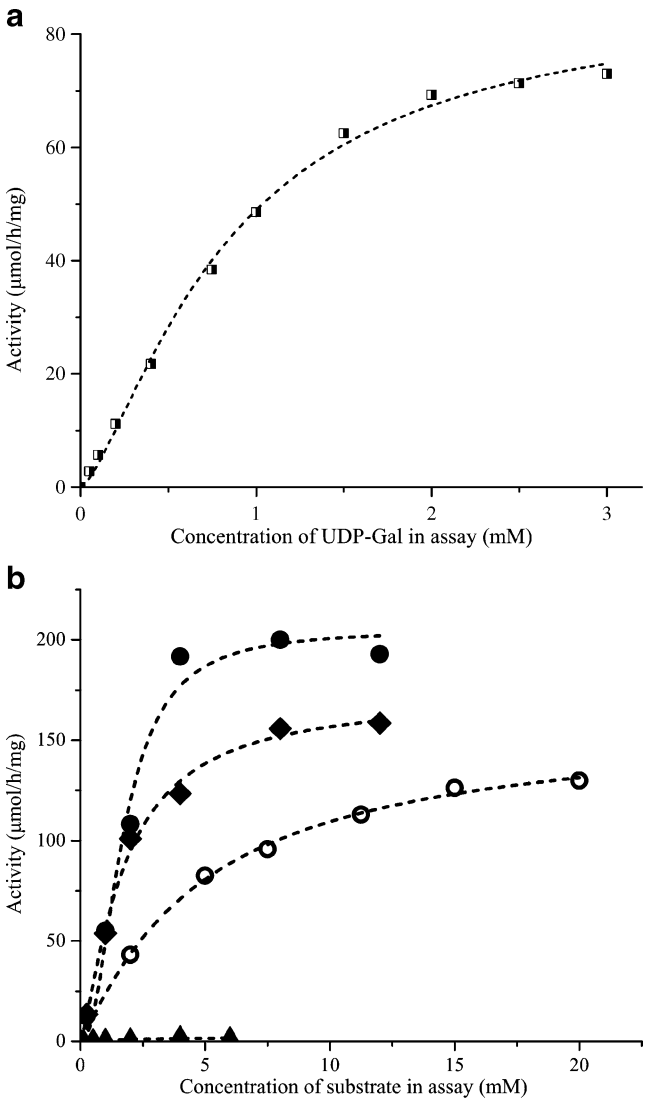

Fig. 2 **a** Kinetics of β3GalT5 activity. The reaction rates of purified β3GalT5 were determined as a function of UDP-Gal concentration with 5 mM GlcNAcβ-Bn as the acceptor substrate. The curves were fitted by non-linear regression with best fit used to determine kinetic mechanism. Apparent K_M=0.8 mM, V_{max}=87 μmol/h/mg. **b** The activities of purified β3GalT5 (in the absence of FBS) are shown. GlcNAcβ-Bn (open circles), GlcNAcβ1-3GalNAcα-pnp (diamonds), GlcNBuβ-NH-2-naphthyl (**629**, triangles) and GlcNAcβ3Galβ-Methyl (closed circles) were used as acceptor substrates, in the presence of 3 mM UDP-Gal concentration in the assay. The curves were fitted by non-linear regression with the best fit used to determine kinetic mechanism. The apparent K_M for GlcNAcβ-Bn was 4.9 mM and V_{max} was 159 μmol/h/mg; the K_M for GlcNAcβ1-3GalNAcα-pnp was 1.7 mM and V_{max} was 172 μmol/h/mg; the K_M for GlcNBuβ-NH-2-naphthyl was 1.8 mM and V_{max} was 1.9 μmol/h/mg, and the K_M for GlcNAcβ1-3Galβ-methyl was 1.7 mM and V_{max} was 205 μmol/h/mg (OriginPro)

The specificity of purified β3GalT5 was also tested using a series of synthetic glycopeptides. At 0.2 mM concentration of acceptors, glycopeptides having GlcNAc only in β1-6 linkage (core 2 and core 6) showed 0.2 to 16% of the activity with GlcNAcβ-Bn. However, glycopeptides having GlcNAc in β1-3 linkage (core 3 and core 4) were very active (113 to 409% of the activity with GlcNAcβ-Bn). This confirms the preference of β3GalT5 for GlcNAcβ1-3Gal(NAc) structures found in O-glycan core 3 and type 1 chains of glycoproteins (Table 5).

