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Cyclic neoglycodecapeptides: how to increase their inhibitory activity and selectivity on lectin/toxin binding to a glycoprotein and cells

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Protein (lectin/toxin)–glycan interaction can be clinically harmful so that the design of inhibitors has become an aim. Cyclic decapeptides are suited as rigid carriers for carbohydrate derivatives. We herein document the bioactivity of sugar headgroups covalently attached to this carrier for the cases of five proteins, i.e. a potent biohazardous plant agglutinin, a leguminous model lectin and three adhesion/growth-regulatory human lectins. They represent the different types of topological organization within the galectin family. The relative inhibitory activities of glycoclusters with the three ligands (galactose, lactose and the disaccharide of the Thomsen-Friedenreich antigen) reflected the affinity of free carbohydrates, hereby excluding an impairment of binding activity by chemical derivatization and conjugation. Headgroup tailoring is thus one route to optimize activity and selectivity of cyclopeptide-based glycoclusters. The increase of ligand density from tetra- to hexadecavalency added a second route. The plant toxin and tandem-repeat-type galectin-4 were especially sensitive to this parameter change. Strategically combining solid-phase assays for screening with analysis of lectin binding to cells in different systems revealed efficient inhibition by distinct glycoclusters, thereby protecting cells from lectin association. Cyclic neoglycodecapeptides thus warrant further study as lectin-directed pharmaceuticals. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: agglutinin; colon cancer; glycocluster; glycopeptide; lectin; oxime ligation; sialylation

Introduction

The growing awareness of the enormous capacity of glycan chains of natural glycoconjugates to serve as versatile biochemical signals has inspired the concept of the sugar code [1]. The natural arrangement of glycan determinants in clusters is a key factor to reach selectivity and avidity toward distinct receptors (lectins), e.g. by forming branches in *N*- and *O*-glycans or at terminal positions of glycan antennae [2–5]. As lectin–glycan recognition is involved in a wide array of clinically relevant processes, e.g. in bacterial/viral infections or regulation of cell adhesion, growth or migration, the design of synthetic glycoclusters with therapeutic potential can benefit from the lessons taught by nature. Consequently, a series of scaffolds was turned into carriers of carbohydrates [6–12]. Glycan attachment to macrocycles or to compounds suited to self-assembly into metal ion-coordinated complexes offers the perspective to establish a rather rigid topology in order to target lectins selectively, and ligand properties of such compounds have been documented in models [13–19]. Along this line, head-to-tail or intramolecular cyclization of linear peptides was exploited to generate spatially constrained glycoclusters, amenable to the set-up of split-and-mix libraries [20–24]. The inclusion of apposing prolylglycine bonds will induce β-type II turns, resulting in an anti-parallel β-sheet with four to six lysine residues for carbohydrate attachment. Thus, the preparation of decapeptides is possible, which enable regioselective functionalization, e.g. with carbohydrate derivatives. Initial testing of this glycocluster design revealed binding to two tested leguminous lectins, i.e. the mannose-specific concanavalin A and peanut agglutinin (PNA) specific for the disaccharide of the Thomsen-Friedenreich (TF) antigen [25–30], and potential to serve as nonimmunogenic vaccine carrier [31–33]. As synthetic glycopeptides or mimetics thereof had been shown to harbour reactivity for medically relevant lectins [34,35], and the sugar part of the TF-antigen can react with human lectins involved in tumor progression [36–38], the next step in testing these cyclic neoglycodecapeptides will be to determine whether they can block cell binding of toxins and human lectins.

For this report, we studied the effect of these glycoclusters on glycan binding of the plant toxin *Viscum album* L. agglutinin (VAA) and three adhesion/growth-regulatory galectins selected to represent the existing types of topological lectin-site arrangement, i.e.

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noncovalent dimerization with galectin-1, chimera-type galectin-3 with the lectin domain connected to a stalk region responsible for noncovalent oligomerization and the covalent connection of two different subunits in tandem-repeat-type galectin-4 by a linker [2,39–41]. To probe into the importance of the sugar headgroup, three structures were tested with galactose (Gal), lactose (Lac) and the disaccharide of the TF-antigen (αTF). To further do so for the parameter of cluster density tetravalency was increased to hexadecavalency by introducing a tetravalent polylysine dendrimeric structure into each lysine side chain of the cyclic core. As it is the therapeutic aim to prevent lectin binding to physiological ligands, whose reactivity may even depend on presentation in microdomains [42], the activity of the resulting set of glycoclusters as inhibitors was determined in test systems of increasing biorelevance. In a solid-phase assay, each of the six types of cyclic neoglycopeptides and free sugars as controls were first challenged to block lectin binding to a surface-presented glycoprotein. The galactose-specific lectins are known to bind to this natural glycoprotein with high affinity and loading of all *N*-glycan antennae [43]. Next, we took the evaluation of the glycoclusters to the level of cells, working in cell models with distinct changes in the glycoprofile and with human colon/pancreatic tumor cells. The glycomic profile of the pancreas carcinoma model has recently been mapped and functional galectin binding with competition between galectins-1 and -3 been detected [44,45]. This strategic combination of cluster design with the two assay methods will answer the question on relative inhibitory activity with respect to sugar headgroup, cluster design and lectin type.

Materials and Methods

Materials and General Procedures

All chemical reagents were purchased from Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France) and were used without further purification. Protected amino acids and Fmoc-Gly-Sasrin resin were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem. For carbohydrate synthesis, progress of reactions was monitored by thin layer chromatography using silica gel 60 F_{254} precoated plates (Merck, Darmstadt, Germany). Spot visualization was performed under UV light and by heating the plate after treatment with a solution of 10% sulfuric acid in ethanol for protected derivatives or 1% ninhydrine in ethanol for hydroxylamine derivatives. Silica gel 60 (0.063–0.2 mm/70–230 mesh, Merck) was used for column chromatography. Optical rotations were measured with a Perkin-Elmer 241 polarimeter, melting points on a Büchi melting point apparatus (model B545). ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC300 spectrometers and chemical shifts (δ) are reported in parts per million (ppm). Spectra were referenced to the residual proton solvent peaks relative to the signal of $CDCl_3$ (δ 7.27 and 77.23 ppm for ^{1}H- and ^{13}C-NMR, respectively) or relative to the signal of D_2O (δ 4.79 ppm for ^{1}H-NMR). Proton and carbon assignments were obtained from GCOSY and GHMQC experiments. The anomeric configuration was established by the determination of the coupling constant (J) between H-1 and H-2. For the synthesis of peptides, progress of reactions was monitored by reverse-phase HPLC on Waters equipment using C_{18} columns. The analytical separation (Nucleosil 120 Å 3 µm C_{18} particles, 30 × 4.6 mm^2) was operated at 1.3 ml/min and the preparative (Delta-Pak 300 Å 15 µm C_{18} particles, 200 × 25 mm^2) at 22 ml/min with UV monitoring at 214 and 250 nm using a linear A–B gradient (buffer A: 0.09% CF_3CO_2H in water; buffer B: 0.09% CF_3CO_2H in 90% acetonitrile; all profiles shown in Supporting Information were recorded at 214 nm). For each synthetic intermediate, mass spectra were recorded either by electrospray ionization (ESI-MS) on a VG Platform II or by chemical ionization (DCI-MS) on a Thermofinnigan Polaris Q in the positive mode. NMR spectra of the compounds **13**–**15** were obtained in D_2O either at 400 or at 500 MHz with a Bruker Avance 400 or Varian Unity Plus spectrometers, respectively.

