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ACCEPTOR SUBSTRATE-BASED SELECTIVE INHIBITION OF GALACTOSYLTRANSFERASES

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Abstract: This paper describes the discovery of glycosyl acceptor analogs as potent and selective inhibitors of α-1,3- and β-1,4-galactosyltransferases. Incorporation of an appropriate aromatic group to the aglycon position of the enzyme's acceptors results in a strong inhibition, representing the first and most potent small uncharged molecules as selective inhibitors of these two enzymes and thus providing a new strategy for the development of selective glycosyltransferase inhibitors.

Galactosyltransferases (GalTs) catalyze the transfer of D-galactopyranose from uridine-5′-diphosphogalactose (UDP-Gal) to a specific hydroxyl of various acceptor sugars.[1] α-1,3-GalT[2–5] has recently attracted much attention due to the problem of organ rejection in xenotransplantation caused by the response of recipient's antibodies to the donor's glycoconjugates bearing a nonreducing terminal α-D-Gal(1,3)-β-D-Gal(1,4)-β-D-GlcNAc-OR.[6–8] β-1,4-GalT catalyzes a number of β-galactosylations involved in intercellular recognitions and is well characterized.[1,9–11] Modification of the glycosyl donor UDP-Gal has been used as a strategy to develop potent β-1,4-GalT inhibitors,[12–17] which inevitably will also inhibit other GalTs, because most GalTs use UDP-Gal as donor substrate. This selectivity problem is common in the development of glycosyltransferase inhibitors as the majority of potent glycosyltransferase inhibitors[18] are based on donor substrates, which contribute most significantly to substrate binding. Inhibitors containing a phosphate or a pyrophosphate group are, however, not readily bioavailable as they are difficult to get into the cell. Consideration of the GalT-substrate specificity,[1,2] however, suggests that the acceptor analogs are better candidates than donor analogs for selective inhibition of GalTs despite that the weak inhibitory activity has been a difficult problem to overcome (Table 1). In this communication, we would like to describe a novel strategy for the development of potent and selective α-1,3- and β-1,4-GalT inhibitors based on acceptors.

The substrate specificity[19–21] and mutagenesis studies[22] suggest an active site model of β-1,4-GalT (Figure 1). The binding site of the donor substrate was built based on the transition state suggested for the enzyme reaction.[14,17] The primary binding affinity and specificity of acceptor substrate are obtained from the specific recognition of GlcNAc by enzymes and lectins.[23–26] (**Site A**) **Site B** interaction affects the substrate binding affinity in a less specific manner.[20,21] In addition, **Site B** prefers bulky aromatic groups[20] to aliphatic[21]

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or sugar residues.[19] This binding mode suggests that the aromatic groups contribute significantly to substrate binding in **Site B**, which normally recognizes a carbohydrate or aromatic amino acid attached to GlcNAc in a natural substrate. This model is also supported by the aromatic–aromatic[28] and aromatic–carbohydrate stacking[29,30] found in crystal structures.