GlcNAcβ-Bn was a better substrate for β3GalT5 than GlcNAcα-Bn. Most β-anomers of GlcNAc showed high activities with the exception of compounds having GlcNAc in β1-2 linkage to Man that showed relatively low activities. Although the specificity of purified β3GalT5 largely resembled that of bovine β4GalT1 [26], there were significant differences. The naphthyl derivatives of GlcNAcβ or GlcNBuβ **581, 583, 589, 612** and **629** showed low activities with β3GalT5 from <4% to

Table 5 Substrate specificities of β3GalT5 towards GlcNAc-terminating O-glycopeptides

Substrate (0.2 mM)	Activity (%)
GlcNAcβ-Bn	100
TTTVTP(Gn3GA)**T**PTG (Core 3)	295
TTTV(Gn3GA)**T**PTPTG (Core 3)	252
TT(Gn3GA)**T**VTPTPTG (Core 3)	113
TTTVTP(Gn6GA)**T**PTG (Core 6)	16
TTTV(Gn6GA)**T**PTPTG (Core 6)	17
TT(Gn6GA)**T**VTPTPTG (Core 6)	1
TTTV(Gn[G]GA)**T**PTPTG (Core 2)	1
TT(Gn[G]GA)**T**VTPTPTG (Core 2)	3
T(Gn[G]GA)**T**TVTPTPTG (Core 2)	11
TTTV(Gn[Gn]GA)**T**PTPTG (Core 4)	279
TT(Gn[Gn]GA)**T**VTPTPTG (Core 4)	380
T(Gn[Gn]GA)**T**PTPTG (Core 4)	409

β3GalT5 was expressed in insect cells without FBS in the medium. For the specificity study 336-fold purified β3GalT5 was used. Glycosyltransferase assays were carried out as described using the AG1x8 method [26]. GlcNAc-terminating O-glycopeptide substrates were present in the complete assays at a final concentration of 0.2 mM. The highlighted Thr residues represent the O-Glycosylation sites. Gn3GA, core 3, GlcNAcβ1-3GalNAcα-; Gn6GA, core 6, GlcNAcβ1-6GalNAcα-; Gn[G]GA, core 2, GlcNAcβ1-6 [Galβ1-3]GalNAcα-; Gn[Gn]GA, core 4, GlcNAcβ1-6 [GlcNAcβ1-3]GalNAcα-

35% (Table 6). This is in contrast to β4GalT1 that was inactive with these compounds. Compound **629** was an acceptor substrate for β3GalT5 (5% activity of that with GlcNAcβ-Bn) with an apparent K_M of 1.8 mM and a V_{max} of 1.9 μmol/h/mg (Figs. 2b, and 3). Compound **612** was a substrate showing 3.5% activity, compared to GlcNAcβ-Bn.

The inhibition of β3GalT5 by **612** was tested with both core 3 and GlcNAcβ-Bn as acceptor substrate. No inhibition was observed using 0.25 mM core 3 acceptor and a 2- or 4-fold higher concentration of **612**; however, at a 10-fold higher concentration of **612**, 23% inhibition was observed, indicating a relatively poor binding of the compound to the enzyme. Using 0.5 mM GlcNAcβ-Bn and 0.5 or 1 mM **612**, 22% and 35% inhibition were obtained, respectively (Table 7). The β4GalT inhibitors **581**, **583** and **589** which were poor substrates showed inhibition between <1 and 16%.

