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Glycocluster Design for Improved Avidity and Selectivity in Blocking Human Lectin/Plant Toxin Binding to Glycoproteins and Cells

Sabine André,[†] Martina Lahmann,[‡] Hans-Joachim Gabius,[†] and Stefan Oscarson*[,§]

Institut für Physiologische Chemie, Tierärztliche Fakultät, Ludwig-Maximilians-Universität, Veterinärstrasse 13, 80539 München, Germany, The School of Chemistry, University of Bangor, Alun Roberts Building, Deiniol Road, Bangor, Gwynedd LL57 2UW, U.K., and Centre for Synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland

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Abstract: Blocking lectin/toxin binding to human cells by suitable inhibitors can therapeutically protect them from harmful effects. Clustered design of ligand presentation holds the promise of affinity increase relative to the free sugar and inherent selectivity among lectin targets. Using first a solid-phase assay with a glycoprotein presenting *N*-glycans as lectin-reactive probe, we assessed the inhibitory potency of bi- to tetravalent clusters on a plant toxin and three human adhesion/growth-regulatory lectins. Enhanced avidity relative to the free sugar was detected together with lectin-type selectivity. These effects were confirmed on the level of cells *in vitro*, also for two leguminous lectins. The lack of toxicity in cell proliferation assays excluded concerns to further work on these compounds. The given cluster design and the strategic combination of the two assay systems of increasing biorelevance will thus be helpful to take the next steps in drug development, e.g. tailoring the sugar headgroup.

Keywords: Agglutinin; colon cancer; glycan branching; glycocluster; multivalency

Introduction

A promising perspective for drug discovery arises from the potency of glycans of cellular glycoconjugates to serve as biochemical signals. This emerging functionality shapes the concept of the sugar code.[1] The interplay with cognate receptors (lectins) can translate the sugar-encoded signals into clinically relevant activities, among them cell adhesion, migration or growth control together with tissue lectins and infections or cell dysfunction with bacterial/viral agglutinins or plant toxins.[1−3] Obviously, a selective interference in this type of binding process has therapeutic potential so that efforts to identify molecular parameters of respective pharmaceuticals blocking lectin binding in model systems are warranted. A key design feature concerns the topological display of ligands, since physiological high-affinity binding is accomplished by an appropriate spatial match between lectin domains and polyvalent ligands. It is most impressively seen in a geometrical affinity enhancement by a numerical valency increase from mono- to triantennary glycans for the haptenic asialoglycoprotein receptor, termed glycoside cluster

* Corresponding author. Mailing address: University College Dublin, Centre for Synthesis and Chemical Biology, Belfield, Dublin 4, Ireland. E-mail: stefan.oscarson@ucd.ie. Phone: +353 17162318. Fax: +353 17162501.

[†] Ludwig-Maximilians-Universität.

[‡] University of Bangor.

[§] University College Dublin.

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effect.[4] To effectively compete with natural binding partners glycoclusters must therefore be tailored to the properties of the respective lectin, exploiting chemical scaffolds which allow bi- to multivalency.[5] That said, it becomes obvious that the assay process must include the aspect of multivalency to ensure biopharmaceutical relevance. In this study, we present a strategic combination of two assay systems meeting this requirement and assess the inhibitory potency of synthetic bi- to tetravalent compounds. A biohazardous plant toxin and three human adhesion/growth-regulatory lectins (galectins) and three galactoside-headgroups in the glycoclusters were selected as test model.

The *Viscum album* L. agglutinin (VAA) belongs to the group of potent plant toxins, also referred to as ribosome-inactivating proteins type 2, including ricin as the most infamous member.[6] As a dimer of two AB complexes with two lectin sites per B-chain, VAA can react with different glycan branches to dock to a target cell.[7,8] Similarly, members of the galectin family are natural cross-linkers, by virtue of either noncovalent dimerization (e.g., galectin-1), oligomerization (e.g., galectin-3) or covalent connection of two subunits by a linker peptide (e.g., galectin-4).[2] The mentioned galectins share rather similar affinity to the unsubstituted *N*-acetyllactosamine terminus of complex-type *N*-glycans.[9] Since aspartate- or lysine/lysyllysine-based compounds have so far given evidence for rather minor, if any, avidity enhancement,[4,10,11] we prepared new clusters using different scaffolds. These scaffolds were propargyl derivatized alcohols based on diethylene glycol (diol), glycerine (triol) and pentaerythritol (tetraol).[12,13] They were chosen for their similarity in polarity, flexibility, and distance (ca. 7 Å) of their linking alkyne groups. The sugar derivatives were coupled to the scaffold as per-acetylated 3-azidopropyl β-glycosides using Huisgen triazole-type click chemistry[14,15] followed by removal of the protecting groups. The resulting glycoclusters were tested as inhibitors of lectin binding in a solid-phase assay with a glycoprotein (asialofetuin) presenting up to nine docking sites.[7,9] Each experimental series included the different lectins to reveal any inter- and intrafamily differences under identical conditions. As the next step toward biomedical relevance, testing was extended to cells *in vitro* to reveal glycocluster efficiency to protect them from lectin binding. In order to prove that the compounds' headgroup can be subjected to modifications to further the activity, structural variations with galactose (Gal), lactose (Lac) and *N*-acetyllactosamine (LacNAc) were implemented. Bioactivity was further ascertained independently for the diglucoside maltose (Malt) as headgroup and two tetrameric glucose/mannose-specific plant lectins, and the demonstration of lack of effect on cell growth of a colon carcinoma line precluded concerns of unspecific harmfulness of the synthetic substances.