O-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 3′)-(4′, 6′-di-*O*-acetyl-2′-azido-2′-deoxy-α-D-galactopyranosyl)-*N*-hydroxyphthalimide (2)

A solution of **1** (1.5 g, 2.33 mmol) in THF (20 ml) was treated with DAST (0.46 ml, 1.5 equiv.) at −30 °C. The mixture was warmed to room temperature and stirred until TLC indicates complete disappearance of the starting material **1** (2 h). After addition of CH_3OH (2 ml) at −30 °C, the solution was evaporated to dryness. The yellowish syrup was dissolved in CH_2Cl_2 (20 ml), washed successively with saturated solution of $NaHCO_3$, then water and the organic layer was dried over Na_2SO_4. After evaporation to dryness, the resulting crude fluoride derivative (R_f = 0.23, eluent: CH_2Cl_2/AcOEt 4 : 1) was dissolved in dry CH_2Cl_2 (20 ml). *N*-Hydroxyphthalimide (0.38 g, 1 equiv.), triethylamine (0.33 ml, 1 equiv.) and $BF_3{\cdot}Et_2O$ (1.2 ml, 4 equiv.) were added and the solution was stirred for 1 h at room temperature. CH_2Cl_2 (20 ml) was then added to the crude mixture. The organic layer was washed twice with 10% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. The α-anomer **2** was finally purified by silica gel chromatography (eluent: ether) and precipitated from CH_2Cl_2/pentane as a white amorphous solid. 0.73 g (41% yield from **1**); R_f = 0.45 (eluent: ether); mp = 103.4–105.7 °C; $[\alpha]^{25}_D$ = 45.7 (c 0.96, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.86–7.74 (m, 4H, Har.), 5.63 (bd, 1H, $^3J_{3',4'}$ = 3.1 Hz, H-4′), 5.59 (d, 1H, $^3J_{1',2'}$ = 3.8 Hz, H-1′), 5.36 (bd, 1H, $^3J_{3,4}$ = 3.3 Hz, H-4), 5.19 (dd, 1H, $^3J_{1,2}$ = 7.8 Hz, $^3J_{2,3}$ = 10.4 Hz, H-2), 5.02 (dd, 1H, $^3J_{5',6a'}$ = 4.3 Hz, $^3J_{5',6b'}$ = 7.3 Hz, H-5′), 5.00 (dd, 1H, H-3), 4.75 (d, 1H, H-1), 4.34 (dd, 1H, $^2J_{6a',6b'}$ = 11.7 Hz, H-6a′), 4.26 (dd, 1H, $^3J_{2',3'}$ = 11.0 Hz, H-3′), 4.16 (dd, 1H, $^3J_{5,6a}$ = 6.7 Hz, $^2J_{6a,6b}$ = 11.3 Hz, H-6a), 4.09 (dd, 1H, $^3J_{5,6b}$ = 6.1 Hz, H-6b), 3.96 (dd, 1H, H-2′), 3.95–3.91 (m, 1H, H-5), 3.83 (dd, 1H, H-6b′), 2.14, 2.12, 2.06, 2.05, 2.02, 1.97 (6s, 18H, 6 × $OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 170.9 (C=O), 170.8 (C=O), 170.6 (C=O), 170.4 (C=O), 169.9 (C=O), 169.8 (C=O), 163.3 (C=O), 135.2 (CHar.), 129.1 (Car.), 124.1 (CHar.), 103.5 (C-1′), 101.9 (C-1), 74.7 (C-3′), 71.4 (C-5), 71.2, 70.2 (C-3, C-5′), 69.8 (C-4′), 69.2 (C-2), 67.3 (C-4), 62.8 (C-6′), 61.6 (C-6), 59.2 (C-2′), 21.1 (CH_3), 21.0 (CH_3), 21.0 (CH_3), 20.9 (CH_3); ESI$^+$-HRMS: calcd for $C_{32}H_{36}N_4O_{18}Na$: 787.1922; found: 787.1933 $[M + Na]^+$. The corresponding β-anomer was also recovered. 0.56 g (31% yield from **1**); R_f = 0.24 (eluent: ether); mp = 104.6–107.1 °C; $[\alpha]^{25}_D$ = 110.4 (c 0.95, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.90–7.76 (m, 4H, Har.), 5.36 (bd, 2H, $^3J_{3,4}$ = 3.4 Hz, $^3J_{3',4'}$ = 3.4 Hz, H-4, H-4′), 5.15 (dd, 1H, $^3J_{1,2}$ = 7.8 Hz, $^3J_{2,3}$ = 10.5 Hz, H-2), 5.01 (dd, 1H, H-3), 4.91 (d, 1H, $^3J_{1',2'}$ = 8.4 Hz, H-1′), 4.77 (d, 1H, H-1), 4.15–4.05 (m, 4H, H-6, H-6′), 3.90 (dd, 1H, $^3J_{2',3'}$ = 10.1 Hz, H-2′), 3.88 (bt, 1H, $^3J_{5,6}$ = 5.7 Hz, H-5), 3.76 (t, 1H, $^3J_{5',6'}$ = 6.2 Hz, H-5′), 3.63 (dd, 1H, H-3′), 2.15, 2.14, 2.09, 2.03, 1.97, 1.94 (6s, 18H, 6 × $OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 170.7 (C=O), 170.6 (C=O), 170.5 (C=O), 170.1 (C=O), 169.7 (C=O), 163.0 (C=O), 135.2 (CHar.), 129.1 (Car.), 124.2 (CHar.), 106.2 (C-1′), 101.8 (C-1), 77.3 (C-3′), 72.5 (C-5′), 71.3 (C-5), 71.0 (C-3), 69.1 (C-2),

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67.9, 67.2 (C-4, C-4′), 61.9, 61.8 (C-6, C-6′), 61.5 (C-2′), 21.0 (CH_3), 20.9 (CH_3); DCI$^+$-MS (NH_3 + isobutane): *m/z* 782.0 [M + NH_4]$^+$.

***O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 3′)-(2′-acetamido-2′-deoxy-4′,6′-di-*O*-acetyl-α-D-galactopyranosyl)-*N*-hydroxyphthalimide (3)**

Compound **2** (0.73 g, 0.96 mmol) was dissolved in a degassed solution of CH_3OH/Ac_2O (9/1, 30 ml) containing 10% Pd/C (0.2 g, 0.3 equiv.). The mixture was stirred for 3 h at room temperature under H_2 before removing the catalyst by filtration over a pad of celite. After evaporation, compound **3** was purified by silica gel chromatography (eluent: EtOAc), then precipitated in CH_2Cl_2/pentane as a white amorphous solid. 0.34 g (45% yield); R_f = 0.38 (eluent : AcOEt); mp = 120.6 °C; $[\alpha]^{25}{}_D$ 120.5 (*c* 0.82, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.85–7.76 (m, 4H, Har.), 6.14 (d, 1H, $^3J_{2',NH}$ = 9.5 Hz, NH), 5.55 (bd, 1H, $^3J_{3',4'}$ = 2.6 Hz, H-4′), 5.37 (d, 1H, $^3J_{1,2}$ = 3.6 Hz, H-1′), 5.36 (bd, 1H, $^3J_{3,4}$ = 2.8 Hz, H-4), 5.14 (dd, 1H, $^3J_{1,2}$ = 7.7 Hz, $^3J_{2,3}$ = 10.3 Hz, H-2), 4.99–4.94 (m, 2H, H-3, H-5′), 4.78 (ddd, 1H, $^3J_{2',3'}$ = 11.3 Hz, H-2′), 4.66 (d, 1H, H-1), 4.37 (dd, 1H, $^3J_{5',6a'}$ = 4.7 Hz, $^3J_{6a',6b'}$ = 11.6 Hz, H-6a′), 4.20–4.09 (m, 3H, H-3′, H-6), 3.94–3.87 (m, 2H, H-5, H-6a′), 2.17 (s, 3H, $OCOCH_3$), 2.16, (s, 3H, $NHCOCH_3$), 2.15, 2.11, 2.08, 2.06, 1.97 (5s, 15H, 5 × $OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.1 (C=O), 170.8 (C=O), 170.7 (C=O), 170.5 (C=O), 170.3 (C=O), 169.9 (C=O), 163.5 (C=O), 135.3 (CHar.), 129.0 (Car.), 124.2 (CHar.), 105.9 (C-1′), 101.2 (C-1), 76.9 (C-2′), 72.5 (C-3′), 71.3 (C-5), 71.1, 70.2 (C-3, C-5′), 69.3 (C-4′), 68.9 (C-2), 67.2 (C-4), 62.8 (C-6′), 61.6 (C-6), 23.7 (CH_3), 21.2 (CH_3), 21.1 (CH_3), 20.9 (CH_3); ESI$^+$-HRMS: calcd for $C_{34}H_{40}N_2O_{19}Na$: 803.2123; found: 803.2117 [M + Na]$^+$.