Table 6 Substrate specificity of β3GalT5. β3GalT5 was expressed in insect cells and purified to study its substrate specificity, as described in the Materials and Methods section. The activities are given relative to the activity with the standard substrate GlcNAcβ-Bn at the same concentration. The abbreviations are listed in Table 1

#	Compounds	Activity (%)
510	GlcNAcβ-Bn	100
537	GlcNAcα-Bn	7
39	GlcNAc	31
511	GlcNBu	29
558	GlcNBuβ-Bn	121
630	GlcNAcβ-thio-methyl	116
611	GlcNAcβ-thio-ethyl	115
616	GlcNAcβ-thio-phenyl	115
589	GlcNAcβ-thio-naphthyl[a]	4
581	GlcNAcβ-naphthyl[a]	35
583	GlcNBuβ-naphthyl[a]	13
612	GlcNBuβ-thio-naphthyl[a]	<4
629	GlcNBuβ-NH-naphthyl[a]	5
588	GlcNAcβ-3-isoquinolinyl[a]	214
592	GlcNAcβ-6-quinolinyl[a]	207
587	GlcNAcβ-8-quinolinyl[b]	321
53	Gnβ2Mα6Glc-β-allyl	22
54	Gnβ2Mα6Glcβ4Glc-β-allyl	49
192	Gnβ2Mα6(Gnβ2Mα3)M-β-octyl	16
197	Gnβ2Mα6(Gnβ2[4-deoxy-]Mα3)(4-*O*-methyl) M-β-octyl	23
121	GlcNAcβ1-3GalNAcα-pnp	462
50	GlcNAcβ1-3Galβ-methyl	564

[a] Assays were carried out in 10% methanol. [b] Assays were carried out in 7% methanol; M, Man; Gn, GlcNAc; GlcNBu, *N*-butyryl-glucosamine. 100% activity=6.7 μmol/h/mg. Activities of 13% or less were confirmed by HPLC

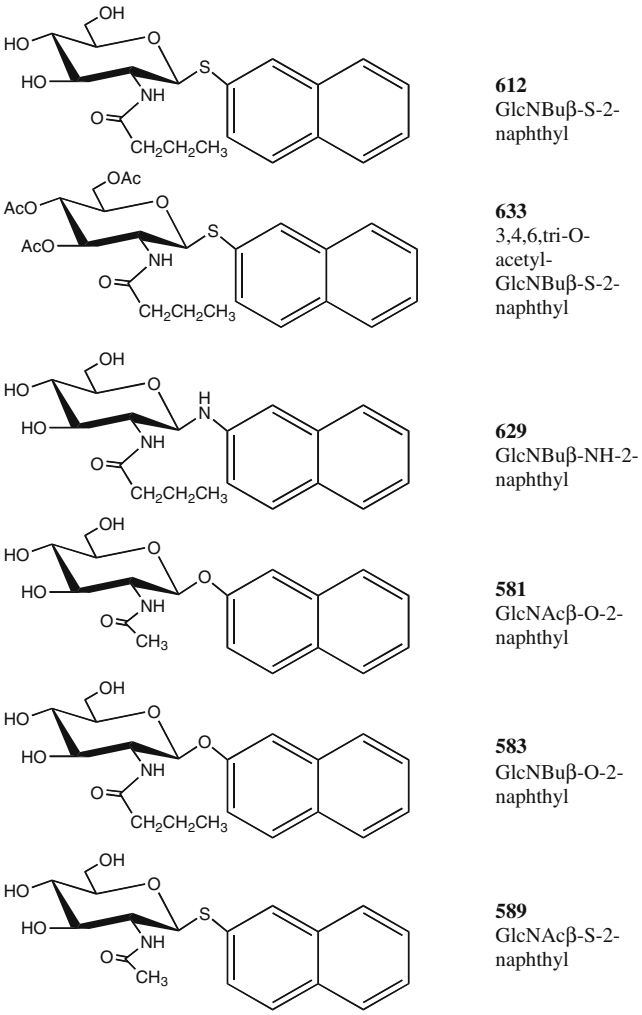

Fig. 3 Structures of analogs of GlcNBuβ-2-naphthyl and GlcNAcβ-2-naphthyl used as acceptor substrates and inhibitor