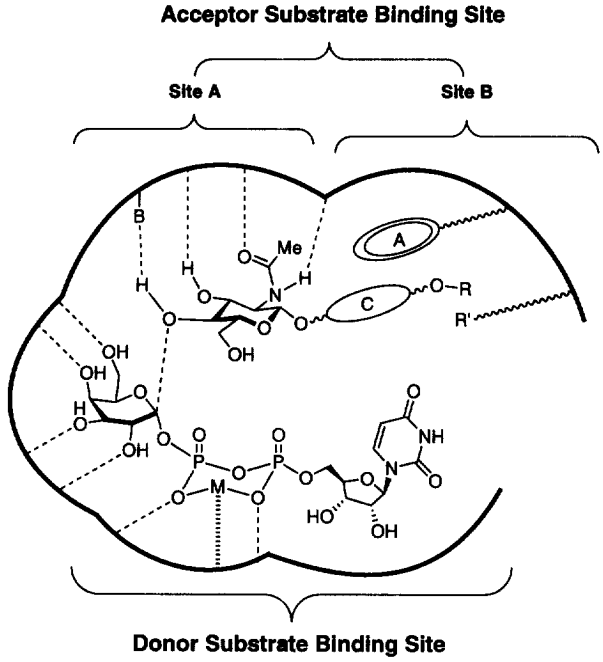
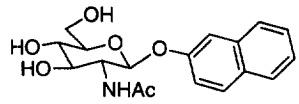
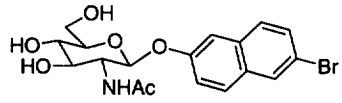
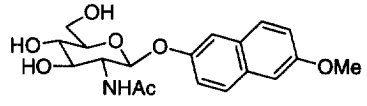
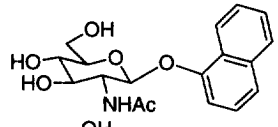
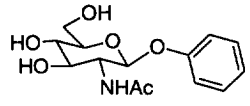
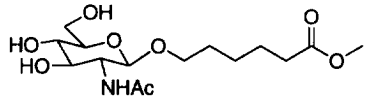
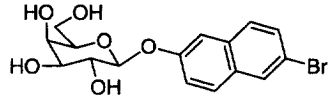
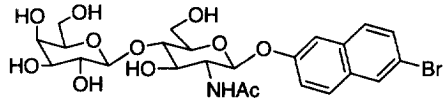
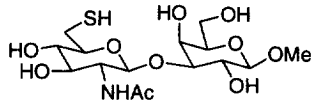
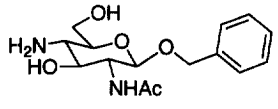
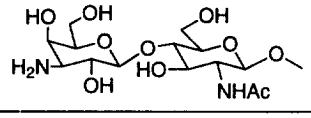


Figure 1. A Proposed Substrate Binding Mode for β-(1,4)-GalT Active Site (A: Aromatic residue; B: Base; C: Carbohydrate; R: Carbohydrate, peptide, alkyl chain etc.; R′: Hydrophobic alkyl chains; X = OH, NHAc; Y = H, OH; M = divalent metal such as Mn^{2+}, Ca^{2+} etc.).

Compounds **1a–c** were designed as model compounds to test the active site model. Compounds **2–4** were selected to define the nature of the hydrophobic site and **5** to verify the carbohydrate specificity. Compound **6** was designed to apply the same concept for the design of an α-1,3-GalT inhibitor. Following the literature method,[31] compounds **1–3** were easily synthesized from 2,3,4,6-tetra-*O*-acetyl-α-GlcNAc chloride by glycosylation with the appropriate naphthol or phenol derivative and deprotection by sodium methoxide, and **9** was prepared from hexa-*O*-acetyl-α-LacNAc chloride.

Compounds **1a–c** were originally evaluated as substrates, but they showed significant substrate inhibition[35] at micromolar concentrations. No enzymatic conversion was observed at 0.1 mM for **1a–c** and **2**. Compounds **1–6** were, thus, evaluated as inhibitors of β-1,4-GalT and α-1,3-GalT, and the results were analyzed by Lineweaver–Burk analysis. As expected, compounds **1a–c** strongly inhibited β-1,4-GalT catalyzed synthesis of LacNAc from UDP-Gal and GlcNAc (Table 1).

Table 1. Known and Newly Developed Inhibitors of GalTs

Entry	Structure	K_i (μM)	
		β-1,4-GalT[a]	α-1,3-GalT[b]
1a		9.5	NI[c]
1b		7.6	NI
1c		3.5	NI
2		22.0	NI
3		NI	–[d]
4		NI	–
5		NI	–
6		NI	9.2
7		1000[e]	–
8		850[f]	–
9		–	104[g]

[a]Recombinant Bovine β-1,4-galactosyltransferase. [b]Porcine α-1,3-galactosyltransferase: The inhibition analysis was carried out in Mess Bufer (pH 6.0, 50 mM) containing 10 mM $MnCl_2$, bovine serum albumin (μg/μL), LacNAc-β-OME (15–120 μM) and inhibitor (0–20 μM). [c]No inhibitory activity. [d]Not tested. [e]Ref 32. [f]Ref 33. [g]Ref 34. NI, no inhibition at 0.2 mM.

