Scope of Multivalent Ligand Function: Lactose-Bearing Neoglycopolymers By Ring-Opening Metathesis Polymerization

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Received 15 April 1999

Abstract: A lactose-bearing norbornene imide template was polymerized using $(Cy_3P)_2Cl_2Ru=CHPh$ to produce a lactose-substituted neoglycopolymer. The resulting polymer showed a 100-fold overall increase in inhibitory potency (5-fold increase on a per saccharide residue basis) compared to monomeric lactose in both a galectin-binding assay and an *Erythrina corallodendrum* hemagglutination assay.

Key words: carbohydrates, lactose, lectins, polyvalency, ring-opening metathesis polymerization

The relatively low affinity and specificity of individual carbohydrate-protein interactions is hard to reconcile with the diversity of oligosaccharide structures that are involved in specific recognition processes. Many scientists resolve this paradox with the assumption that multiple carbohydrate-protein interactions can cooperate in each recognition event to give the necessary functional affinity.[1,2] By changing the number of individual interactions as well as controlling their spatial arrangement, a multivalent ligand can become specific to a target or targets. Because protein–carbohydrate complexation is important in a wide range of medically significant interactions including inflammation and bacterial and viral pathogenesis, an understanding of multivalent recognition events is critical for designing therapeutics that target these processes.[3]

Multivalent interactions are paramount in a variety of physiological processes, yet relatively little is known about these binding events. For example, much work has been done in elucidating the specific monovalent interactions between carbohydrate ligands with specific proteins such as the cell-surface selectins,[4] the galectins,[5] and various plant lectins,[6] yet an understanding of the contribution of polyvalent interactions to the functional affinity and specificity of these processes is only beginning to emerge. To build on our studies of multivalent saccharide derivatives,[7–10] we decided to explore the inhibitory properties of polymeric ligands that display a high local concentration of saccharide epitopes in assays with two different lectins.

Several mechanisms may operate to enhance the functional affinity (i.e., the measured activity) of a particular multivalent ligand. Two such mechanisms include the chelate effect and the statistical effect due to high local concentration. In the chelate effect, a multidentate ligand occupies multiple binding sites within a protein (Figure 2A). The statistical effect we refer to is that in which ligands that present a high local concentration of saccharide epitopes to a single binding site exhibit slower off rates (Figure 2B). To test the contributions of these effects to saccharide recognition, we chose to explore galectin-3 (Gal-3) and the lactose-binding lectin from *Erythrina corallodendrum* (EcorL). The galectins, like the selectins, have been implicated in the inflammatory response. Some of the naturally occurring ligands that bind the galectin family of proteins consist of polylactosamine units found in glycoconjugates such as fibronectin and laminin.[11] The structures of naturally occurring galectin ligands suggests multidentate inhibitors could function through the chelate effect. In contrast, multivalent ligands are only modestly more effective than their monovalent counterparts in EcorL inhibition.[17] Both Gal-3 and EcorL are proposed to be dimeric under certain conditions. Therefore, we expected these lectins and a lactose-bearing polymer (Figure 1) to be a good test of statistical effects versus chelate effects in polyvalent binding.
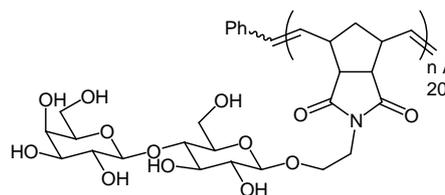
1 Lactose Neoglycopolymer

Figure 1

The ring-opening metathesis polymerization (ROMP) is a versatile method to generate multivalent, biologically active displays;[1] consequently, we applied this method to generate a lactose-substituted array. A suitable monomer could be obtained from the known peracetylated β-allyl lactopyranoside[12] upon ozonolysis followed by sodium borohydride reduction to unmask the primary alcohol **3**. This alcohol underwent a Mitsunobu reaction with the cyclic imide template **2**[13] to produce **4** (Scheme). The lactose derivative was then fully deacetylated to give **5** under mildly basic conditions that precluded opening of the imide. For the preparation of the desired lactose-conjugated neoglycopolymer **1**, the ruthenium alkylidene catalyst $(Cy_3P)_2Cl_2Ru=CHPh$, developed by Grubbs and co-workers, was chosen for several of its properties. The catalyst is highly reactive, readily tolerates polar functionality,