***O*-(β-D-Galactopyranosyl)-(1 → 3′)-2′-acetamido-2′-deoxy-α-D-galactopyranosyl hydroxylamine (4)**

Compound **3** (55 mg, 0.07 mmol) was dissolved in EtOH (2 ml) containing methylhydrazine (0.3 ml, 80 equiv.). After stirring for 4 h at room temperature, the solvent was evaporated to dryness. The fully deprotected compound **4** was purified by precipitation in CH_3OH/CH_2Cl_2, then lyophilization. 20 mg (72% yield); $[\alpha]^{25}{}_D$ 93.9 (*c* 0.6, H_2O); ^{1}H NMR (300 MHz, D_2O): δ = 4.95 (d, 1H, $^3J_{1',2'}$ = 4.0 Hz, H-1′), 4.45 (d, 1H, $^3J_{1,2}$ = 7.7 Hz, H-1), 4.39 (dd, 1H, $^3J_{2',3'}$ = 11.4 Hz, H-2′), 4.24 (bd, 1H, $^3J_{3',4'}$ = 2.8 Hz, H-4′), 4.02 (bt, 1H, $^3J_{5',6'}$ = 6.3 Hz, H-5′), 3.97 (dd, 1H, H-3′), 3.91 (bd, 1H, $^3J_{3,4}$ = 3.2 Hz, H-4), 3.80–3.72 (m, 4H, H-6, H-6′), 3.66–3.64 (m, 1H, H-5), 3.61 (dd, 1H, $^3J_{2,3}$ = 9.9 Hz, H-3), 3.50 (dd, 1H, H-2), 2.03 (s, 3H, $NHCOCH_3$); ^{13}C NMR (75 MHz, D_2O) δ = 175.0 (C=O), 105.0 (C-1), 101.1 (C-1′), 77.3 (C-3′), 75.3 (C-5), 72.9 (C-3), 71.1, 70.9 (C-2, C-5′), 69.1, 68.9 (C-4, C-4′), 61.5, 61.3 (C-6, C-6′), 48.2 (C-2′), 22.3 (CH_3); ESI$^+$-HRMS: calcd for $C_{14}H_{26}N_2O_{11}Na$: 421.1434; found: 421.1435 [M + Na]$^+$.

Synthesis of Tetravalent Glycoclusters 8–10

The following oxime ligation procedure is typical. A solution of R(4CHO) **7** [26] and glycopyranosylhydroxylamine **4**–**6** (10 equiv.) was stirred in 10% AcOH in H_2O overnight at 37 °C. The crude reaction mixture was next processed by reverse-phase HPLC to obtain tetravalent glycoclusters **8**–**10** as white powders after lyophilization. R(4βGal) **8**: 7 mg (yield: 55%); analytical RP-HPLC: R_t = 5.99 min (5–40% B in 15 min, λ = 214 and 250 nm); ESI$^+$-MS: calcd for $C_{76}H_{122}N_{18}O_{38}$: 1895.9, found: *m/z* 1896.7 [M + H]$^+$. R(4βLac) **9**: 9 mg (yield: 60%); analytical RP-HPLC: R_t = 5.30 min (5–40% B in 15 min, λ = 214 and 250 nm); ESI$^+$-MS: calcd for $C_{100}H_{162}N_{18}O_{58}$: 2544.5, found: *m/z* 2544.9 [M + H]$^+$. R(4αTF) **10**: 7 mg (yield: 61%); analytical RP-HPLC: R_t = 6.87 min (5–40% B in 15 min, λ = 214 and 250 nm); ESI$^+$-MS: calcd for $C_{108}H_{174}N_{22}O_{58}$: 2708.7, found: *m/z* 2709.3 [M + H]$^+$.

Synthesis of R(16CHO) 12

The procedure described for **8**–**10** was followed from R(4CHO) **7** and PolyK(4Ser)ONH$_2$ **11** [46]. The resulting compound (16 mg; 2.3 μmol) was dissolved in water (10 ml), and sodium periodate (79 mg; 160 equiv.) was added to the solution. After stirring at room temperature for 30 min, the aldehyde-containing conjugate R(16CHO) **12** was purified by preparative RP-HPLC. 10 mg (yield: 92%); analytical RP-HPLC: R_t = 8.34 min (5–40% B in 15 min, λ = 214 and 250 nm); ESI$^+$-MS: calcd for $C_{200}H_{306}N_{58}O_{74}$: 4707.0; found: *m/z* 4707.8 [M + H]$^+$.

Synthesis of Hexadecavalent Glycoclusters 13–15

The procedure described for **8**–**10** was then followed from R(16CHO) **12** and glycopyranosylhydroxylamine **4**–**6**. We thus obtained hexadecavalent glycoclusters **13**–**15** after semi-preparative HPLC. R(16βGal) **13**: 6 mg (yield: 73%); analytical RP-HPLC: R_t = 7.98 min (5–40% B in 15 min, λ = 214 and 250 nm); ^{1}H NMR (500 MHz, D_2O): δ = 7.88–7.77 (m, 20H, 20 × Hox), 5.15–5.12 (m, 16H, 16 × H-1), 4.76–4.71 (m, 8H, 4 × $COCH_2$–O), 4.23–4.45 (m, 28H, 28 × H_α), 4.12 (d, 2H, $^2J_{H\alpha,H\alpha'}$ = 17.8 Hz, 2 × H_α Gly), 4.03–3.99 (m, 16H, 16 × H-4), 3.89–3.70 (m, 86H, 16 × H-2, 16 × H-3, 16 × H-5, 32 × H-6, 2 × $H_{\alpha'}$ Gly and 2 × $CH_{2\delta}$ Pro), 3.35–3.20 (m, 40H, 20 × $CH_{2\varepsilon}$ Lys), 2.35–1.32 (m, 146H). R(16βLac) **14**: 10 mg (yield: 77%); analytical RP-HPLC: R_t = 8.03 min (5–40% B in 15 min, λ = 214 and 250 nm); ^{1}H NMR (400 MHz, D_2O): 7.91–7.79 (m, 20H, 20 × Hox), 5.27–5.22 (m, 16H, 16 × H-1′), 4.76–4.74 (m, 8H, 4 × $COCH_2$–O), 4.52 (d, 16H, $^3J_{1,2}$ = 7.7 Hz, 16 × H-1), 4.47–4.26 (m, 28H, 28 × H_α), 4.07–3.96 (m, 34H, 16 × H-6a′, 16 × H-4 and 2 × H_α Gly), 3.93–3.75 (m, 118H, 16 × H-3′, 16 × H-4′, 16 × H-5, 16 × H-5′, 16 × H-6b′, 32 × H-6′, 2 × $H_{\alpha'}$ Gly and 2 × $CH_{2\delta}$ Pro), 3.73 (dd, 16H, $^3J_{3,4}$ = 3.2 Hz, $^3J_{2,3}$ = 10.0 Hz, 16 × H-3), 3.68–3.59 (m, 32H, 16 × H-2 and 16 × H-2′), 3.37–3.24 (m, 40H, 20 × $CH_{2\varepsilon}$ Lys), 2.36–1.27 (m, 146H). R(16αTF) **15**: 6 mg (yield: 65%); analytical RP-HPLC: R_t = 7.85 min (5–40% B in 15 min, λ = 214 and 250 nm); ^{1}H NMR (500 MHz, D_2O): δ = 7.80–7.76 (m, 20H, 20 × Hox), 5.62–5.59 (m, 16H, 16 × H-1′), 4.73–4.69 (m, 8H, 4 × $COCH_2$–O), 4.56 (td, 16H, $^3J_{1',2'}$ = 3.8, $^3J_{2',3'}$ = 11.5 Hz, 16 × H-2′), 4.51 (d, 16H, $^3J_{1,2}$ = 7.6 Hz, 16 × H-1), 4.41–4.22 (m, 44H, 16 × H-4′ and 28 × H_α), 4.15–4.07 (m, 18H, 16 × H-3′ and 2 × H_α Gly), 4.02 (bt, 16H, $^3J_{5',6'}$ = 6.0 Hz, 16 × H-5′), 3.93 (bd, 16H, $^3J_{3,4}$ = 3.0 Hz, 16 × H-4), 3.83–3.62 (m, 102H, 16 × H-3, 16 × H-5, 32 × H-6, 32 × H-6′, 2 × $H_{\alpha'}$ Gly and 2 × $CH_{2\delta}$ Pro), 3.58–3.52 (m, 16H, 16 × H-2), 3.33–3.18 (m, 40H, 20 × $CH_{2\varepsilon}$ Lys), 2.36–1.27 (m, 194H).