Materials and Methods

Synthetic Procedures. *General Methods.* Organic reagents and anhydrous $CHCl_3$ stabilized with amylene was used as purchased (ACROS, Chloroform 99.9%, extra dry over molecular sieves, stabilized). DMSO was stored over 3 Å molecular sieves. Dry THF was distilled over sodium−benzophenone ketyl radical and CH_2Cl_2 over calcium hydride. All reactions except for the click and deacylation reactions were carried out under an inert atmosphere (nitrogen or argon). Organic solutions were dried over $MgSO_4$ before concentration under reduced pressure at <40 °C (bath). TLC was performed on silica gel F_{254} (E. Merck) with detection by UV light and/or charring with 8% sulfuric acid. Silica gel (0.041−0.063 mm, Amicon) was used for column chromatography. Size exclusion chromatography (P2 gel, BioRad, polyacrylamide, fine) was done using 1% *n*-butanol in water at a flow rate recommended by the

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manufacturer. NMR spectra were recorded at 25 °C at 400 MHz (^{1}H) or 100 MHz. All proton and carbon NMR spectra in $CDCl_3$ were referenced to the chloroform signal (^{1}H δ 7.26 ppm, ^{13}C δ 77.17 ppm). No internal reference was used in D_2O (^{1}H δ 4.79 ppm). Numerical coupling constants are given without further assignment. MALDI-Tof spectra were recorded on a Bruker Reflex III using 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) as matrix. For synthesis and characterization data of compounds **1**−**5** see the Supporting Information.

3-Azidopropyl Hepta-O-acetyl-β-D-maltoside **6.** 1-Chloropropanol (600 μL, 7.2 mmol) and $BF_3 \cdot Et_2O$ (1.0 mL, 5.2 mmol) were added to a solution of octa-*O*-acetyl-β-D-maltose (2.2 g, 3.2 mmol) in anhydrous CH_2Cl_2 (20 mL). After 2 h stirring at room temperature, toluene (30 mL) was added. The reaction mixture was concentrated and coevaporated twice with toluene (2 × 20 mL). Flash chromatography (toluene → toluene/EtOAc, 3:1) gave 3-chloropropyl hepta-*O*-acetyl-β-D-maltoside (1.6 g, 2.2 mmol, 69%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41 (1 H, d, *J* 4.0, H-1α), 5.36 (1 H, dd, *J* 10.4, 9.7), 5.26 (1 H, t, *J* 9.2), 5.05 (1 H, t, *J* 9.9), 4.85 (2 H, dd, *J* 10.1, 3.6), 4.82 (1 H, dd, *J* 9.1, 7.5), 4.53 (1 H, d, *J* 7.9, H-1β), 4.48 (1 H, dd, *J* 12.1, 2.7), 4.24 (2 H, ddd, *J* 12.3, 8.4, 4.2), 4.04 (4 H, dd, *J* 12.5, 2.3), 4.01 (1 H, d, *J* 9.5), 3.96 (2 H, dd, *J* 10.0, 4.9), 3.69 (2 H, ddd, *J* 9.7, 8.0, 4.6), 3.58 (2 H, dd, *J* 7.2, 5.4), 2.14 (3 H, s), 2.10 (3 H, s), 2.04 (3 H, s), 2.03 (3 H, s), 2.02 (3 H, s), 2.00 (3 H, s), 2.00 (3 H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 170.6, 170.3, 170.1, 169.8, 169.6, 100.7, 95.7, 75.5, 72.8, 72.3, 72.3, 70.15, 69.5, 68.7, 68.2, 66.5, 62.9, 61.7, 41.4, 32.4, 21.1, 21.0, 20.8, 20.8, 20.7. The mixture of 3-chloropropyl hepta-*O*-acetyl-β-D-maltoside (1.6 g, 2.2 mmol) and KI (0.7 g, 4.2 mmol), dissolved in DMF (15 mL), was stirred at 50 °C for 30 min. NaN_3 (1.4 g, 22 mmol) was added, and the reaction mixture was stirred (oil bath 130 °C) for 1 h. The heating source was removed. After an additional 5 min, the still hot reaction mixture was poured onto ice−water (ca. 50 mL) and EtOAc (200 mL) was added. The organic phase was transferred into a separation funnel, washed with water (3 × 100 mL), dried and concentrated. Flash chromatography (toluene → toluene/EtOAc 2:1) of the residue gave **6** (1.5 g, 2.1 mmol, 93%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (1 H, d, *J* 4.0, H-1α), 5.35 (1 H, dd, *J* 10.4, 9.7), 5.24 (1 H, t, *J* 9.2), 5.04 (1 H, t, *J* 9.9), 4.85 (1 H, dd, *J* 10.5, 4.0), 4.81 (1 H, dd, *J* 9.4, 7.9), 4.52 (1 H, d, *J* 7.9, H-1β), 4.48 (1 H, dd, *J* 12.1, 2.8), 4.23 (2 H, m), 4.04 (1 H, dd, *J* 12.4, 2.3), 4.01−3.87 (3 H, m), 3.67 (1 H, ddd, *J* 9.6, 4.3, 2.8), 3.59 (1 H, ddd, *J* 9.8, 7.4, 5.2), 3.34 (2 H, td, *J* 6.5, 2.6), 2.13 (3 H, s), 2.09 (3 H, s), 2.03 (3 H, s), 2.02 (3 H, s), 2.01 (3 H, s), 1.99 (3 H, s), 1.99 (3 H, s), 1.89−1.75 (2 H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 170.6, 170. 3, 170.1, 169.8, 169.5, 100.4, 95.7, 75.5, 72.8, 72.3, 72.3, 70.1, 69.5, 68.6, 68.2, 66.6, 62.9, 61.6, 48.1, 29.1, 21.0, 21.0, 20.8, 20.8, 20.7, 20.7.

