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Synthesis of bivalent lactosides and their activity as sensors for differences between lectins in inter- and intrafamily comparisons

Sabine André [a], Dilip V. Jarikote [b], Dandan Yan [b,c], Lisa Vincenz [b], Guan-Nan Wang [b], Herbert Kaltner [a], Paul V. Murphy [b,*], Hans-Joachim Gabius [a]

[a] Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich, Veterinärstr. 13, 80539 Munich, Germany
[b] School of Chemistry, National University of Ireland, Galway, University Road, Galway, Ireland
[c] School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland

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ABSTRACT

The synthesis of nine bivalent lactosides (based on ditriazoles, diamides, a glycocyclophane and an acyclic analogue of the glycocyclophane) and one monovalent lactosyl triazole facilitated the assessment of the sensitivity of plant/animal lectins to this type of ligand display. The inhibitory potency of the compounds was determined in two assays of increasing biorelevance. These were solid-phase and cell binding set-ups. Hereby, the ability of the compounds to inhibit the binding of two plant agglutinins and the entire set of adhesion/growth-regulatory galectins from one organism (chicken) to a glycoprotein or to cell surfaces was systematically evaluated. Differential sensitivities were detected between plant and animal lectins and also between distinct galectin forms within the chicken series. Two of the bivalent probes can be considered as sensors for interlectin differences. Most pronounced were the selectivities of *N*-glycosyl 1,2,3-triazole derivatives for the chimera-type galectin and its proteolytically truncated version.

A broad range of physiological functions is emerging for the glycan chains of cellular glycoconjugates via carbohydrate-protein (lectin) recognition.[1] Distinct carbohydrate structures serve as target sites for receptors (exogenous agglutinins from plants or bacteria, tissue lectins) and their interaction begins the conversion of sugar-encoded information into cellular responses. If consequences of this interplay are harmful for the organism, for example, toxicity exerted by plant lectins or immune dysregulation/tumor invasion by endogenous effectors,[2] then blocking of the docking onto cells by custom-made inhibitors becomes an attractive goal for medicinal chemistry. In principle, two general structural parameters can be varied with the aim of accomplishing high-level specificity: (i) the carbohydrate headgroup including any modification (additions such as sulfation or synthetic modifications at distinct sites to enhance complementary binding to target receptors) and (ii) the valency of presentation. Of course, interpretation of experimental results benefits from a clear design approach, avoiding too many parameter changes. Also, such studies gain impact if—besides revealing affinity of a carbohydrate derivative in a binding assay for a single lectin—further comparative binding analyses with a range of relevant targets are performed to assess selectivity. Ideally, this should be accomplished with cell assays where natural ligands are in their physiological presentation.

To meet these criteria we herein focused on characterizing the impact of different modes of bivalent ligand presentation of a common sugar headgroup (lactose). Although the linker length (distance between two lactose residues) cannot always bridge the distance between contact sites of bi- to oligovalent lectins in an intramolecular sense, such a topological (bivalent) ligand display has nonetheless previously indicated its potential for targeting galactoside-specific lectins to varying extents.[3] Three ditriazoles, four diamides, a glycocyclophane and an acylic analogue of the latter were synthesized as the test panel (Fig. 1, **1**–**4**, **6**–**10**). Also included was a control monomer (**5**), which contains structural features of the aglycone found in **3** and **4**, where lactose is connected via a triazole to a benzylated glucuronic acid derivative. A galactoside-binding plant toxin (*Viscum album* L., VAA; with low (Tyr)- and high (Trp)-affinity sites in the dimer separated by 87 Å or 15 Å, respectively, in the β-trefoil fold[4]), a leguminous β-sandwich agglutinin (*Erythrina crystagalli* agglutinin, ECA) and adhesion/growth-regulatory galectins were purified by affinity chromatography[3a] to give a range of suitable lectins for the biochemical/cell biological part of the study. Of note, the family of galectins encompasses three topological modes for binding-site presentation (Fig. 2). Since it is appealing to comprehensively determine the structure–activity profiles of these three types for proteins from one organism, we have run the assays on the five chicken galectins (CGs).[5] Our panel thus comprises the three homodimeric proto-type galectins (CG-1A, CG-1B, CG-2), the chimera-type CG-3 along with its proteolytically truncated variant

* Corresponding author. Tel.: +353 91 492465; fax: +353 91 495576.
E-mail address: paul.v.murphy@nuigalway.ie (P.V. Murphy).