Lectins

Purification from extracts of dried mistletoe leaves or nuts and pellets of bacteria after recombinant galectin production by affinity chromatography on lactosylated Sepharose 4B as crucial step and quality controls by one- and two-dimensional gel electrophoresis and mass spectrometry were performed as described [18,34,47]. Proteolytic truncation of human galectin-3 by digestion with collagenase and product analysis by mass spectrometry were performed as described [48]. Labeling using the *N*-hydroxysuccinimide ester derivative of biotin (Sigma, Munich, Germany) under activity-preserving conditions, determination of

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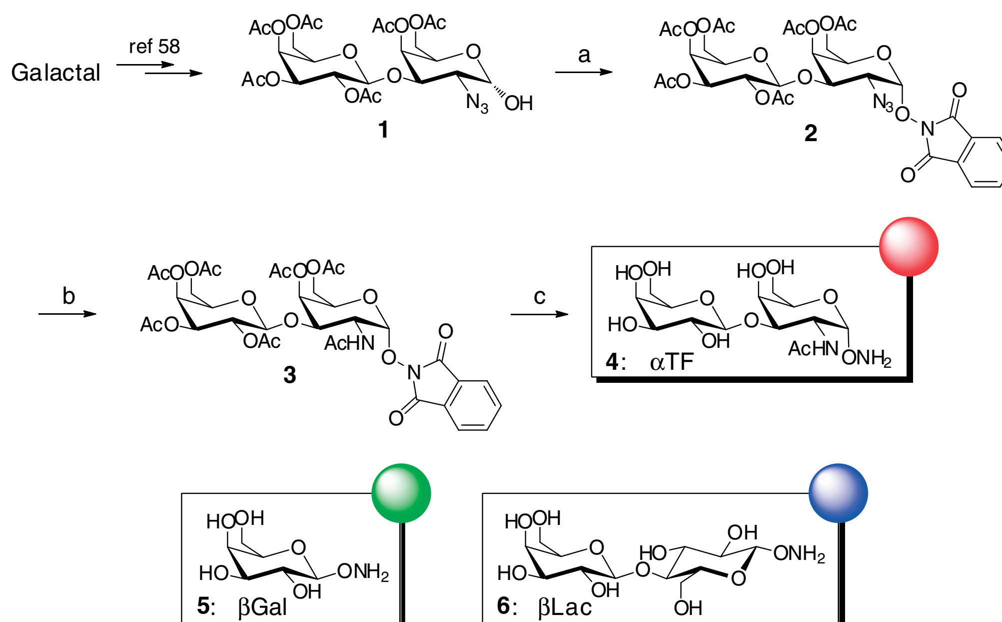

Scheme 1. Reagents and conditions: (a) i: DAST, THF, rt; ii: *N*-hydroxyphthalimide, TEA, BF_3. Et_2O, CH_2Cl_2, rt; (b) H_2, Pd/C, MeOH/Ac_2O (9 : 1), rt and (c) methylhydrazine/EtOH (1 : 1), rt.

degree of substitution by two-dimensional gel electrophoresis and activity controls of labeled lectins followed established protocols [49,50].

Inhibition Assays

Solid-phase assays were routinely done in microtiter plate wells, whose surface was coated with the glycoprotein asialofetuin (0.5 µg/well), under conditions to yield signal intensity in the linear range when systematically increasing the concentration of the labeled lectins (VAA: 0.4 µg/ml; galectins-1 and -3: 5 µg/ml; truncated galectin-3: 30 µg/ml and galectin-4: 3 µg/ml), measuring the effect of competition between *N*-glycans of the glycoprotein and the inhibitor (free sugar or glycocluster; all given concentrations are always normalized to the molarity of the sugar) spectrophotometrically [18,51]. Assays were done in triplicates with up to five independent series with standard deviations not exceeding 13.1%. 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 and Capan-1 pancreatic carcinoma cells, reconstituted for expression of the tumor suppressor $p16^{INK4a}$ (kindly provided by K. M. Detjen, Berlin, Germany), was performed in duplicates with up to six independent series using aliquots of cell suspensions of the same or the next passage with standard deviations not exceeding 12.1% [44,52,53]. Controls for carbohydrate-independent binding were rigorously performed in both assay types by omitting the incubation step with the lectin and by titrations with cognate and noncognate sugars, the latter used as osmolarity control.

Results and Discussion

Synthesis of Cyclic Neoglycopeptides

Chemoselective ligations build a versatile bridge between the scaffold (carrier) and the carbohydrate headgroup to prepare glycoclusters [18,54]. In our context, an oxime-based strategy reported recently was found to be very efficient for this purpose [9,55]. This conjugation strategy starts with the incorporation of an anomeric hydroxylamine functionality into carbohydrates to facilitate reactivity with the cyclopeptide scaffold containing clustered aldehydic acceptors.

Synthesis of glycosylhydroxylamines

Stereoselective synthesis of β-glycosylhydroxylamines has previously been described using phase-transfer catalysis [56]. We have then reported a complementary strategy that enables the formation of both α- and β-glycosylhydroxylamines from the corresponding glycosyl fluorides [57]. In this study, the latter strategy was followed to prepare several types of glycosylhydroxylamine based on the three selected carbohydrate determinants, i.e. the disaccharide of the TF-antigen termed αTF **4**, βGal **5** and βLac **6** (Scheme 1). Briefly, the aminooxy derivative of the disaccharide of the TF-antigen **4** was obtained from triacetylated galactal following a multi-step synthetic route leading to the disaccharide **1** [58]. This compound was next activated with DAST in tetrahydrofuran, then the resulting crude glycosyl fluoride was glycosylated with *N*-hydroxyphthalimide to obtain phthalimido derivatives in α/β-anomeric mixture. The expected α-anomer **2** was isolated by silica gel chromatography in 41% yield and fully characterized by NMR spectroscopy. Multi-dimensional experiments (GCOSY, GHMQC) enabled complete proton and carbon assignment of compound **2**. The α-anomer configuration was unambiguously confirmed by the coupling constant value of H-1 ($J_{1,2} = 3.8$ Hz). After the reductive acetylation of the azido group of **2** by catalytic hydrogenation in methanol/acetic anhydride, the *N*-acetylated compound **3** was fully deprotected by treatment with methylhydrazine in ethanol. The aminooxy αTF disaccharide **4** was finally recovered in 72% yield after precipitation from methanol/dichloromethane. A similar strategy was followed to prepare βGal **5** and βLac **6** derivatives in good yields [25].