3-Azidopropyl 2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-3,6-di-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside **7.** TMSOTf (160 μL, 0.83 mmol) was added to a solution of peracetylated *N*-acetyllactosamine (500 mg, 0.74 mmol) in 1,2-dichloroethane (10 mL). The reaction mixture was stirred at 50 °C for 10 h and then cooled (ice-bath) before Et_3N (600 μL) was added. After removal of the solvent by evaporation, flash chromatography (toluene/EtOAc/Et_3N 200:200:1→ toluene/EtOAc/Et_3N 100:200:1) afforded 3-azidopropyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-1,2-dideoxy-D-glucopyrano[2,1-*d*]oxazoline (410 mg, 0.66 mmol, 90%). Pyridinium tosylate (23 mg, 91 μmol) was added to a solution of oxazoline (560 mg, 0.91 mmol) and 3-azidopropanol (800 mg, 3.1 mmol) in absolute chloroform (10 mL), and the mixture was kept under reflux conditions overnight. Then, the reaction (TLC: Et_2O/MeOH 15:2, $R_{f[\text{oxazoline}]} = 0.55$, $R_{f[\text{glycoside}]} = 0.47$) was quenched by addition of Et_3N and the solvent removed by evaporation. Flash chromatography (toluene/EtOAc/1:3→ toluene/EtOAc 1:6) of the residue afforded **7** (560 mg, 0.64 mmol, 70%): ^{1}H NMR (400 MHz, $CDCl_3$) δ (400 MHz, $CDCl_3$) 5.99 (1 H, d, *J* 9.5), 5.29 (1 H, dd, *J* 3.3, 0.8), 5.06−4.96 (2 H, m), 4.91 (1 H, dd, *J* 10.5, 3.4), 4.45 (1 H, d, *J* 7.8), 4.44 (1 H, dd, *J* 11.9, 2.6), 4.38 (1 H, d, *J* 7.9), 4.12−3.93 (4 H, m), 3.89−3.80 (2 H, m), 3.72 (1 H, t, *J* 8.7), 3.59−3.53 (1 H, m), 3.49 (1 H, ddd, *J* 9.8, 7.9, 4.9), 3.30 (2 H, t, *J* 6.5), 2.08 (3 H, s), 2.05 (3 H, s), 2.01 (3 H, s), 1.99 (3 H, s), 1.98 (2 H, s), 1.90 (3 H, s), 1.89 (2 H, s), 1.83−1.67 (2 H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 170.4, 170.3, 170.2, 170.1, 170.0, 169.3, 101.1, 101.0, 75.9, 72.7, 72.6, 70.8, 70.7, 69.1, 66.7, 65.9, 62.2, 60.8, 53.2, 48.0, 28.9, 23.2, 20.9, 20.8, 20.6, 20.6, 20.5.

Click Reaction (General Procedure). Except for the peracetylated 3-azidopropyl sugar derivatives, solutions (0.1 M) were prepared from all reagents prior to the click reactions. The scaffold solutions were prepared in DMF, $CuSO_4 \cdot 5H_2O$ was dissolved in deionized water, and sodium ascorbate was dissolved in one part of water and then nine parts of DMF were added (9:1, v/v, DMF/H_2O). To a sample vial (1.5−5 mL) containing the sugar derivative (70−180 μmol, 1.5 equiv per triple bond) and a micro mixing bar, an aliquot of the alkyne solution, $CuSO_4$ solution (0.3 equiv per triple bond) and sodium ascorbate solution (0.3 equiv per triple bond) were added. DMF was added to achieve a final concentration of approximately 0.1 M with respect to the sugar derivative. The reaction mixture was left stirring for 2−3 h at room temperature and monitored by TLC and MALDI-Tof (TLC 9:1, DCM/MeOH). MALDI-Tof samples were obtained by treating a sample of the reaction mixture with brine and EtOAc. The organic phase was then used for the MALDI-Tof analysis. For workup, brine was added (1−2 mL) and the reaction mixture extracted with EtOAc (3 mL). The organic phase was washed with brine (3 × 1 mL), dried over $MgSO_4$ and concentrated. The product was isolated by flash chromatography. During the first column (4 mL of silica gel; cluster **8**−**16**, DCM/EtOAc 9:1; cluster **17**−**19**, DCM/MeOH 20:1) the major portion of excess azido-spacer glycoside was removed, while a second column (4 mL of silica gel, DCM/MeOH 9:1) was necessary to remove small

amounts of defect conjugates and residual azido-spacer glycoside. For yields and characterization data of compounds **8**−**19** see the Supporting Information.

Deacetylation (General Procedure). The peracetylated glycoconjugate was dissolved in MeOH (5 mL), and NaOMe (1 M, 250 μL) was added. The reaction mixture was stirred overnight, producing partially deacetylated conjugates insoluble in the reaction mixture. Deionized water was added until a clear solution was obtained again. Stirring was continued until MALDI-Tof showed the formation of a unified product. The reaction mixture was neutralized using Dowex-H^+ ion-exchange resin, concentrated. The crude material was purified using reversed phase chromatography and size exclusion chromatography (P2). Reversed phase chromatography was carried out with a stepwise gradient starting with water (C18 silica gel, eluent system: H_2O/MeOH, 20% steps). Product-containing fractions were collected, concentrated, dissolved in deionized water and freeze-dried to afford clusters **20**−**31**. For yields and characterization data of compounds **20**−**31** see the Supporting Information.