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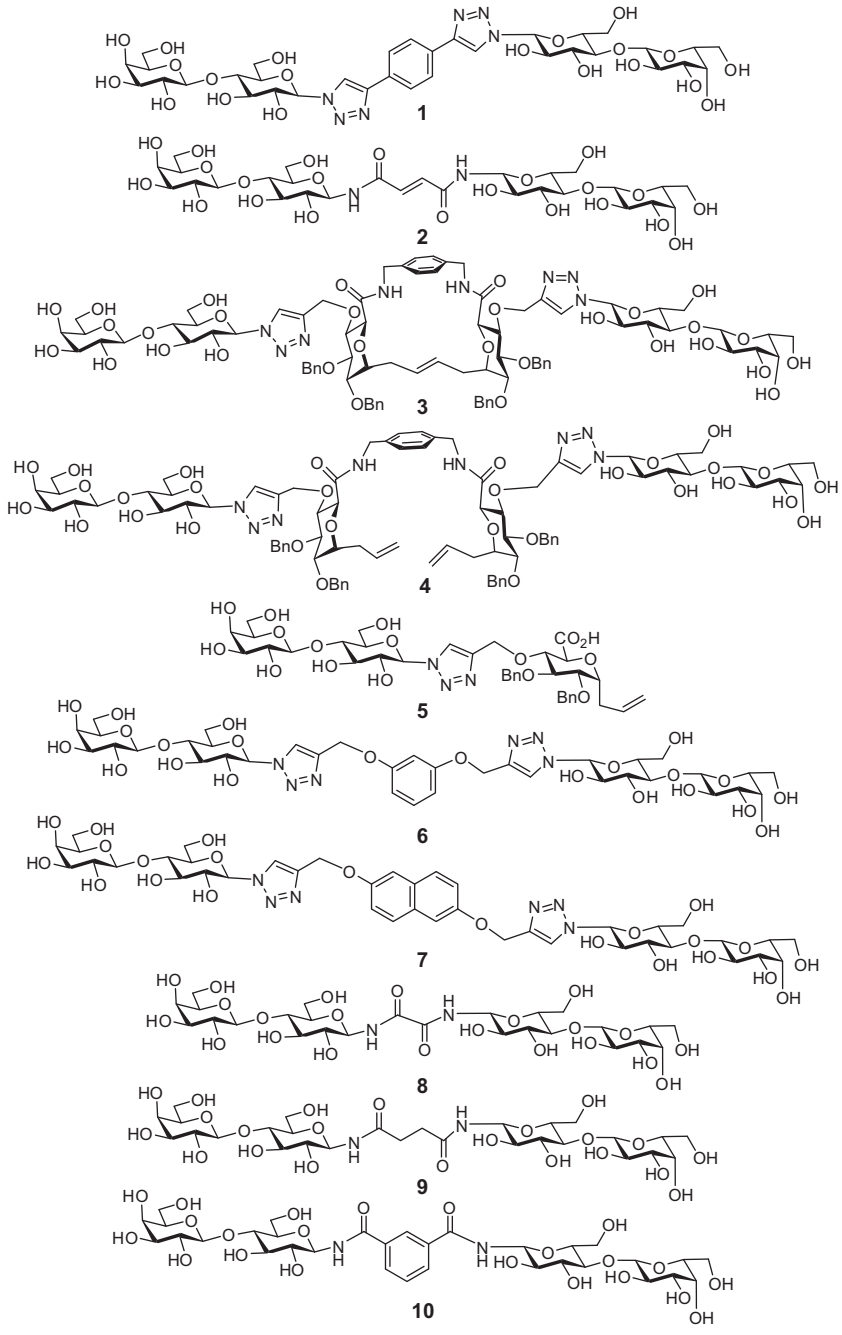


Figure 1. Compounds **1**–**10**.

(trCG-3) and the two versions of tandem-repeat-type CG-8 with natural variations in linker length (either 9 or 28 amino acids) which arises from alternative splicing, together with a separate domain (the N-terminal domain, termed Gal-8N) (Fig. 2). Orthologs of proteins from each group are present in mammals, and the potential for functional competition, documented between proto- and chimera-type galectins,[6] makes this broad screening approach mandatory. Regarding potential for aggregation of the proteins in solution, CG-8 behaves as monomer in gel filtration and ultracentrifugation, as does galectin-3, which can oligomerize in the presence of multivalent ligands.[3c,5d] The affinity of **1**–**10** was assessed in inhibitory assays in two steps: firstly a measure of their potency in interfering with lectin binding to a glycoprotein matrix (asialofetuin (ASF), a pan-galectin ligand) was evaluated; secondly their ability to inhibit lectin binding to cultured cells was evaluated. The latter experiment provides an in vitro setting with increased relevance for the clinical situation.

The synthesis of ditriazoles began from the lactosyl azide **11** (Scheme 1), which was prepared as described previously[3e] and reacted with a variety of dialkynes (**12**–**14**) using copper-catalysed azide alkyne cycloaddition reactions[7,8] to give protected intermediates. Subsequent deacetylation using methoxide in methanol gave compounds **1**, **6** and **7**.