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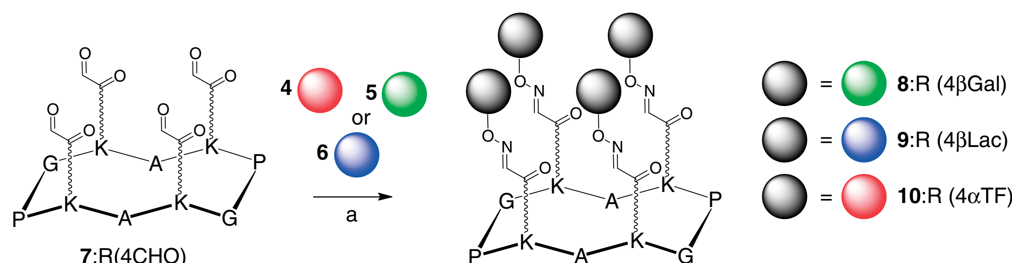
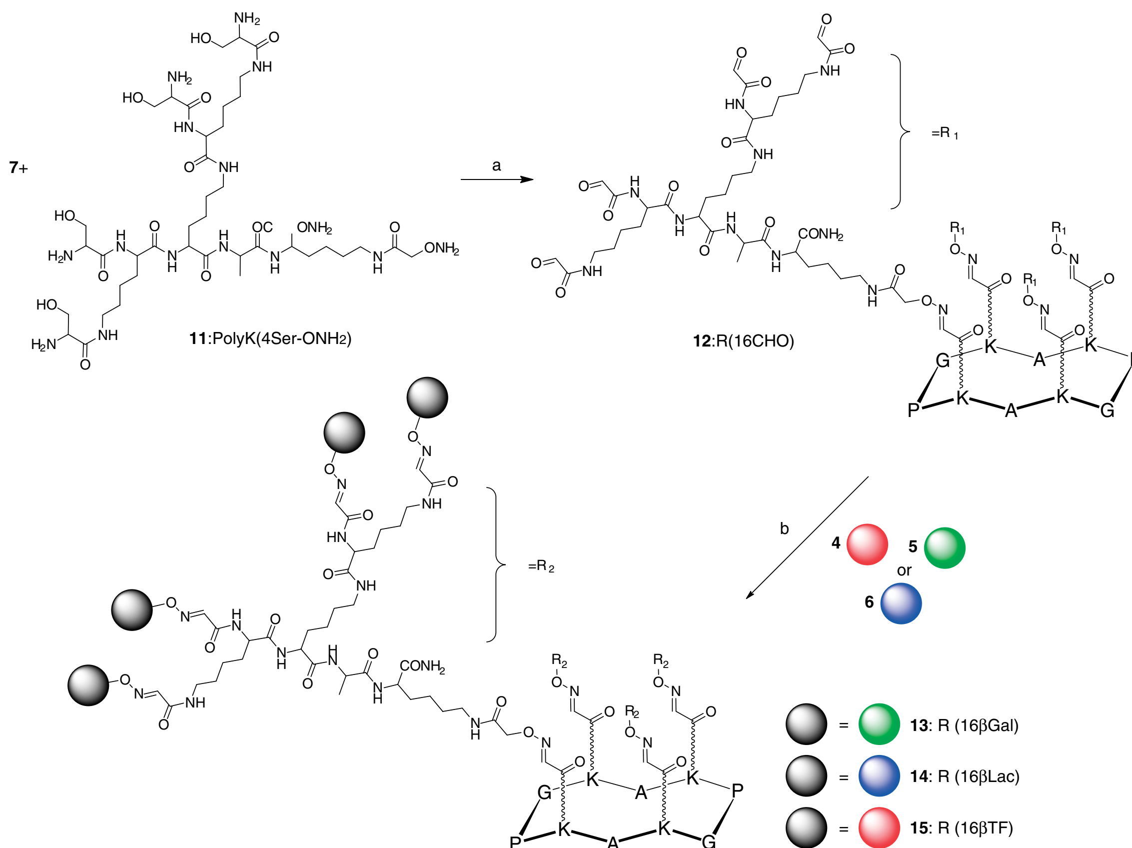


Scheme 2. Reagents and conditions: (a) AcOH 10% in H_2O, 37 °C.



Scheme 3. Reagents and conditions: (a) i: AcOH 10% in H_2O, 37 °C; ii: $NaIO_4$, H_2O, rt and (b) AcOH 10% in H_2O, 37 °C.

Molecular assembly to neoglycocyclopeptides

Having in hand each carbohydrate building block, the molecular assembly of tetravalent glycoclusters was achieved by a simple and efficient oxime ligation protocol. Typically, the R(4CHO) **7** was treated with a tenfold excess of glycosylhydroxylamine **4**–**6** under mild aqueous acidic conditions in aqueous acetic acid (Scheme 2) [26,59]. After one night at 37 °C, RP-HPLC analyses attested clean crude reaction mixtures and complete conversion of **7** into the corresponding tetravalent glycoclusters **8**–**10** (see Supporting Information). The excess of free carbohydrate derivative was finally removed by semi-preparative HPLC to afford **8**–**10**, as confirmed using electrospray ionisation mass spectrometry.

In order to increase both glycocluster valency and density, a polylysine dendrimeric peptide PolyK(4Ser-ONH_2) **11** [46] was incorporated to the tetravalent scaffold R(4CHO) **7** under experimental conditions described earlier (Scheme 3). Aldehydic anchoring sites were next generated using oxidative cleavage of serines by treatment with a large excess of sodium periodate in water. The resulting conjugate R(16CHO) **12** was isolated by semi-preparative HPLC, the expected presence of the 16 aldehyde functions was ascertained by ESI-MS. This compound **12** displaying 16 aldehyde functions was subjected to oxime coupling with glycosylhydroxylamines **4**–**6**. Product formation by the final oxime ligation between the clustered aldehyde scaffold **12** and carbohydrates **4**–**6** was studied by high-field NMR spectroscopy in D_2O. Although the relative structural asymmetry of the resulting high-molecular-weight hexadecavalent glycoclusters (from 7.5 to 10.7 kDa) strongly impacts the signals' resolution and multiplicity in ^1H NMR spectral data, COSY experiments have enabled the unambiguous assignment of characteristic signals in each molecular structure. In particular, the integration of both anomeric and oxime protons signals (e.g. δ 4.51, $16H_1$, 5.62 ppm, $16H_{1'}$ and δ 7.80 ppm, $20H_{ox}$, respectively, shown in Figure 1 for compound **15**) is in accord with presence of 16 carbohydrate units attached