Studies of recombinant β3GalT1 and 2 in the presence of FBS

In order to examine if **612** could inhibit β3GalT1 and 2, these enzymes were each expressed as soluble enzymes in Sf9 insect cells in the presence of FBS in the cell growth medium. To confirm the β3GalT activity, large scale enzyme products for structure analysis were prepared. T1 and T2 enzyme products were purified by HPLC using a C18 column, and analyzed by 1D-NMR as well as correlation spectroscopy (Table 4). However, the spectra showed that most of the GalT products had the structure Galβ1-4GlcNAcβ-Bn, with a signal of H-1 (4.42 ppm) and C-1 (102.6 ppm) of Gal, and H-4 (3.67 ppm) and C-4 (78.2 ppm) of GlcNAcβ, determined by HMBC (Table 4, Fig. 1). Enzyme products of β3GalT1 and 2 were minor components of the total enzyme product. This suggested that the major GalT activity in the insect cell supernatant

Table 7 Inhibition of β3GalT5 by **612** (2-naphthyl2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside)

Substrate	Inhibitor	% Inhibition
0.25 mM GlcNAcβ1-3GalNAcα-pnp	0.5 mM **612**	<1
0.25 mM GlcNAcβ1-3GalNAcα-pnp	1.0 mM **612**	<1
0.25 mM GlcNAcβ1-3GalNAcα-pnp	2.5 mM **612**	23
0.5 mM GlcNAcβ-Bn	0.5 mM **612**	22
0.5 mM GlcNAcβ-Bn	1.0 mM **612**	35
0.5 mM GlcNAcβ-Bn	0.5 mM **581**	<1
0.5 mM GlcNAcβ-Bn	1.0 mM **581**	<2
0.5 mM GlcNAcβ-Bn	0.5 mM **589**	10
0.5 mM GlcNAcβ-Bn	1.0 mM **589**	16
0.5 mM GlcNAcβ-Bn	0.5 mM **583**	8
0.5 mM GlcNAcβ-Bn	1.0 mM **583**	11

Glycosyltransferase assays were carried out as described in the Materials and Methods section. To measure inhibition, GlcNAcβ-Bn or GlcNAcβ1-3GalNAcα-pnp was utilized as acceptor substrate. Inhibitor **612** was present in the assay at the final concentration indicated in the table. Assays contained 10% methanol in the reaction mixture. The abbreviations are listed in Table 1

was β4GalT. This high background made it very difficult to characterize the β3GalT activities.

A GalT from fetal calf serum that acted on GlcNAc and ovalbumin had previously been reported by Turco and Heath [40] and it was suggested that the enzyme may be similar or identical to the bovine milk enzyme. We therefore characterized GalT in the FBS-containing media of insect cells expressing β3GalT1 and 2, as well as in FBS alone. Media and FBS showed a high activity of β4GalT towards GlcNAcβ-Bn, while BSA was void of activity. The properties and specificities of these GalT activities (data not shown) of FBS-containing preparations showed a striking similarity to those of the previously characterized bovine milk β4GalT [26]. In addition, the GalT activities of FBS-containing insect cell supernatants, and of pure FBS, were strongly inhibited by **612**, suggesting that the FBS enzyme is β4GalT.

Discussion

In this work we showed that the inhibitor **612** is specific for β4GalT. The compound did not inhibit membrane-bound and purified polypeptide GalNAc-transferases and several GlcNAc-transferases but inhibited GalT activities in all human and mouse cell homogenates tested and in bovine serum (FBS), as well as the activity of purified bovine β4GalT1. In contrast, β3GalT5 only showed minimal or no inhibition by **612**. The major difference between β4GalT1 and β3GalT5 was shown to be the preferred recognition of β1-3-linked GlcNAc by β3GalT5, and the binding of naphthyl derivative **612**, which is an acceptor substrate for β3GalT5 with no significant inhibition, while it strongly inhibits β4GalT1. Other properties and the cofactor requirements of β4GalT1 and β3GalT5 were found to be similar.