and can effect a living polymerization.[14] Lactose monomer **5** was polymerized using the ruthenium catalyst in a mixture of water, methanol, and dichloroethane. The monomer and polymer were soluble in the more polar solvents, while the catalyst had to be added as a solution in dichloroethane. After termination of the reaction with excess ethyl vinyl ether, the resulting material was washed with methanol to remove unreacted monomer, which provided polymer **1** in 60% yield. The ^{13}C NMR spectrum of this polymer shows broad peaks as in the proton spectrum, but only for the backbone carbons. The saccharide resonances are sharper, indicating the sugar residues are not significantly constrained by the polymer backbone. The average number of repeating units of the polymer was estimated by comparison of the ^{1}H NMR integration of the aromatic protons, derived from the initiating catalyst, to the backbone alkene protons. This comparison showed an average polymer length of twenty monomer units.

To characterize the binding properties of lactose neoglycopolymer **1**, it was tested against two known lactose-binding proteins — Gal-3 and EcorL. Gal-3 is usually monomeric in solution, and therefore no large increase in functional affinity for multivalent ligands would be expected, unlike the large effect previously seen with the tetrameric lectin concanavalin A (Con A).[15] Still, Gal-3 can agglutinate cells and it can dimerize under certain circumstances;[16] therefore, the outcome of the assay could not be predicted. In contrast, EcorL is known to be divalent, and only a slight enhancement of hemagglutination inhibition by a lactose-bearing acrylamide polymer over lactose has been seen.[17] These results suggest multivalent ligands do not react with EcorL through the chelate effect.

Lactose neoglycopolymer **1** was tested in an inhibition assay against Gal-3.[18] Binding of human Gal-3 to a glycosylated antibody was monitored by a horseradish peroxidase reporter system in the presence of lactose (Lac), N-acetyllactosamine (LacNAc), the lactose neoglycopolymer **1**, and the corresponding mannose neoglycopolymer.[19] Concentrations required for 50% inhibition were calculated on a saccharide residue basis for each substrate tested. The N-acetyllactosamine epitope is known to bind with higher affinity than lactose to the galectins; this difference in relative affinities was reproduced in this assay. Mannose res-

idues do not interact with the galectins, and the mannose-substituted polymer shows no inhibition in this assay. This result suggests that the observed effects are due to lactose presentation and are not due to a non-specific effect of the sugars or the polymer backbone. The lactose neoglycopolymer **1**, however, does show concentration-dependent inhibition. The polymer overall has a 100-fold greater inhibition potency over monomeric lactose, which translates into a 5-fold greater potency on a saccharide residue basis.

The increase in functional affinity observed with the galectins was compared with that obtained for EcorL, a dimeric protein with binding sites oriented such that it is unable to benefit from the chelate effect (Figure 2B). Inhibition of human red blood cell hemagglutination[15] by EcorL by monomeric and polymeric lactose showed an effect similar to that seen with the galectin: The polymer **1** was 6-fold more effective than the monomeric lactose as an inhibitor on a saccharide residue basis.

The slightly increased inhibitory effect of a polymer for a monovalent protein such as Gal-3 is best explained by a statistical effect. When one lactose residue within a multivalent display dissociates from the galectin, the local concentration of lactose residues is large resulting in an increased probability that a new lactose residue from the same polymer chain will bind the galectin (Figure 2B). A similar explanation can explain the lactose-binding dimeric plant lectin results. Although this mechanism is due to multivalent display of recognition elements, it is distinct from the chelate effect. The chelate effect can give rise to an exponential increase in binding affinity, as seen with neoglycopolymers in the inhibition of tetrameric Con A-mediated hemagglutination.[10,15,19] The large activity enhancement for the polymers likely results from their ability to chelate two or more binding sites on the same protein complex (Figure 2A).

Neither our neoglycopolymers nor lactose-derivatized acrylamide polymers[17] exhibited a large increase in inhibitory potency over monovalent lactose for the dimeric plant lectin from *Erythrina corallodendron*. The binding sites of this lectin appear to be in the wrong orientation for polymers to span them. X-ray crystallographic analysis of
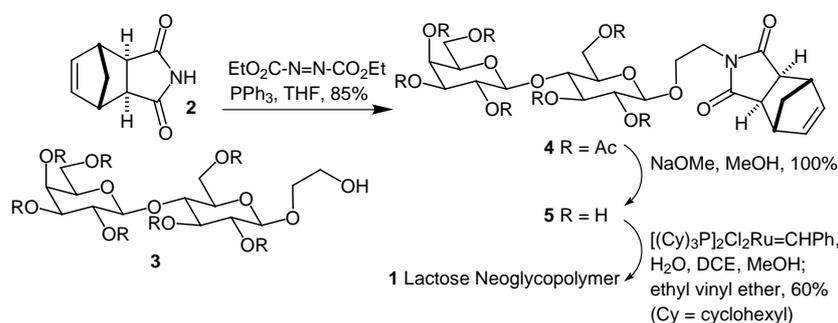
Scheme