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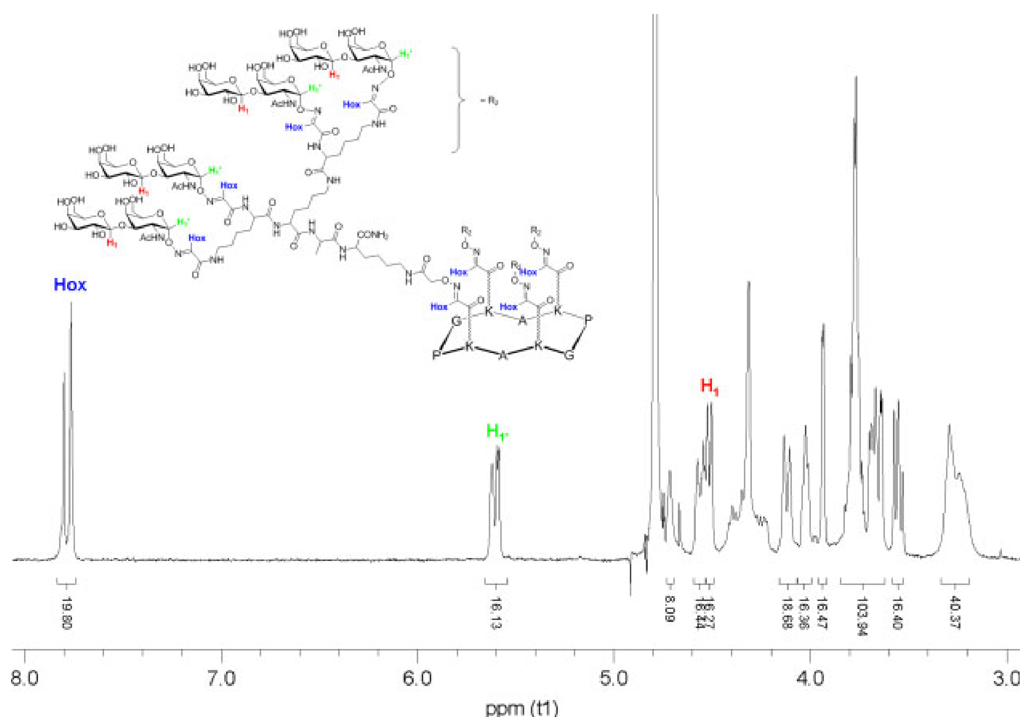

Figure 1. ^{1}H NMR spectrum of hexadecavalent glycoclusters R(16αTF) **15** and assignment of characteristic signals. The experiment was performed at 500 MHz (298 K, D_2O) without presaturation of the HOD signal. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

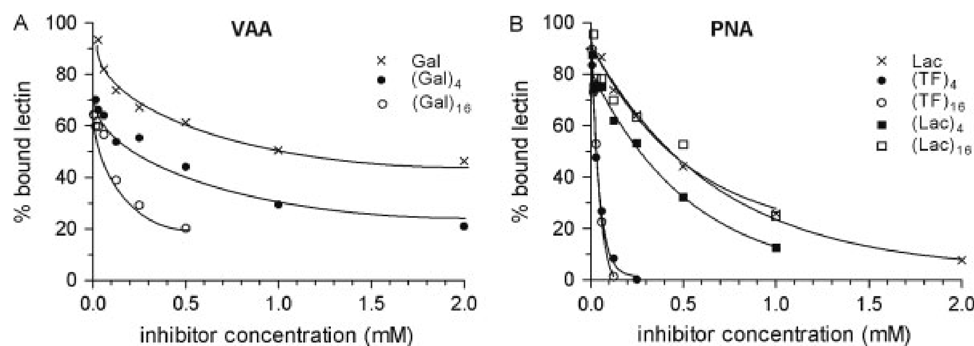

Figure 2. Inhibition of binding of VAA (A) and PNA (B) to surface-immobilized asialofetuin by free galactose (×) and this sugar presented by cyclic peptides as scaffold at a density of 4 (●) and 16 (○) headgroups per carrier (A) as well as by free lactose (×) and carbohydrates presented by cyclic peptides as scaffold at a density of 4 (■, ●) and 16 moieties (□, ○), using lactose (■, □) and the TF-antigen disaccharide (●, ○) as headgroup. Concentration is normalized to the sugar in all cases.

to the scaffold through oxime linkages. In addition, no major trace of side product originating from undesired transoximation or partial conjugation was observed in HPLC runs (see Supporting Information), reflecting the efficiency of the iterative oxime ligation process in glycocluster assembly. These synthetic procedures gave access to a panel of cyclic neoglycodecapeptides with constant backbone structure as well as variations in the structure of the sugar headgroup and its local density. The effects of these two parameter changes were tested in lectin inhibition assays, first using a surface-presented glycoprotein as ligand. As in the clinical situation, the lectin was always in solution.

Inhibition Assays (Glycoprotein)

Asialofetuin, a glycoprotein with three *N*-glycosylation sites and up to nine lectin-reactive glycan antennae [43,60], was adsorbed to the surface of microtiter plate wells. Lectin binding to this matrix was saturable and inhibitable by haptenic sugars. Systematic titrations with inhibitors resulted in the determination of the concentration that reduced the extent of binding to 50%, termed IC_{50}-value (Figure 2). The compilation of these numbers answers the questions on relative bioactivity of free to conjugated sugars and impact of valency as follows: derivatization of the mono- and disaccharides did not impair their reactivity to lectins and marked differences in inhibitory capacity were seen depending on the type of sugar headgroup and protein (Table 1). Grading of binding activity was maintained for the free ligands, when comparing their activities, e.g. for the two plant agglutinins [61–63]. Of note, scaffold attachment will not automatically raise lectin reactivity (Table 1). Selective enhancements upon valency increase were disclosed for VAA and the tandem-repeat-type galectin-4 (Table 1). With respect to the interprotein comparison between the human lectins, no inhibition was seen in the case of the homodimeric galectin-1 up to a concentration of 1 mM (not

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Table 1. IC_{50}-values of free and glycocluster-presented mono- and disaccharides for blocking binding of biotinylated lectins to surface-immobilized ASF (in mM)

Lectin inhibitor	VAA[a]	PNA	Gal-3	Gal-4
R(4βGal) **8**	0.3	1 mM (11%)	4 mM (21%)	4 mM (26%)
R(4βLac) **9**	0.75	0.25	0.25	0.25
R(4αTF) **10**	1 mM (36%)[b]	0.03	0.4	1.0
R(16βGal) **13**	0.02	1 mM (11%)	4 mM (24%)	4 mM (30%)
R(16βLac) **14**	0.2	0.5	0.25	0.03
R(16αTF) **15**	1.0	0.03	0.3	0.125
Gal	3.5	2.0	44	32 mM (39%)
Lac	2.0	0.4	0.8	3.0

[a] The concentrations of lectins were: VAA, 0.5 µg/ml; PNA, 3.5 µg/ml; Gal-3, 5 µg/ml and Gal-4, 3 µg/ml.
[b] Extent of inhibition at the given concentration.

shown). The presence of a linker peptide between the two subunits, distinguishing the tandem-repeat-type protein galectin-4 from the noncovalently associated subunits in galectin-1, appears to implement selectivity for these clustered ligands. If this is the case, then disruption of lectin bivalency should negatively effect the inhibitor activity. Indeed, when testing the separate lectin domains of galectin-4, a decrease of sensitivity was noted with an IC_{50}-value of about 0.1 mM for the *N*-domain or a mixture of *N*- and *C*-domains, using the hexadecavalent neoglycopeptides presenting lactose. Hydrodynamically, the diffusion constants of galectins-1 and -4 were not notably different despite the structural divergence established by the linker [64]. But the two structural types of bivalent lectin organization reacted differently to the clusters. This result signifies a remarkable sensor functionality of the glycoclusters for the topological displays of lectin sites.

Bivalency of the tandem-repeat-type protein is a key factor for high sensitivity to hexadecavalent clusters. The dimeric VAA shows a similar susceptibility, whereas ligand tetravalency already suffices for tetrameric PNA (Table 1). In contrast, the inhibition of binding of proteolytically truncated galectin-3 was rather similar to that of the full-length protein (not shown). Evidently, the cluster design affected lectin binding depending on the relative topology of the contact sites. However, the emerging potential of degree of glycan branching of the glycoprotein in the matrix to affect the extent of inhibition should be noted, a factor precluding general extrapolations [65]. Also, any medical relevance will need to be based on the efficiency of inhibitors to protect cells from lectin/toxin binding, and glycomic profiles are known to reflect dynamic changes in branching and end-group presentation [44,66,67]. Consequently, it is essential to proceed to respective assays *in vitro*. Toward this end, human cells are a suitable test system. We also took advantage of the availability of glycosylation mutants to probe into the relation between distinct defects of glycan tailoring and lectin binding in order to determine the inhibitory potency of the cyclic neoglycodecapeptides. The results of the cell assays will also enable to answer the question whether the solid-phase tests are useful for screening.