There is a very high degree (85%) of sequence identity between human and bovine β4GalT. The human β3GalT5 protein showed 34%, 27%, 31% and 23% sequence identity with human β3GalT1, T2, T3 and T4, respectively [18]. β4GalT2, T3, T4, T5, T6 and T7 have 50%, 40%, 36%, 37%, 34% and 25% sequence identity, respectively, to β4GalT1. β4GalT activities from all cell and tissue homogenates studied are strongly inhibited by **612**; it is likely therefore that other members of the β4GalT family are inhibited by **612**. However, this needs to be confirmed with the individual enzymes of the β4GalT family. **612** will be useful in GalT assays of tissue or cell homogenates as a blocker of β4GalT, thus allowing β3GalT activity to proceed.

Among the members of the β3GalT family, β3GalT5 appears to be mainly responsible for type 1 chain synthesis and the extension of the GlcNAcβ1-3 residue of O-glycan cores 3 and 4. The enzyme is highly expressed in small intestine, pancreas and testis [18]. Our specificity studies suggest significant differences in the acceptor binding sites of β4GalT1 and β3GalT5. The specific amino acids responsible for inhibitor binding of β4GalT1 and the binding of **612** to β3GalT5 remain to be determined. The β3-linked GlcNAc may fit into the acceptor binding site of β3GalT5 tighter than β1-2 or β1-6 linked GlcNAc residues. The 1-6 linkage is very flexible, which may prevent tight substrate binding [41]. GlcNAcβ1-3GalNAc-, GlcNAcβ1-2Man- and GlcNAcβ1-6GalNAc- containing compounds have similar efficiencies as acceptor substrates for bovine milk β4GalT [26, 32]. Thus, the extension of N-glycans is less likely to be catalyzed by β3GalT5 than by other members of the β3GalT and β4GalT families.

A comparison of the sequences of human β3GalT and human β4GalT shows that human β3GalTs lack the GWGG sequence, which may be responsible for binding hydrophobic residues. This may explain why the naphthyl containing compound **612** binds to human β4GalT and prevents catalysis, but poorly binds to human β3GalT5, and is a poor substrate for the latter enzyme but not an inhibitor. In contrast to naphthyl derivatives, the quinolinyl compounds that have a nitrogen within the aromatic ring structure are good β3GalT5 and β4GalT1 substrates, suggesting that the bulky ring structure does not represent a steric hindrance for binding and catalysis. However, the naphthyl group is an important structural element of the **612** inhibitor, and promotes inhibition while other hydrophobic groups, such as benzyl and phenyl groups, promote binding but do not inhibit catalysis. We determined here that not only GlcNAc and the naphthyl moiety but also other structural features are

responsible for binding and inhibiting the enzyme, and no single group is solely responsible for inhibition. Several GlcNAc-naphthyl analogs having oxygen or sulfur in the glycosidic linkage inhibit β4GalT; however, the introduction of nitrogen into the anomeric linkage (compound **629**), which adds to the hydrophilic properties of the compound resulted in a GalT substrate with little inhibitory properties. The design of a more potent inhibitor needs to consider the properties of the inhibitor as well as the binding site in the enzyme.

Since β3GalT5 likely competes with β4GalT in the synthesis of the backbones of glycan chains, lowering the activity of β4GalT and maintaining the activity of β3GalT5 with **612** treatment could suppress the formation of the type 2 chains and consequently reduce the abundance of sialyl-Lewis x [27]. This strategy may be beneficial as an anti-cancer or anti-inflammatory strategy.