Analytical Procedures. *Lectins.* Extracts of dried mistletoe leaves, beans of *Canavalia ensiformis* (for ConA) and *Pisum sativum* (for PSA) and pellets of bacteria after recombinant galectin production were the sources for the lectins. They were purified by affinity chromatography on lactose- or mannose-bearing Sepharose 4B resin as key step. The affinity resin was produced after divinyl sulfone activation to enable optimal lectin yields.[16] The extracts to obtain the lactose-binding lectins were prepared in 20 mM phosphate buffer (pH 7.2) containing 150 mM NaCl, using 100 mM lactose for elution of the galectins and plant toxin after thorough washes to remove any unbound material from the slurry in the columns.[16,17] Beans were homogenized in 50 mM Tris buffer (pH 8.0) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. Prefractionation of proteins was done by addition of 1 M acetic acid up to a final pH of 5. After centrifugation the lectin-containing supernatant was applied to mannose-presenting Sepharose 4B, the resin was thoroughly washed and the two plant agglutinins were eluted with 200 mM D-mannose (ConA) or 200 mM D-glucose (PSA). The product quality was routinely checked by one- and two-dimensional gel electrophoresis, gel filtration to ascertain the quaternary structure and mass spectrometry including peptide mapping to exclude any modifications or sequence deviations.[8,17] Galectin-3 was proteolytically trimmed by collagenase D from *Clostridium histolyticum* (Roche Diagnostics, Mannheim, Germany) with cleavage at the Tyr106/Gly107 and Glu229/Ile230 peptide bonds to obtain the truncated derivative.[18] The lectins were biotinylated under activity-preserving conditions with the *N*-hydroxysuccinimide ester derivative of biotin (Sigma, Munich, Germany), the extent and site of incorporation of the label were monitored by gel electrophoretic and mass spectrometric product analyses, and the activity was checked by solid-phase and cell assays.[18,19]

Inhibition Assays. Adsorption of asialofetuin (0.5 μg) to the surface of microtiter plate wells from phosphate-buffered saline (50 μL) at 4 °C overnight generated the lectin-reactive matrix, on which binding of the biotinylated probes (VAA, 0.5 μg/mL; galectins-1, -3 and -4, 5 μg/mL; truncated galectin-3, 40 μg/mL) was quantitated spectrophotometrically as described.[11] Cytofluorometric lectin binding to the Chinese hamster ovary (CHO) glycosylation mutants Lec2 (reduced sialylation) and Lec4 (reduced β1,6-branching of *N*-glycans; kindly provided by P. Stanley (New York, USA)), human SW480 colon adenocarcinoma cells, additionally treated for 24 h with 150 μM of the mannosidase I inhibitor 1-deoxymannojirimycin, and human Croco II B-lymphoblastoid cells was performed in duplicates with up to five independent series using aliquots of cell suspensions of the same or the next passage with standard deviations not exceeding 12.7% after normalization of the data.[20] Cell growth in the absence and presence of test substance in aliquots of suspensions of SW480 colon adenocarcinoma cells was measured after 48 h using the blue chromogen MTT (0.5 mg/mL; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).[21]

Results and Discussion

Synthesis of the Glycoclusters. The syntheses of the glycoclusters started with commercially available ethylene glycol, glycerine and pentaerythritol, which were alkylated

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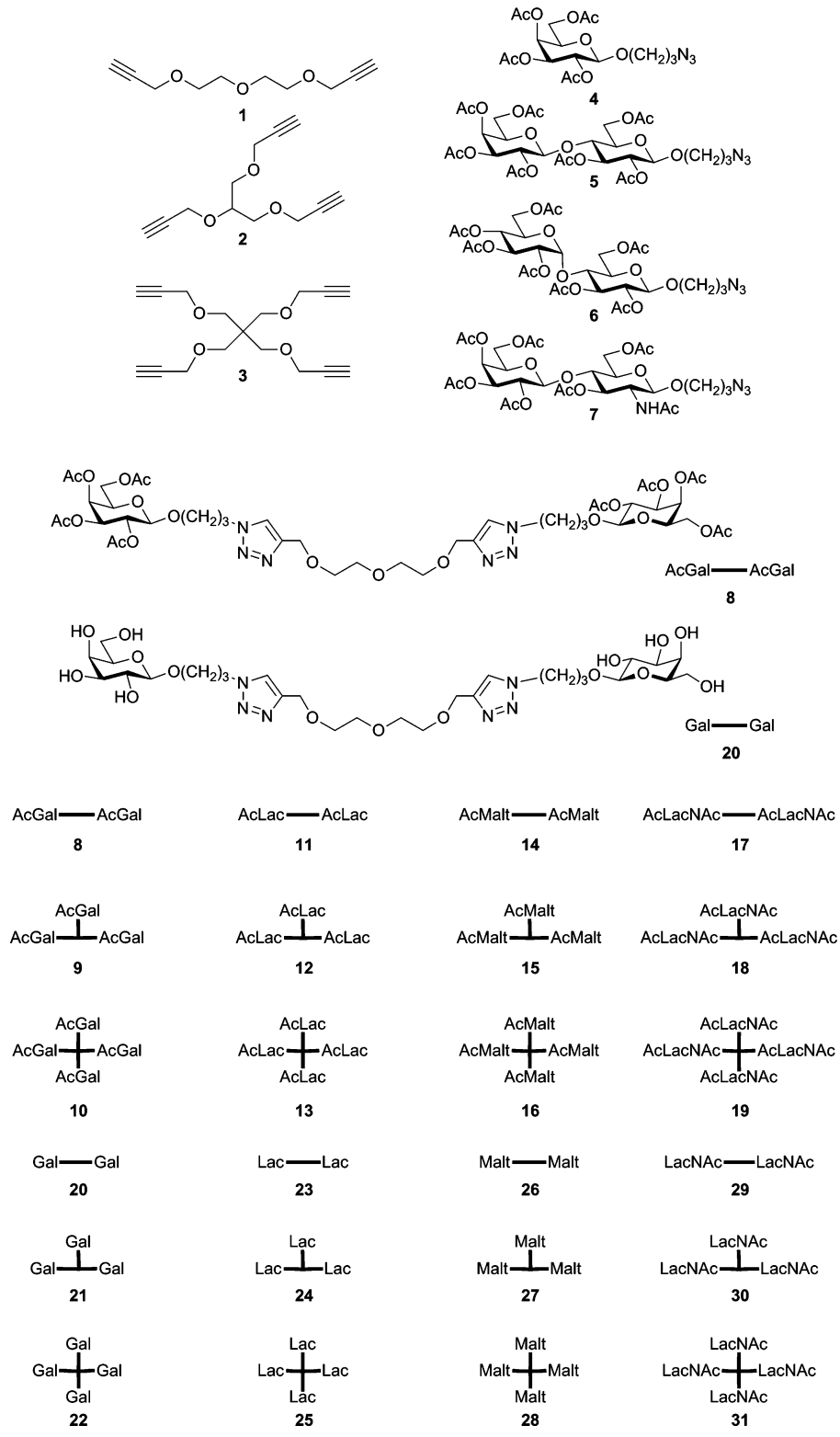