Compounds **1a–c** are competitive inhibitors of GlcNAc with K_i of 3.5~9.5 μM, and **2** is also a competitive inhibitor with K_i of 22.0 μM. Compounds **3–6**, however, showed no inhibitory activity at concentrations up to 200 μM. Compounds **3** and **4** are indeed very poor substrates. The K_m values were not determined as at higher substrate concentrations the enzyme was inhibited. Comparison of **1a–c** with **4** suggests that the aromatic ring is responsible for the drastic increase in the binding affinity of inhibitors. Compounds **2** and **3** show the importance of the geometry and size of the aromatic ring to binding. No inhibition with **5**, **6**, and 6-bromo-2-naphthol was observed, indicating that the recognition of GlcNAc by **site A** is responsible for the acceptor substrate specificity of β-1,4-GalT. When **1a–c** and **2** were tested against α-1,3-GalT, none of them showed any significant inhibition, while donor analog inhibitors showed inhibition of both α-1,3- and β-1,4-GalTs. Compound **6** was designed as a potential specific inhibitor of α-1,3-GalT based on the assumption that the active site of α-1,3-GalT might contain a region similar to that of β-1,4-GalT, because β-1,4-GalT catalyzes the synthesis of LacNAc, which is the acceptor substrate of α-1,3-GalT. The potent inhibition of α-1,3-GalT by **6** suggests that the assumption is correct. In addition, specificity study based on site-directed mutagenesis of β-1,4-GalT suggests the involvement of aromatic group in acceptor binding,[22] which could be used to design inhibitors of the less characterized α-1,3-GalT. Compounds **1a–c** should only bind to the acceptor sugar binding site of GalT because **1b** showed a noncompetitive inhibition for UDP-Gal with K_i of 8.56 μM (K_m of UDP-Gal was 3.86 μM).

In summary, selective inhibitors of α-1,3- and β-1,4-GalT have been developed on the basis of a proposed active site model of β-1,4-GalT. Incorporation of an aromatic group to the aglycon moiety of acceptor could result in a strong inhibition of the enzyme. The results obtained in this study provide a new strategy for the development of selective galactosyltransferase inhibitors.

Experimental Section

Synthesis of **1b**, To a stirred solution of 2-acetamido-2-deoxy-3,4,6-triacetyl-α-D-glucopyranosyl chloride (3.66 g, 10 mmol), 6-bromo-2-naphthol (4.46 g, 20 mmol), and tetra-*n*-butylammonium bromide (3.22 g, 10 mmol) in dichloromethane (40 mL) was added dropwise aqueous solution of 1 N NaOH (40 mL) and the resulting mixture was vigorously stirred at room temperature for 40 min. The mixture was diluted with ethyl acetate (100 mL). The organic phase was washed with aqueous 1 N NaOH (2 × 30 mL), water (30 mL), brine (30 mL), and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on a silica gel column (chloroform/acetone, 20~10/1). Recrystallization of collected product from ethyl acetate-hexane give 3, 4, 6-tri-*O*-acetylated precursor of **1b**: 3.19 g (57.8%). To a solution of 3, 4, 6-tri-*O*-acetylated precursor of **1b** (3 g, 5.43 mmol) in methanol (100 mL)-CH_2Cl_2 (100 mL) was added a catalytic amount of sodium methoxide (~100 mg) and stirred overnight at rt. The resulting white precipitate was filtered, washed with dichloromethane, and dried in vacuo to give **1b** (2.24 g, 96.8%) as white powder. ^{1}H NMR (DMSO-d_6 + D_2O): δ 8.10 (1H, s), 7.83 (1H, d), 7.77 (1H, d), 7.56 (1H, dd), 7.43 (1H, s), 7.20 (1H, dd), 5.12 (1H, d), 3.74