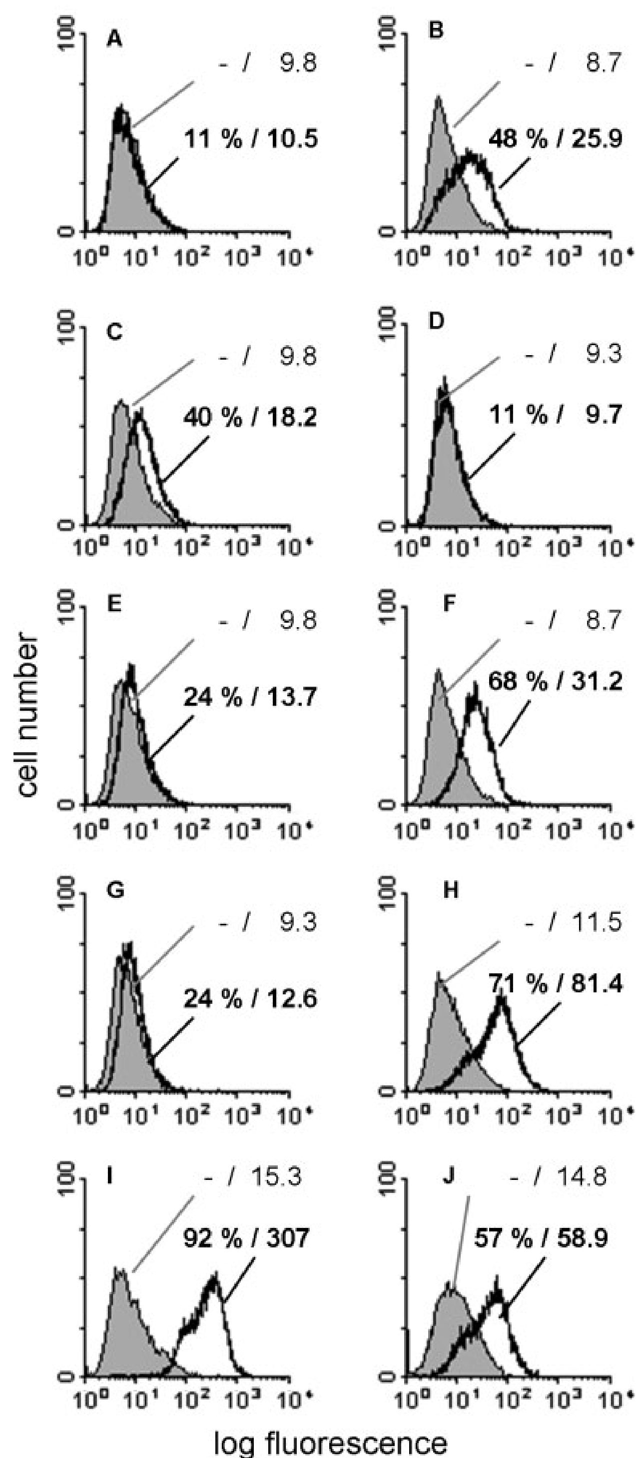

Figure 3. Semilogarithmic representation of fluorescent surface staining of CHO wild-type and mutant cells by biotinylated lectins. The control value representing lectin-independent staining by the fluorescent second-step reagent after processing without the incubation step with lectin is routinely included as shaded area. Quantitative data on percentage of positive cells (%) and mean fluorescence intensity are given for each curve in each panel. Staining profiles of wild-type (A) and the Lec2 mutant cells (B) by PNA (1 µg/ml), of wild-type (C) and the Lec4 mutant cells (D) by phytohemagglutinin (PHA-L; 1 µg/ml), of wild-type (E), Lec2 mutant (F) and Lec4 mutant cells (G) by VAA (1 µg/ml) as well as of wild-type (H), Lec2 mutant (I) and Lec4 mutant cells (J) by human galectin-3 (5 µg/ml).

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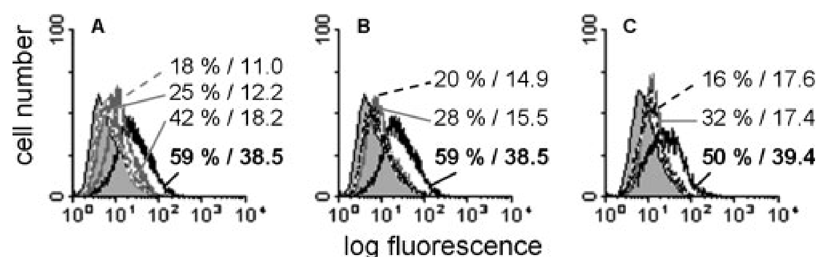

Figure 4. Effect of haptenic sugar (lactose) and glycoclusters on lectin binding to CHO Lec2 mutant cells (black line: positive control without inhibitor; shaded area: negative control without lectin but with fluorescent second-step reagent). Staining with biotinylated VAA (1 µg/ml) when coincubated with either 1, 5 or 10 mM free galactose (A) or with 0.5 mM galactose presented in glycoclusters with 4 (solid grey line) or 16 (dashed black line) sugar headgroups (B). Staining with biotinylated PNA (1 µg/ml) when coincubated with 0.05 mM TF-antigen disaccharide presented in glycoclusters with 4 (solid grey line) or 16 (dashed black line) sugar headgroups (C). All quantitative data are inserted into each panel in the order of listing, starting at the bottom with the positive control, then moving upward with increasing inhibitor concentration for the free sugar (A) or the type of glycocluster (B and C).

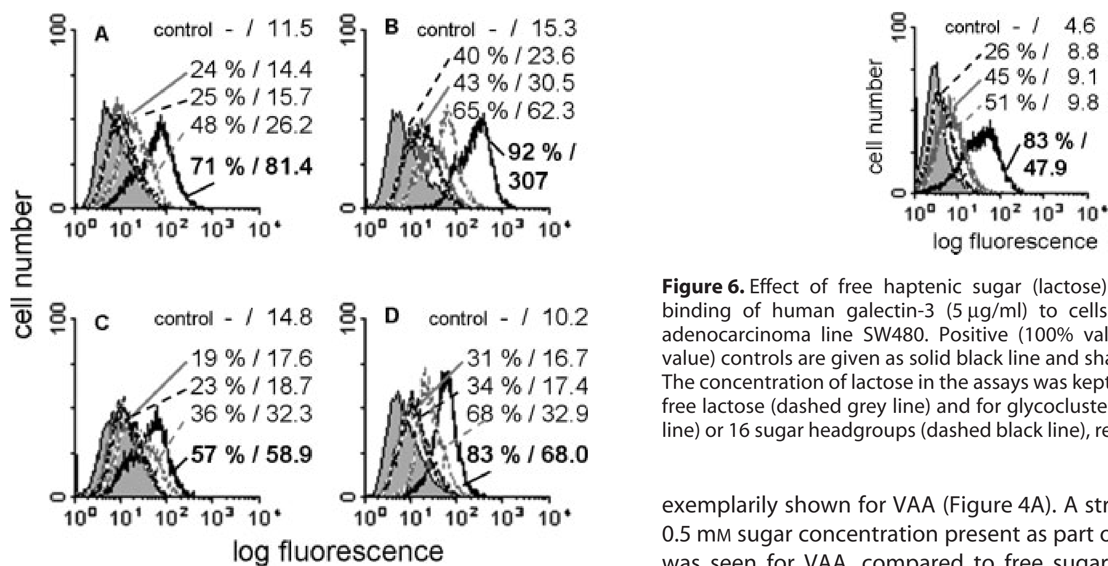

Figure 5. Effect of free haptenic sugar (lactose) and glycoclusters on binding of human full-length galectin-3 (5 µg/ml; please see also Figure 2 H–J for positive and negative controls) to CHO wild-type (A) as well as Lec2 (B) and Lec4 mutant cells (C). The concentration of free lactose and lactose presented in glycoclusters was kept constant at 0.5 mM, data on lactose inhibition presented using a dashed grey line, on glycoclusters bearing 4 sugar headgroups using a solid grey line and on glycoclusters bearing 16 sugar headgroups using a dashed black line. Staining profile of Lec2 mutant cells by proteolytically truncated human galectin-3 (10 µg/ml) and its inhibition are illustrated in panel D (please compare to panel B showing respective data for full-length galectin-3).