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References

1. Brockhausen, I.: Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. EMBO Rep. **7**, 599–604 (2006)
2. Galvan, M., Tsuboi, S., Fukuda, M., Baum, L.G.: Expression of a specific glycosyltransferase enzyme regulates T cell death mediated by galectin-1. J. Biol. Chem. **275**, 16730–16737 (2000)
3. Nakahara, S., Raz, A.: Biological modulation by lectins and their ligands in tumor progression and metastasis. Anticancer Agents Med. Chem. **8**, 22–36 (2008)
4. Rabinovich, G.A., Toscano, M.A., Ilarregui, J.M., Rubinstein, N.: Shedding light on the immunomodulatory properties of galectins: novel regulators of innate and adaptive immune responses. Glycoconj. J. **19**, 565–573 (2004)
5. Ochieng, J., Furtak, V., Lukyanov, P.: Extracellular functions of galectin-3. Glycoconj. J. **19**, 527–535 (2002)
6. Perillo, N.L., Marcus, M.E., Baum, L.G.: Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. J. Mol. Med. **76**, 402–412 (1998)
7. Jørgensen, T., Berner, A., Kaalhus, O., Tveter, K.J., Danielsen, H. E., Bryne, M.: Up-regulation of the oligosaccharide sialyl LewisX: a new prognostic parameter in metastatic prostate cancer. Cancer Res. **55**, 1817–1819 (1995)
8. Fuster, M.M., Brown, J.R., Wang, L., Esko, J.D.: A disaccharide precursor of sialyl Lewis X inhibits metastatic potential of tumor cells. Cancer Res. **63**, 2775–2781 (2003)
9. Brown, J.R., Yang, F., Sinha, A., Ramakrishnan, B., Tor, Y., Qasba, P.K., Esko, J.D.: Deoxygenated disaccharide analogs as specific inhibitors of β1-4- galactosyltransferase 1 and selectin-mediated tumor metastasis. J. Biol. Chem. **284**, 4952–4959 (2009)
10. Barthel, S.R., Wiese, G.K., Cho, J., Opperman, M.J., Hays, D.L., Siddiqui, J., Pienta, K.J., Furie, B., Dimitroff, C.J.: Alpha 1,3 fucosyltransferases are master regulators of prostate cancer cell trafficking. Proc. Natl Acad. Sci. USA **106**, 19491–19496 (2009)
11. Hennet, T.: The galactosyltransferase family. Cell. Mol. Life Sci. **59**, 1081–1095 (2002)
12. Amado, M., Almeida, R., Carneiro, F., Levery, S.B., Holmes, E. H., Nomoto, M., Hollingsworth, M.A., Hassan, H., Schwientek, T., Nielsen, P.A., Bennett, E.P., Clausen, H.: A family of human beta 3-galactosyltransferases. Characterization of four members of a UDP-galactose: beta-N-acetyl-glucosamine/beta-nacetyl- galactosamine beta-1, 3-galactosyltransferase family. J. Biol. Chem. **273**, 12770–12778 (1998)
13. Almeida, R., Amado, M., David, L., Levery, S.B., Holmes, E.H., Merkx, G., van Kessel, A.G., Rygaard, E., Hassan, H., Bennett, E., Clausen, H.: A family of human beta4-galactosyltransferases. Cloning and expression of two novel UDP-galactose:beta-n-acetylglucosamine beta1, 4-galactosyltransferases, beta4Gal-T2 and beta4Gal-T3. J. Biol. Chem. **272**, 31979–31991 (1997)