this glycosylated, dimeric lectin bound to lactose indicates that its tertiary structure is related to that of Con A.[20] EcorL, however, is glycosylated at a site that sterically prevents it from adopting the tetrameric quaternary structure of Con A. The resulting dimer has two carbohydrate binding sites positioned in opposite directions, rather than on the same face (Figure 2C).[21] A multivalent display of carbohydrate epitopes, therefore, could not place saccharide groups in both carbohydrate binding sites within the lectin dimer, but it could crosslink two protein dimers.

Because Gal-3 can agglutinate cells,[22] it was presumed to act as a dimer or oligomer, even though it exists as a monomer in solution. Our data suggests that Gal-3 interacts in a mode similar to that of EcorL. We hypothesize that the Gal-3 dimer has a quaternary structure similar to that of EcorL, which would orient its carbohydrate-binding sites in opposite directions (Figure 2B). Recent data by Liu and co-workers[23] supports this model with the finding that Gal-3 dimerizes at the *N*-terminal domain in the presence of its carbohydrate ligand but at the C-terminal carbohydrate-binding domain in its absence. These combined findings suggest Gal-3 can act as a switch: it dimerizes at the C-terminal domain in solution until one unit binds a saccharide ligand. Then the dimer switches to an *N*-terminal domain association, which places the two individual carbohydrate-binding domains on opposite faces. The second orientation facilitates crosslinking of two cells or the binding of one cell to laminin.
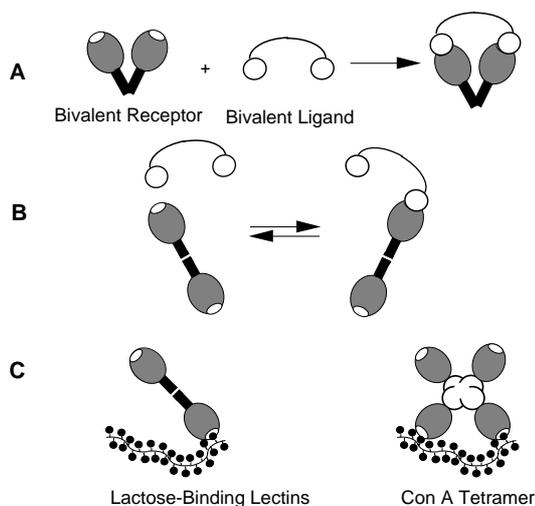

Figure 2. Multivalent modes of binding. A. Binding enhancement due to simultaneous spanning of two binding sites (the chelate effect). B. Enhancement of binding due to increased local concentration of available ligand (statistical effect). C. Both modes of inhibition are available to tetrameric Con A, yet only B is available to the dimeric *E. corrallodendron* lectin and galectin-3 with their carbohydrate binding pockets on opposite faces of the dimer.

Some general principles for choosing lectin targets as well as for designing inhibitors emerge from this analysis. With regard to target selection, soluble lectins with multiple carbohydrate binding sites oriented in a similar direc-

tion or membrane-anchored lectins are excellent candidates for inhibition by multidentate ligands. Conversely, dimeric lectins that crosslink two different surfaces are unlikely to be potently inhibited by multivalent carbohydrate displays. Multivalent inhibitors of tetrameric Con A are highly effective.[15] Multivalent ligands targeted at Gal-3 or the EcorL are only slightly more active than monovalent lactose.[17] Some increase (typically 5- to 10-fold), however, may be seen with a polyvalent over a monovalent ligand because the increased local concentrations of a recognition epitope decreases the rate of dissociation of the ligand from the lectin. An understanding of both monovalent and multivalent carbohydrate-protein interactions is required for the design of effective inhibitors of protein–saccharide interactions. Access to defined synthetic, multidentate displays through ROMP provides tools to illuminate biologically and medically important multivalent recognition events.