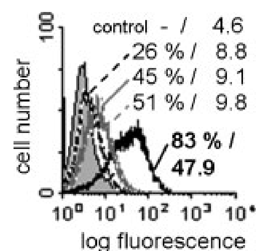

Figure 6. Effect of free haptenic sugar (lactose) and glycoclusters on binding of human galectin-3 (5 µg/ml) to cells of the human colon adenocarcinoma line SW480. Positive (100% value) and negative (0% value) controls are given as solid black line and shaded area, respectively. The concentration of lactose in the assays was kept constant at 0.5 mM for free lactose (dashed grey line) and for glycoclusters bearing 4 (solid grey line) or 16 sugar headgroups (dashed black line), respectively.

Inhibition Assays (Cells)

In this assay type, the labeled lectin was incubated with cells in the absence or presence of inhibitors. Lectin binding to the cell surface was quantitated by cytofluorometry measuring percentage of positive cells and signal intensity in each case. Experimental series were routinely done with aliquots of cell suspensions to ensure comparability. First, cells with marked lectin binding were identified. Using the CHO cell system with wild-type and two mutant lines, binding properties of PNA and VAA were strongly elevated by reducing the abundant α2,3-sialylation, VAA mostly targeting branched *N*-glycans (Figure 3A–G). Galectin-3, in contrast, can tolerate α2,3-sialylation and also targets *N*-glycans with β1,6-branching (Figure 3H–J), as shown previously by solid-phase assays using neoglycoproteins [68]. Binding was consistently dependent on lectin concentration (not shown) and inhibited by cognate sugar, exemplarily shown for VAA (Figure 4A). A strong cluster effect at 0.5 mM sugar concentration present as part of the cyclic peptides was seen for VAA, compared to free sugar (Figure 4A, B). Also in accord with the solid-phase data, the glycopeptides proved strongly active for the leguminous lectin PNA (Figure 4C).

Galectin-3 was tested in the three CHO cell lines at the same sugar concentration of 0.5 mM. Irrespective of the alteration in sialylation (Lec2) and *N*-glycan branching (Lec4) grading was rather similar (Figure 5A–C). The removal of the collagenase-sensitive stalk did not significantly alter the lectin's susceptibility to the presence of glycoclusters (Figure 5B, D). The reactivity pattern was also not altered when testing human SW480 colon adenocarcinoma cells with galectin-3 (Figure 6).

Similarly, the potency of glycoclusters to block galectin-4 binding was tested in two cell systems. Conspicuous cluster reactivity was revealed in this case. Even at 10-fold (Figure 7A) or 20-fold concentration difference (Figure 7B) the clusters readily surpassed the free sugar's inhibitory capacity. When comparing galectin-1, such a sensitivity was recorded only at identical sugar concentrations (Figure 7C). The cell assays thus not only extend the documentation of the efficiency of the cyclic neoglycodecapeptides to block lectin/toxin binding with protein-type selectivity but also afford the perspective of physiologic relevance using glycomic profiles *in vitro*.

Conclusions

The presented design of cyclic neoglycodecapeptides is shown to maintain bioactivity of the carbohydrate part to a plant toxin and

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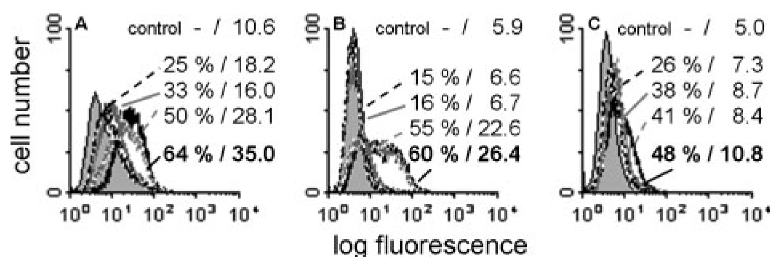

Figure 7. Effect of free haptenic sugar (lactose) and glycoclusters on binding of human chimera-type galectin-4 to cells of the CHO Lec2 mutant line (A; 20 μg/ml) and of the human pancreatic carcinoma line Capan-1, reconstituted for expression of the tumor suppressor $p16^{INK4a}$ (B; 30 μg/ml) and on binding of human homodimeric proto-type galectin-1 (C; 30 μg/ml) to the pancreatic carcinoma cells. Positive (100% value) and negative (0% value) controls are given as solid black line and shaded area, respectively. The concentration of lactose was 100 μM for free sugar (dashed grey line) and 1 μM for glycoclusters bearing 4 (solid grey line) and 16 sugar headgroups (dashed black line) in panel A, 2 mM for free and 0.1 mM for glycocluster-presented lactose at a density of 4 (solid grey line) and 16 headgroups (dashed black line) in panel B as well as 2 mM for free and glycocluster-presented lactose in panel C.

medically relevant human lectins. Our experiments underscore that the type of sugar headgroup can thus be tailored to modulate glycocluster efficiency and selectivity. As a further favorable means toward selectivity, the degree of local ligand density was disclosed. Human galectin-4 and the plant toxin, here already with galactose as ligand, were especially responsive to this factor. Methodologically, the strategic combination of solid-phase and cell assays underscored both the suitability of using a glycoprotein for screening and glycocluster efficiency to protect cells from toxin/lectin binding. Of note, fluorescent forms of these glycoclusters were markedly less suited than respective neoglycoproteins, e.g. exposing the disaccharide of the T-antigen [36,69–71], to visualize cellular binding sites. The recently described conformational restraints of parts of a cyclic neoglycopeptide [72] can contribute to explain this observation. Although matching the spacings between carbohydrate headgroups and lectin sites is ideal for the distinct polyvalent plant agglutinin tested in that report [72], such rather rigid preformation is less suitable for screening binding capacity. Used as sensor for topological lectin-site display, these glycoclusters, along with other tetravalent scaffolds such as calix [4]arenes, pentaerythritol tetrakis (*m*/*p*-iodobenzyl)ether or a low-generation poly(amidoamine)dendrimer [18,73–74], can be useful as molecular rulers.

Toward the aim of developing lectin blockers, valency, topology of presentation and headgroup parameters are the parameters to be merged. Taking into account fine-specificity differences among lectins not detectedable in systematic binding studies with natural glycoproteins or synthetic oligosaccharides, e.g. for galectins-3 and -4 [38,75–77], or with galactoside libraries differing in the structure of the aglyconic substitution, e.g. between the plant toxin and galectins [78,79], will be helpful to optimize inhibitory potency and interprotein selectivity. Based on the presented results this approach using cyclic neoglycopeptides as scaffold appears to hold promise also for the design of inhibitors against other clinically relevant lectins, e.g. bacterial/viral agglutinins, siglecs or lectins involved in viral entry, inflammation or metastasis [80–82].

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Supporting information

Supporting information may be found in the online version of this article.

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