(2H, t), 3.43–3.54 (3H, m), 3.21 (1H, dd), 1.84 (3H, s); ^{13}C NMR (DMSO-d_6 + D_2O): δ 170.59, 155.90, 132.99, 130.71, 129.80, 129.77, 129.71, 129.19, 120.27, 117.46, 111.01, 99.34, 77.42, 74.22, 70.54, 61.02, 55.72, 23.34; HRMS (FAB, M + Na^+) calcd for $C_{18}H_{20}BrNO_6Na$ 450.0351, found 450.0367. Compounds **1c** and **6** were prepared similarly. **1c**: ^{1}H NMR (DMSO-d_6 + D_2O) δ 7.69–7.74 (2H, m), 7.34 (1H, s), 7.24 (1H, s), 7.10–7.13 (2H, m), 5.04 (1H, d), 3.80 (3H, s), 3.73–3.77 (2H, m), 3.42–3.55 (3H, m), 3.24 (1H, t), 1.85 (3H, s); ^{13}C NMR (DMSO-d_6 + D_2O): δ 172.34, 157.00, 154.36, 131.28, 130.04, 129.54, 129.26, 120.04, 119.95, 111.99, 106.88, 100.19, 77.47, 74.53, 70.91, 61.51, 56.27, 56.08, 23.61; HRMS (FAB, MH^+) calcd for $C_{19}H_{24}BrNO_7$ 378.1553, found 378.1565. **6**: ^{1}H NMR (DMSO-d_6 + D_2O) δ 8.12 (1H, s), 7.86 (1H, d), 7.81 (1H, d), 7.60 (1H, d), 7.47 (1H, s), 7.24 (1H, d), 5.20 (1H, d), 4.31 (1H, d), 3.84–3.91 (2H, m), 3.65–3.75 (4H, m), 3.53–3.57 (4H, m), 3.39 (2H, m) 1.88 (3H, m); ^{13}C NMR (DMSO-d_6 + D_2O) δ 171.93, 156.06, 133.37, 131.24, 130.39, 130.19 (2C), 129.74, 120.72, 118.12, 111.44, 104.33, 99.45, 80.69, 76.24, 75.81, 73.64, 72.77, 71.40, 68.99, 61.42, 60.67, 55.47, 23.53; HRMS (FAB, M + Cs^+) calcd for $C_{24}H_{30}BrNO_{11}Cs$ 720.0057, found 720.0079.

Determination of K_i for β-1,4-galactosyltransferase

To an assay mixture of $MnCl_2$ (100 mM, 5 μL), UDP-[6-^{3}H]-galactose (2 mM, 5 μL), GlcNAc (20, 10, 5, 2.5 mM , each 5 μL), a solution of inhibitor in DMSO (0, 100, 200 μM, each 2.5 μL) , bovine serum albumin (10 mg/1 mL, 5 μL), and HEPES buffer (pH 7.4, 100 mM, 27.5 μL) was added β-1,4-galactosyltransferase (340 μU, 5 μL) to give 50 μL of total volume. The assay mixture was incubated for 50 min at 25 °C, and then stopped by the addition of deionized water (300 μL) and immediately subjected to a column of Dowex 1X8 (0.6 × 2 mm). The column was washed with water (3 × 300 μL) and the eluent was collected in a vial containing ScintiVersa I scintillation cocktail. A control reaction without GlcNAc was used to establish the background count. Each initial rate was obtained in a consuming period of less than 7% of UDP-[6-^{3}H]-galactose. K_m of GlcNAc was measured as 1.8 mM.

Determination of K_i for α-1,3-galactosyltransferase

To an assay mixture of $MnCl_2$ (100 mM, 5 μL), UDP-[6-^{3}H]-galactose (500 μM, 5 μL), LacNAc-β-OMe (1.2, 0.6, 0.3, 0.15 mM , each 5 μL), a solution of inhibitor in DMSO (0, 100, 200, 400 μM, each 2.5 μL) , bovine serum albumin (10 mg/1 mL, 5 μL), and MES buffer (pH 6.0, 500 mM, 27.5 μL) was added α-1,3-galactosyltransferase (340 μU, 5 μL) to give 50 μL of total volume. The assay mixture was incubated for 16 min at 25 °C, and then stopped by the addition of deionized water (300 μL) and immediately subjected to a column of Dowex 1X8 (0.6 × 2 mm). The column was washed with water (3 × 300 μL) and the eluent was collected in a vial containing ScintiVersa I scintillation cocktail. A control reaction without GlcNAc was used to establish the background count. Each initial rate was obtained in a consuming period of less than 8.3 % of UDP-[6-^{3}H]-galactose. K_m of LacNAc-β-OMe was measured as 59.5 μM.

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