Figure 1. Structures of synthesized scaffolds (**1**−**3**), acetylated azidopropyl glycosides (**4**−**7**), and acetylated and product clusters (**8**−**19** and **20**−**31**, respectively).

with propargyl bromide in the presence of NaH or NaOH according to the literature to give compounds **1**−**3** (Figure 1).[12,13] Peracetylation of Gal, Lac and LacNAc as well as Malt followed by treatment with chloropropanol and BF_3-etherate afforded the corresponding 3-chloropropyl β-glycosides, in which azide displacement of the chloride yielded the 3-azidopropyl β-glycosides **4**−**7** (Figure 1), of which derivatives **4** and **5** were known before.[22−24] Formation of the glycoclusters was then accomplished by Cu(I)-catalyzed cyclization[14,15] of the carbohydrate azide group and the scaffold acetylene group to produce triazole-coupled structures **8**−**10** (AcGal), **11**−**13** (AcLac), **14**−**16** (AcMalt), and

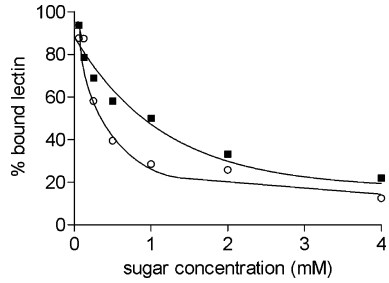

Figure 2. Concentration dependence of extent of inhibition of binding of labeled VAA (0.5 μg/mL) to the surface-immobilized glycoprotein asialofetuin (0.5 μg) by Lac (■) and the tetravalent Lac-presenting glycocluster (○). In each case, the concentration is given with respect to the sugar to ensure direct comparability. The concentration to yield an inhibition level of 50% (IC_{50} value) is determined by this graph (for comparisons between IC_{50} values for all compounds and lectins tested, please see Figure 3).

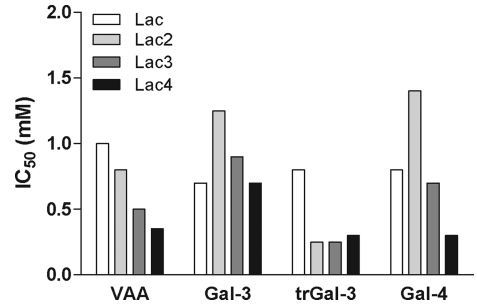

Figure 3. Relative potency of lactose (Lac) and this sugar as part of bi-, tri- and tetravalent glycoclusters (Lac_2, Lac_3, Lac_4 **23**−**25**) as inhibitors of binding of labeled lectins (VAA (0.5 μg/mL), human galectin-3 (5 μg/mL) and its proteolytically truncated derivative termed trGal-3 (40 μg/mL) and galectin-4 (5 μg/mL)) to the surface-immobilized glycoprotein asialofetuin, expressed in IC_{50} values. Concentration is given with respect to sugar in all cases. Assays were routinely done in triplicates for up to five independent series with standard deviations not exceeding 13.8%.

17−**19** (AcLacNAc) in 44−88% yield. Final deacetylation using Zemplen conditions (NaOMe/MeOH) generated the target clusters **20**−**22** (Gal), **23**−**25** (Lac), **26**−**28** (Malt), and **29**−**31** (LacNAc) in 44−100% yield after purification by size exclusion gel chromatography.

Inhibitory Capacity in Solid-Phase Assays. The matrix for carbohydrate-dependent lectin binding was established in microtiter wells by coating the plastic surface with the glycoprotein asialofetuin. Extent of lectin binding was saturable and nearly completely abolished by the presence of the cognate but not on unrelated sugar such as mannose. In each case, assays were performed at a subsaturating lectin concentration to characterize inhibitory capacity in the linear range of lectin-dependent signal increase. Titrations with increasing inhibitor concentrations normalized to the sugar content facilitated to determine the inhibitory concentration which yielded 50% inhibition (IC_{50} value), as shown for VAA (Figure 2). This parameter is a relative measure of affinity, which allows discernment of enhancements by the display of a distinct ligand on a scaffold. No precipitation of glycocluster−lectin complexes, which would confound the interpretation, was observed under these conditions. The plotting of the data for the lactose-presenting scaffolds revealed different patterns of reactivity, i.e. a stepwise increase for the plant toxin surpassing the free sugar's activity at all steps, a constant enhancement for truncated galectin-3 irrespective of valency and stepwise improvements finally markedly surpassing the free sugar's activity for the tandem-repeat-type galectin-4 (Figure 3). In contrast, full-length galectin-3, with its ability to pentamerize in the presence of a multivalent ligand,[25] was not susceptible to the tested clusters relative to the free sugar. This result highlights the importance of the collagenase-sensitive stalk region for reactivity to clusters. Of pharmaceutical interest, these glycoclusters can thus distinguish between the two natural versions of galectin-3, facilitating to envision a selective inhibition of the truncated form generated by proteolytic degradation *in situ*. Equally important, binding of homodimeric galectin-1 was only slightly affected by the presence of these glycocompounds compared to free lactose, the tetravalent compound reaching an IC_{50} value of about 4 mM relative to about 1 mM for lactose (please see the Supporting Information). In view of the rather similar ΔG values for binding to the disaccharide and to asialofetuin,[9] these assays also detected a conspicuous difference in reactivity with glycoclusters between the homodimeric galectin-1 and galectin-4, in which the two lectin domains are covalently linked by a connecting peptide. Despite rather similar hydrodynamic behavior of galectins-1 and -4 in fluorescence correlation spectroscopy,[26] both lectins presenting two lectin sites, their relative positioning is apparently different, and the tested glycoclusters can sense this difference. Overall, these two cases demonstrate the potential of

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cluster design to implement selectivity among closely related proteins as well as between two physiological forms of a lectin prior to and after proteolytic truncation. It can further be enhanced by modifying the sugar headgroup. To substantiate such a potential we tested the monosaccharide.