2-Hydroxyethyl *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (3)
A solution of allyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside[12] in MeOH (3.0 mL), and CH_2Cl_2 (13.0 mL) at –78 °C was treated with ozone until the solution turned blue. The solution was sparged with N_2 to remove excess ozone. Solid $NaBH_4$ (23 mg, 610 μmol, 5.9 equiv) was then added and the reaction was warmed to 0 °C. After TLC suggested the reduction was complete, the resulting solution was quenched with aq 2 M HCl (5 mL) and the reaction was extracted with EtOAc (20 mL). The organic layer was washed with additional 2 M HCl (2 ↔ 5 mL) and brine (5 mL). The solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography (silica, 60% EtOAc/CH_2Cl_2) to yield **3** as an amorphous white solid (49 mg, 70%).

IR (NaCl, thin film): ν = 3520 (br), 1750, 1230 cm^{-1}.

^{1}H NMR (300 MHz, CDCl₃): δ = 1.97 (s, 3 H), 2.05 (s, 3 H), 2.06 (s, 6 H), 2.07 (s, 3 H), 2.13 (s, 3 H), 2.16 (s, 3 H), 2.55 (m, 1 H), 3.90–3.64 (m, 7 H), 4.17–4.03 (m, 3 H), 4.49 (d, 1 H, *J* = 7.7 Hz), 4.51 (d, 1 H, *J* = 7.9 Hz), 4.55 (dd, 1 H, *J* = 13.6, 2.0 Hz), 4.92 (dd,

1 H, J = 9.5, 7.9 Hz), 4.96 (dd, 1 H, J = 10.4, 3.5 Hz), 5.12 (dd, 1 H, J = 10.4, 7.7 Hz), 5.22 (dd, 1 H, J = 9.5, 8.8 Hz), 5.35 (dd, 1 H, J = 3.5, 1.1 Hz).

^{13}C NMR (75 MHz, CDCl$_3$, DEPT 135): δ = 20.4 (CH$_3$), 20.5 (CH$_3$), 20.7 (CH$_3$), 60.8 (CH$_2$), 61.9 (CH$_2$), 62.0 (CH$_2$), 66.6 (CH), 69.1 (CH), 70.7 (CH), 70.9 (CH), 71.6 (CH), 72.6 (CH), 72.7 (CH), 73.3 (CH$_2$), 76.3 (CH), 101.0 (CH), 101.2 (CH), 169.0 (C), 169.6 (C), 169.7 (C), 169.9 (C), 170.0 (C), 170.2 (C), 170.3 (C).

MS (LSIMS, 3-NBA): m/z = 681.2 [C$_{28}$H$_{40}$O$_{19}$+H requires 681.3].

4-[2-Hydroxyethyl *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-Tri-*O*-acetyl-β-D-glucopyranoside]-*exo*-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-dione (4)

A solution of **3** (139 mg, 204 μmol), the imide **2**^{13} (47 mg, 288 μmol, 1.41 equiv) and PPh$_3$ (63 mg, 240 μmol, 1.18 equiv) in THF (1.20 mL) was treated dropwise with diethyl azodicarboxylate (38 μL, 240 μmol, 1.18 equiv). After 6 h, the reaction solvent was removed under a stream of N$_2$. The resulting compound was purified by repeated flash chromatography (silica gel, 50% EtOAc/CH$_2$Cl$_2$) to isolate the product as a white solid (143 mg, 85%).

IR (NaCl, thin film): ν = 1750, 1700, 1370, 1220, 1060 cm^{-1}.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 1.28 (br d, 1 H, J = 9.9 Hz), 1.48 (br d, 1 H, J = 9.9 Hz), 1.96 (s, 3 H), 2.03 (s, 3 H), 2.04 (s, 3 H), 2.06 (s, 6 H), 2.13 (s, 3 H), 2.15 (s, 3 H), 2.66 (s, 2 H), 3.26 (br s, 2 H), 3.57–3.96 (m, 7 H), 4.04–4.14 (m, 3 H), 4.47 (dd, 1 H, J = 12.7, 1.9 Hz), 4.47 (d, 1 H, J = 7.9 Hz), 4.48 (d, 1 H, J = 7.7 Hz), 4.82 (dd, 1 H, J = 9.4, 7.9 Hz), 4.95 (dd, 1 H, J = 10.5, 3.3 Hz), 5.10 (dd, 1 H, J = 10.5, 7.7 Hz), 5.16 (dd, 1 H, J = 9.1 Hz), 5.34 (d, 1 H, J = 2.6 Hz), 6.28 (s, 2 H).