14. Renkonen, J., Paavonen, T., Renkonen, R.: Endothelial and epithelial expression of sialyl Lewis(x) and sialyl Lewis(a) in lesions of breast carcinoma. Int. J. Cancer **74**, 296–300 (1997)
15. Seko, A., Ohkura, T., Kitamura, H., Yonezawa, S., Sato, E., Yamashita, K.: Quantitative Differences in GlcNAc:beta} 1->3 and GlcNAc:{beta} 1->-4 Galactosyltransferase Activities between Human Colonic Adenocarcinomas and Normal Colonic Mucosa. Cancer Res. **56**, 3468–3473 (1996)
16. Isshiki, S., Kudo, T., Nishihara, S., Ikehara, Y., Togayachi, A., Furuya, A., Shitara, K., Kubota, T., Watanabe, M., Kitajima, M., Narimatsu, H.: Lewis type 1 antigen synthase (beta3Gal-T5) is transcriptionally regulated by homeoproteins. J. Biol. Chem. **278**, 36611–36620 (2003)
17. Salvini, R., Bardoni, A., Valli, M., Trinchera, M.: beta 1, 3-Galactosyltransferase beta 3Gal-T5 acts on the GlcNAcbeta 1–> 3Galbeta 1–>4GlcNAcbeta 1–>R sugar chains of carcinoembryonic antigen and other N-linked glycoproteins and is down-regulated in colon adenocarcinomas. J. Biol. Chem. **276**, 3564–3573 (2001)
18. Zhou, D., Berger, E.G., Hennet, T.: Molecular cloning of a human UDP- galactose:GlcNAcbeta1, 3GalNAc beta1, 3 galactosyltransferase gene encoding an O- linked core3-elongation enzyme. Eur. J. Biochem. **263**, 571–576 (1999)
19. Isshiki, S., Togayachi, A., Kudo, T., Nishihara, S., Watanabe, M., Kubota, T., Kitajima, M., Shiraishi, N., Sasaki, K., Andoh, T., Narimatsu, H.: Cloning, expression, and characterization of a novel UDP-galactose:beta-N-acetylglucosamine beta1, 3-galactosyltransferase (beta3Gal-T5) responsible for synthesis of type 1 chain in colorectal and pancreatic epithelia and tumor cells derived therefrom. J. Biol. Chem. **274**, 12499–12507 (1999)
20. Lin, C.H., Fan, Y.Y., Chen, Y.Y., Wang, S.H., Chen, C.I., Yu, L.C., Khoo, K.H.: Enhanced expression of beta 3-galactosyltransferase 5 activity is sufficient to induce *in vivo* synthesis of extended type 1 chains on lactosylceramides of selected human colonic carcinoma cell lines. Glycobiology **19**, 418–427 (2009)
21. Odunsi, K., Ghamande, S., Chandrasekaran, E.V., Ta, A., Moysich, K.B., Driscoll, D., Matta, K., Lele, S.: Evaluation of beta1, 4-galactosyltransferase as a potential biomarker for the detection of subclinical disease after the completion of primary therapy for ovarian cancer. Am. J. Obstet. Gynecol. **187**, 575–580 (2002)
22. Ichikawa, T., Nakayama, J., Sakura, N., Hashimoto, T., Fukuda, M., Fukuda, M.N., Taki, T.: Expression of N-acetyllactosamine and beta1, 4-galactosyltransferase (beta4GalT-I) during adenoma-