Bioactivity of modifications of the sugar headgroup is ascertained by measuring a rather comparable reactivity of the galactose-containing clusters on VAA binding (not shown). This result was based on the relative affinities of galactose and lactose.[10] Glycoclusters with galactose, in contrast, failed to inhibit galectin binding, in line with the corresponding experiments with free galactose. Selectivity for the lectin type, here a plant toxin vs human galectins, can thus be established by using galactose. Maltose-bearing glycoclusters were not active, excluding an effect by carbohydrate-independent binding.

In summary, the increase in local ligand density is thus most effective for the plant toxin and galectin-4. Removal of the collagenase-sensitive stalk region from galectin-3 makes the truncated version reactive already for the bivalent compound. Of note, this feature is selective when compared to full-length galectin-3 as well as galectins-1 and -4. The solid-phase assay thus revealed inhibitory activity of glycoclusters with selective properties in interlectin comparison. Since it is the aim of glycocluster application to protect cells from lectin binding, the solid-phase assay can be considered as a screening tool to identify active compounds to be taken to cell assays. On the cell surface, the labeled lectin will target glycans with appropriate structure and spatial presentation. As in the solid-phase setting, the glycocluster will compete with the glycan(s) for the binding site of the lectin. The resulting inhibition is quantitated by cytofluorometric analysis measuring the percentage of positive cells and the mean fluorescence intensity. These parameters are presented in each panel of the figures mentioned in the following paragraph.

Inhibitory Capacity in Cell Assays. Each panel presents the measurements in semilogarithmic scaling. It includes the control for probe-independent binding as shaded area as well as the data on percentage of positive cells and mean fluorescence intensity for each scan (for further details, please see legend to Figure 4). To take the analysis through different steps we took advantage of the availability of glycosylation mutants. We started with a line presenting mostly unsubstituted β-galactoside-terminated glycans, i.e. the CHO Lec2 mutant. The glycoclusters will in this case compete with unsubstituted LacNAc termini. Binding of the plant toxin to the cells was dependent on the lectin concentration (Figure 4A) and inhibitable by the haptenic sugar (Figure 4B), enabling definition of conditions in the linear range (i.e., 0.2 μg VAA/mL, 2 mM disaccharide as inhibitor). Testing the glycoclusters under identical conditions, routinely with aliquots of the same cell batch and passages, revealed increasing potency with number of valency up to values of 30%/14.5 for the tetravalent substance relative to 40%/19.0 for free lactose at the same concentration of sugar (Figure

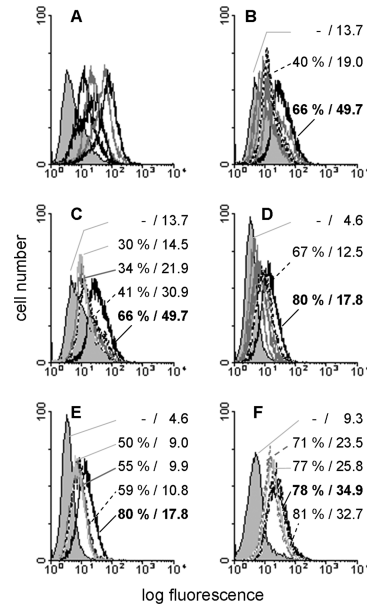

Figure 4. Semilogarithmic representation of fluorescent surface staining of cells of the Chinese hamster ovary glycosylation mutant line Lec2 defective in sialylation (A−C), the human colon adenocarcinoma line SW480 (D, E) and the human B-lymphoblastoid line Croco II (F) by labeled VAA. The control value illustrating staining by second-step reagent in the absence of lectin is always given as shaded area (negative control: 0%). Quantitative data on the percentage of positive cells and mean fluorescence intensity are given in each panel. The dependence of signal distribution and intensity on lectin concentration was tested at concentrations of 0.1 μg/mL, 0.2 μg/mL, 0.5 μg/mL, 1 μg/mL and 2 μg/mL (A), and the extent of inhibition by the cognate sugar lactose was determined at the constant lectin concentration of 0.2 μg/mL with Lac concentrations of 1 mM, 2 mM (dashed line), 5 mM, 10 mM and 20 mM, quantitative data given for the control in the absence of inhibitor (100%-control, numbers printed in bold for curve given as black line), for presence of 2 mM Lac (dashed line) and the 0%-control without lectin (B). Di- to tetravalent glycoclusters were tested for comparison at the sugar concentration of 2 mM, presenting quantitative data from bottom to top for the control value in the absence of inhibitor (black line), the bi-, tri- and tetravalent compounds and the negative control without lectin (C). Extent of inhibition of VAA binding to the colon cancer cells at the constant concentration of 2 μg/mL by galactose at concentrations of 1 mM, 2 mM, 5 mM, 10 mM and 20 mM, quantitative data given for the controls and for presence of 1 mM galactose (D), and extent of inhibition at this concentration of the sugar for the bi-, tri- and tetravalent compounds under otherwise identical conditions, quantitative data presented in this order from bottom to top (E). Extent of inhibition of VAA binding to the B-lymphoblastoid cells at the constant lectin concentration of 0.4 μg/mL for the sugar concentration of 1 mM tested with free lactose (bottom) as well as Lac and LacNAc presented as tetravalent compounds (Lac_4 **25**, $LacNAc_4$ **31**) listed in this order from bottom to top. The control in the absence of sugar is presented as a black line, the numbers printed in bold (F).