^{13}C NMR (75 MHz, CDCl$_3$, DEPT 135): δ = 20.4 (CH$_3$), 20.5 (CH$_3$), 20.6 (CH$_3$), 20.7 (CH$_3$), 20.8 (CH$_3$), 38.0 (CH$_2$), 42.7 (CH$_2$), 45.2 (CH), 47.7 (CH), 60.7 (CH$_2$), 61.9 (CH$_2$), 65.0 (CH$_2$), 66.6 (CH), 69.1 (CH), 70.7 (CH), 70.9 (CH), 71.4 (CH), 72.6 (CH), 72.7 (CH), 76.2 (CH), 100.1 (CH), 101.0 (CH), 137.7 (CH), 137.8 (CH), 169.0 (C), 169.5 (C), 169.6 (C), 169.9 (C), 170.0 (C), 170.2 (C), 170.3 (C), 177.6 (C), 177.7 (C).

MS (LSIMS, 3-NBA): m/z = 848.2 [C$_{37}$H$_{47}$O$_{20}$N+Na requires 848.3].

4-[2-Hydroxyethyl *O*-(β-D-Galactopyranosyl)-(1→4)- β-D-glucopyranoside]-*exo*-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-dione (5)

A solution of the protected lactose monomer **4** (66 mg, 80 μmol) in MeOH (1.5 mL) was treated with 0.05 M NaOH in MeOH (0.20 mL) to bring the pH of the solution to 8–9. After 2 h, the reaction was neutralized with Amberlyst 15 acidic ion exchange resin and filtered through a plug of cotton. The filtrate solvent was evaporated under reduced pressure and the resulting compound was purified by flash chromatography (silica gel, 20% MeOH/CHCl$_3$) to yield **5** as a white solid (42 mg, 100%).

^{1}H NMR (300 MHz, D$_2$O): δ = 1.30 (br d, 1 H, J = 10.5 Hz) 1.46 (br d, 1 H, J = 10.1 Hz), 2.82 (br s, 2 H), 3.18–4.04 (m, 18 H), 4.36 (d, 2 H, J = 7.7 Hz), 4.38 (d, 2 H, J = 7.7 Hz), 6.32 (s, 2 H).

^{13}C NMR (75 MHz, D$_2$O, DEPT 135): δ = 39.1 (CH$_2$), 42.9 (CH$_2$), 45.6 (CH), 48.6 (CH), 60.8 (CH$_2$), 61.6 (CH$_2$), 67.2 (CH$_2$), 69.2 (CH), 71.6 (CH), 73.1 (CH), 73.3 (CH), 74.9 (CH), 75.3 (CH), 76.0 (CH), 79.0 (CH), 102.5 (CH), 103.6 (CH), 138.5 (CH), 182.4 (C), 182.5 (C).

Lactose Neoglycopolymer (1)

The lactose monomer (**5**) (19 mg, 36 μmol) was dissolved in MeOH (42 μL) and H$_2$O (11 μL) and the resulting solution was deoxygenated by freeze-pump-thaw cycles (3 x). The Grubbs ruthenium alkylidene catalyst (3 mg, 4 μmol, 0.1 equiv) was dissolved in

dichloroethane (280 μL, passed through a column of alumina and deoxygenated as above) and added to the monomer. The reaction went from purple to green with vigorous stirring. Additional degassed MeOH (225 μL) and degassed H$_2$O (75 μL) were added to keep the monomer in solution. After 2 h, the reaction was quenched with ethyl vinyl ether (0.5 mL), heated to 45 °C, and stirred for 3 h. The solvent was evaporated under a stream of N$_2$. The resulting syrup was taken up in MeOH, and the polymer was filtered off and washed with MeOH (3 × 5 mL) to remove unreacted monomer and ruthenium, leaving neoglycopolymer **1**, as a pale brown solid (11.4 mg, 60%).

^{1}H NMR (300 MHz, D$_2$O): δ = 1.50–2.20 (br, 1 H), 2.62–2.90 (br, 1 H), 3.15–3.34 (m, 2 H), 3.50–4.13 (m, 18 H), 4.44 (br, 2 H), 5.55–5.87 (br, 2 H), 7.25–7.50 (br, 0.25 H).

^{13}C NMR (125 MHz, D$_2$O, DEPT 135): δ = 38.6 (m), 45.2, 45.8 (m), 48.2, 49.1, 51.0 (m), 60.4 (m), 61.3, 66.1 (m), 68.8, 71.2, 72.8, 72.9, 73.0, 74.9, 75.0, 75.6, 78.7 (m), 102.3 (m), 103.2, 132.4 (m), 138.0, 181.5 (m).

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