carcinoma sequence in the human colorectum. J. Histochem. Cytochem. **47**, 1593–1602 (1999)
23. Xu, S., Zhu, X., Zhang, S., Yin, S., Zhou, L., Chen, C., Gu, J.: Over-expression of beta- 1, 4-galactosyltransferase I, II, and V in human astrocytoma. J. Cancer Res. Clin. Oncol. **127**, 502–506 (2001)
24. Yang, X., Lehotay, M., Anastassiades, T., Harrison, M., Brockhausen, I.: The effect of TNF-alpha on glycosylation pathways in bovine synoviocytes. Biochem. Cell Biol. **82**, 559–568 (2004)
25. García-Vallejo, J.J., van Dijk, W., van Die, I., Gringhuis, S.I.: Tumor necrosis factor-alpha up-regulates the expression of beta1, 4-galactosyltransferase I in primary human endothelial cells by mRNA stabilization. J. Biol. Chem. **280**, 12676–12682 (2005)
26. Brockhausen, I., Benn, M., Bhat, S., Marone, S., Riley, J.G., Montoya-Peleaz, P., Vlahakis, J.Z., Paulsen, H., Schutzbach, J.S., Szarek, W.A.: UDP-Gal: GlcNAc-R beta1, 4-galactosyltransferase–a target enzyme for drug design. Acceptor specificity and inhibition of the enzyme. Glycoconj. J. **23**, 525–541 (2006)
27. Yang, X., Newmarch, K., Szarek, W.A., Brockhausen, I.: Modification of glycosylation alters the adherence of Pseudomonas aeruginosa and Burkholderia cenocepacia to human lung cancer cells. Glycoconj. J. **26**, 7 (2009)
28. Schutzbach, J., Brockhausen, I.: Inhibition of glycosyltransferase activities as the basis for drug development. Methods Mol. Biol. **534**, 359–373 (2009)
29. Brockhausen, I., Carran, J., McEleney, K., Lehotay, M., Yang, X., Yin, L., Anastassiades, T.: N-Acyl derivatives of glucosamine as acceptor substrates for galactosyltransferase from bone and cartilage cells. Carbohydr. Res. **340**, 1997–2003 (2005)
30. Möller, G., Reck, F., Paulsen, H., Kaur, K.J., Sarkar, M., Schachter, H., Brockhausen, I.: Control of glycoprotein synthesis: substrate specificity of rat liver UDP-GlcNAc: Manα3R β2-N-acetylglucosaminyl-transferase I using synthetic substrate analogues. Glycoconj. J. **9**, 180–190 (1992)
31. Reck, F., Meinjohanns, E., Springer, M., Wilkens, R., Dorst, J. A.L.M., Paulsen, H., Möller, G., Brockhausen, I., Schachter, H.: Synthetic substrate analogues for UDP- GlcNAc: Manα1-6R β (1-2)-N-acetylglucosaminyltransferase II. Substrate specificity and inhibitors for the enzyme. Glycoconj. J. **11**, 210–216 (1994)
32. Brockhausen, I., Dowler, T., Paulsen, H.: Site directed processing: role of amino acid sequences and glycosylation of acceptor glycopeptides in the assembly of extended mucin type O-glycan core 2. Biochim. Biophys. Acta **1790**, 1244–1257 (2009)
33. Brockhausen, I., Reck, F., Kuhns, W., Khan, S., Matta, K.L., Meinjohanns, E., Paulsen, H., Shah, R.N., Baker, M.A., Schachter, H.: Substrate specificity and inhibition of UDP- GlcNAc: GlcNAcβ1-2Manβ1-6R β6-N-acetylglucosaminyltransferase V using synthetic substrate analogues. Glycoconj. J. **12**, 371–379 (1995)
34. Homa, F.L., Baker, C.A., Thomsen, D.R., Elhammer, A.P.: Conversion of a bovine UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase to a soluble, secreted enzyme, and expression in Sf9 cells. Protein Expr. Purif. **6**, 141–148 (1995)
35. Toki, D., Sarkar, M., Yip, B., Reck, F., Joziasse, D., Fukuda, M., Schachter, H., Brockhausen, I.: Expression of stable human O-glycan core 2 beta-1, 6-N- acetylglucosaminyltransferase in Sf9 insect cells. Biochem. J. **325**(Pt 1), 63–69 (1997)
36. Kost, T.A., Condreay, J.P., Jarvis, D.L.: Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat. Biotechnol. **23**, 567–575 (2005)
37. Ponticelli, F., Trendafilova, A., Valoti, M., Saponara, S., Sgaragli, G.: Synthesis and antiperoxidant activity of new phenolic O-glycosides. Carbohydr. Res. **330**, 459–468 (2001)
38. Qasba, P.K., Ramakrishnan, B., Boeggeman, E.: Structure and Function of β-1, 4- Galactosyltransferase. Curr. Drug Targets **9**, 292–309 (2008)
39. Ramakrishnan, B., Boeggeman, E., Ramasamy, V., Qasba, P.K.: Structure and catalytic cycle of beta-1, 4-galactosyltransferase. Curr. Opin. Struct. Biol. **14**, 593–600 (2004)
40. Turco, S.J., Heath, E.C.: The molecular and catalytic properties of galactosyltransferase from fetal calf serum. Arch. Biochem. Biophys. **176**, 352–357 (1976)
41. Pollex-Krüger, A., Meyer, B., Stuike-Prill, R., Sinnwell, V., Matta, K.L., Brockhausen, I.: Preferred Conformations and Dynamics of five core structures of mucin-type O-glycans determined by NMR spectroscopy and force field calculations. Glycoconj. J. **10**, 365–380 (1993)