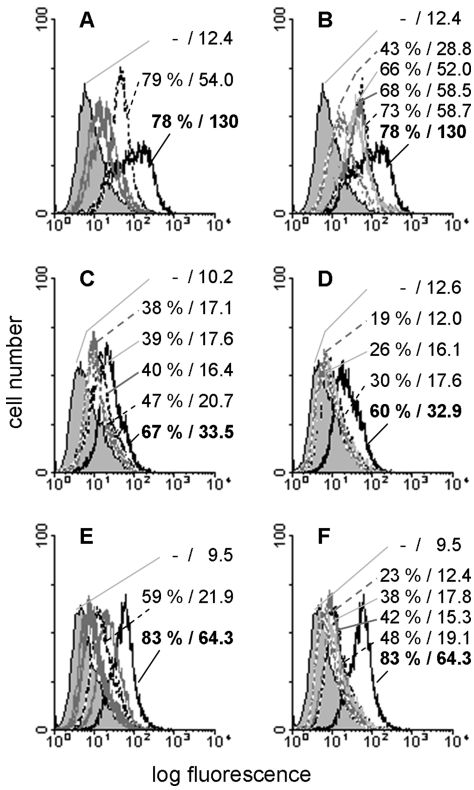

Figure 5. Semilogarithmic representation of fluorescent surface staining of cells of the Chinese hamster ovary glycosylation mutant line Lec2 (A−C), the respective wild-type cells (D) and the mutant line Lec4 defective in β1,6-branching of *N*-glycans (E, F) by human galectin-3 (A, B, D−F) and its proteolytically processed form (C) (for further details on control values and quantitative data, please see legend to Figure 3). Extent of inhibition by lactose at the constant lectin concentration of 5 μg/mL with concentrations of 0.5 mM (dashed line), 2 mM and 5 mM (A) and inhibitory capacity of the bi-, tri- and tetravalent glycoclusters as well as the tetravalent compound presenting LacNAc ($LacNAc_4$ **31**) at this sugar concentration (from bottom to top, control in the absence of sugar given at the bottom, printed in bold) (B). For comparison, the truncated derivative of galectin-3 (10 μg/mL) was tested under otherwise identical conditions, documenting the control in the absence of sugar (bottom) and profiles in the presence of 0.5 mM lactose as well as in the presence of tri-, bi- and tetravalent glycoclusters containing *N*-acetyllactosamine (**29**−**31**) at this sugar concentration (from bottom to top) (C). Galectin-3-dependent staining of the wild-type cells with normal status of sialylation was tested at 10 μg/mL in the absence of sugar (control) and in the presence of 0.5 mM lactose as well as 0.2 mM sugar presented in tetravalent glycoclusters (Lac **25** and LacNAc **31**) (from bottom to top) (D). Effect of absence of β1,6-branching in *N*-glycans on extent of inhibition was tested with galectin-3 (5 μg/mL) using Lac concentrations at 0.2 mM, 0.5 mM (dashed line), 1 mM, 2 mM and 5 mM (E) as well as 0.5 mM lactose presented in bi-, tri- and tetravalent glycoclusters and 0.5 mM LacNAc in the tetravalent glycocluster ($LacNAc_4$ **31**) (from bottom to top) (F).

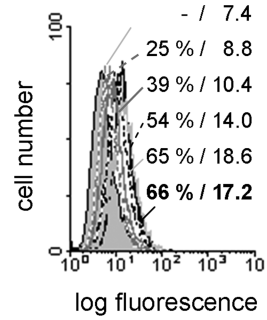

Figure 6. Fluorescent staining of cells of the Chinese hamster ovary glycosylation mutant line Lec2 by human galectin-4 (10 μg/mL) in the absence of inhibitor (bold numbers, black line for profile) and in the presence of 0.5 mM Lac as bivalent glycocluster (**23**), as carrier-free sugar, as tri- (**24**) and as tetravalent glycocluster (**25**) (from bottom to top; for further details on control values and quantitative data, please see legend to Figure 4).

4B,C). Proceeding to glycoprofiles with the physiological complexity of glycan branches, i.e. human tumor cells, the pattern with increased activity correlating with valency was maintained, already with galactose as headgroup, in line with the data from the solid-phase assays (Figure 4D,E). The tetravalent substance consistently reached the highest level of inhibition, regardless of the cell type (carcinoma or lymphoma) tested, and tailoring of the headgroups from Gal to Lac and finally LacNAc improved the reactivity (Figure 4F).

The influence of valency and headgroup tailoring was also seen for galectin-3, corroborating the solid-phase data, as a rather minor extent of dependence was measured for its proteolytically truncated derivative (Figure 5A−D). In order to identify a contribution of β1,6-branching of *N*-glycans with its characteristic occurrence of galectin-reactive LacNAc repeats we used the Lec4 glycosylation mutant. Diminution of this structural feature of *N*-glycans had no major influence on compound reactivity (Figure 5E,F). The incorporation of LacNAc instead of Lac was favorable, yielding a significant increase in inhibition (Figure 5F). The strong susceptibility of galectin-4 in solid-phase assays was also seen on the level of cells; in fact grading of activity followed the same course (Figure 3, Figure 6).

To further explore the relationship between valency and quaternary structure of lectins we added work with two tetrameric leguminous lectins and maltose-presenting clusters. In these cases, glycocluster testing led to a rather weak effect (Figure 7A−C). Its extent may be altered, if the density of cell surface ligands is changed. In order to answer the question whether a shift in the glycoprofile to high-mannose-type *N*-glycans to increase lectin binding can have an impact on cluster activity for the tetrameric proteins, we treated the cells with an appropriate inhibitor of glycan processing (i.e., 1-deoxymannojirimycin) and ascertained the predicted increase in ligand density (Figure 7D). Following this treatment a slight activity enhancement for glycoclusters was detectable

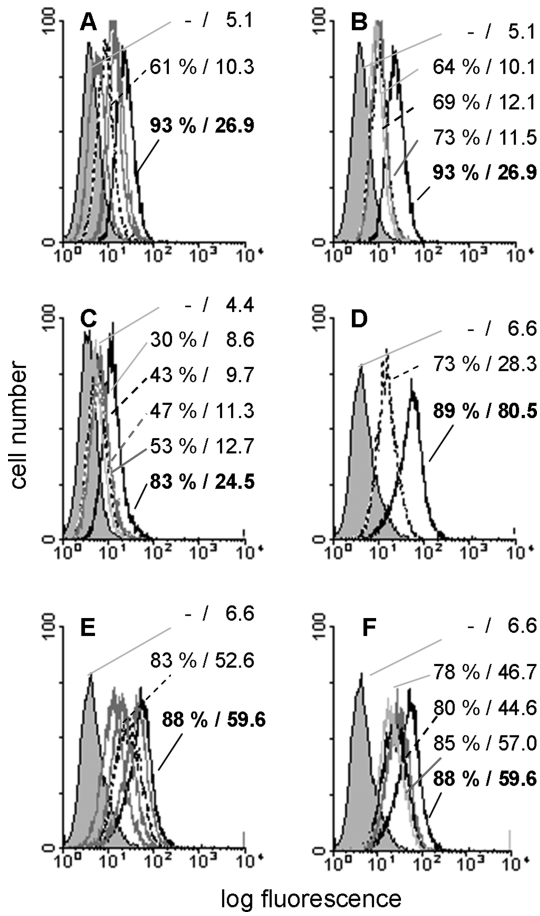

Figure 7. Fluorescent staining of cells of the human colon adenocarcinoma line SW480 by concanavalin A (1 μg/mL) in the absence of inhibitor (black line) and in the presence of increasing concentrations of maltose from 1 mM to 2 mM and 5 mM (dashed line) up to 20 mM (A) and in the presence of 5 mM Malt presented in the tri-, bi- and tetravalent glycoclusters **26**−**28** (from bottom to top; for further details on control values and quantitative data, please see legend to Figure 4) (B). The effect of type of sugar presentation was also tested with *Pisum sativum* agglutinin (0.2 μg/mL) in the absence of sugar (given in bold) and in the presence of 5 mM sugar presented in the tri- (**27**) and bivalent (**26**) glycoclusters, as carrier-free sugar and in the tetravalent glycocluster (**28**) (from bottom to top) (C). Treatment of cells with 1-deoxymannojirimycin increased reactivity to concanavalin A (1 μg/mL) as shown by reactivity of control cells (dashed line) and of treated cells (black line) (D). Staining profiles of the treated cells using this plant lectin in the presence of increasing concentrations of Malt from 2 mM to 5 mM (dashed line) and 10 mM up to 20 mM (E) and 5 mM sugar presented in the tri-, bi- and tetravalent glycoclusters (**27**, **26**, and **28**) (from bottom to top) (F).

(Figure 7E,F). Evidently, ligand density on cell surfaces, together with the quaternary structure, can have a bearing on lectin reactivity to glycoclusters. Since any consideration to pursue these scaffolds for drug development would be precluded, if the compounds were toxic, we finally exposed human colon adenocarcinoma cells to the compounds. Up to a concentration of 10 mM, normalized to sugar, no evidence for reduced cell growth was detected after a period of 48 h.

Conclusions

The design of glycoclusters pursues the aim to achieve an optimal inhibitory potency. To address the challenge to likewise reach an optimal level of selectivity between plant toxins/human lectins and members of a human lectin family, assays need to be performed in a comparative setting. Bioactivity of bi- to tetravalent compounds was revealed for three classes of lectins and different sugar headgroups. Toxin binding was already reduced by galactose, which, notably, is not effective for galectins. These lectins can physiologically target ligands organized in microdomains, and their recently reported ability for functional competition makes the quest for selective inhibitors especially attractive.[27−29] In this lectin class, homodimeric galectin-1 was rather insensitive to the test panel, galectins-3 and -4 reactive particularly with the tetravalent compound, while the physiological process of proteolytic truncation made galectin-3 already susceptible to the bivalent compound. The reactivity profiles thus showed selective properties. This finding may facilitate to shift the balance to medically favorable outcomes in cases of functional competition, e.g. between pro-anoikis galectin-1 and anti-anoikis galectin-3 seen in a model for pancreatic cancer.[19,29] The direct comparison of the results from solid-phase and cell assays proved the validity of the solid-phase system as initial test tool applicable in high-throughput screening. That toxicity assays revealed no effect on cell growth of the tested line qualifies this scaffold for further rounds of ligand design. Tailoring of the headgroup, e.g. introducing 6-deoxy galactose, α2,6-sialylated DiLacNAc or the respective section of a sulfatide including its hydroxylation in the bioinspired spacer, can help increase selectivity to the plant toxin or to galectins-3 and -4, respectively.[30−32] Considering the aglyconic part for scaffold conjugation and a carrier such as phenyl-bis-alanine may be a source for further advances toward targeting individual

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members of this lectin family.[10,33−36] Strategic combination of synthetic carbohydrate and cluster chemistry[5,37] with the documented screening procedures of increasing biorelevance is thus a solid basis for efforts to advance the development of glycoclusters as pharmaceuticals.

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Supporting Information Available: Experimental procedures and characterization data for compounds **1**−**5** and **8**−**31** as well as a figure showing a titration for human galectin-1 and lactose/Lac_4 glycocluster in the solid-phase assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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