Next, the glycocyclophane **3** was prepared, also from the azide **11** (Scheme 2). Reaction of **11** with glucuronic acid derivative **15** by copper-catalysed azide alkyne cycloaddition reaction gave triazole **16**. The coupling of **16** with *p*-xylylenediamine, subsequent ring closure metathesis and removal of the acetates gave the final

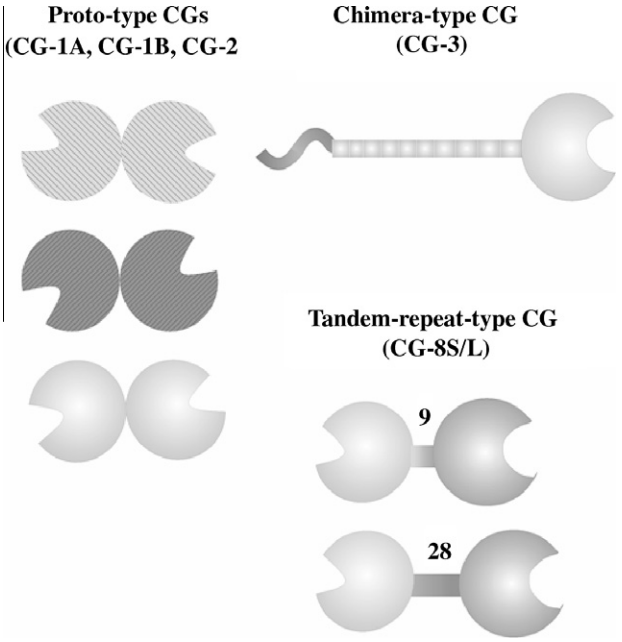


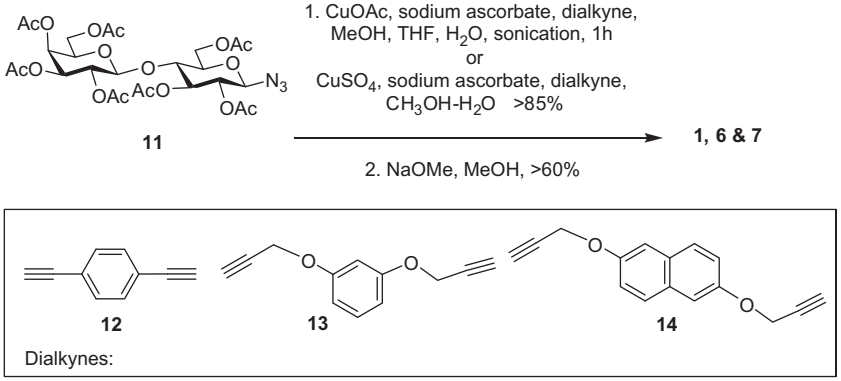
Figure 2. Representation of the three types of structural organization of chicken galectins based on crystallographic data,[18b,c] experimental evidence in solution and homology considerations.[5c–e] Proteolytic degradation of the collagen-like stalk of CG-3 with its 10 Gly/Pro-rich sequence repeats consisting of either five (one repeat), seven (five repeats) or eight (four repeats) amino acids turns the full-length version (CG-3) into trCG-3. The numbers of amino acids constituting the linker is given for CG-8S/L.

product **3**. The comparatively flexible analogue **4** was also prepared by coupling and deacetylation, avoiding the macrocyclisation step. Deacetylation of **16** gave the monomer **5**.[9]

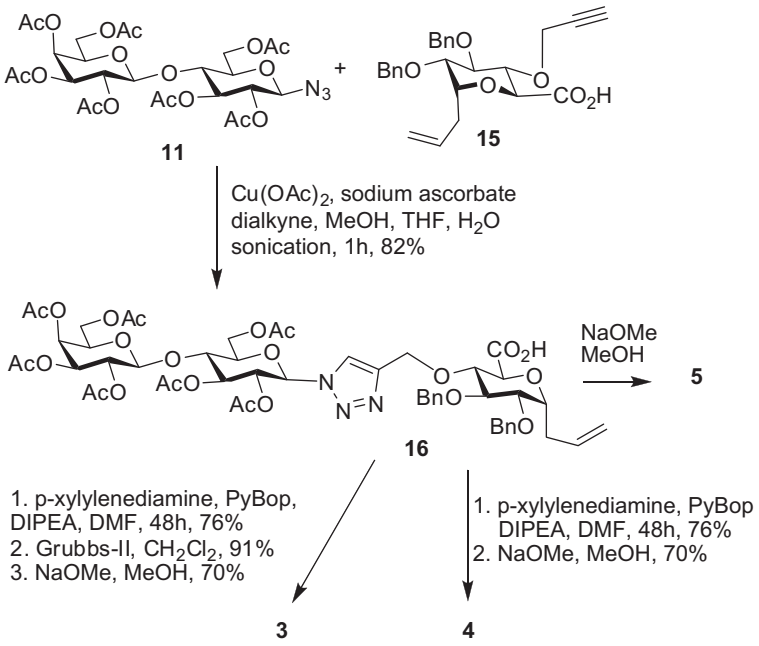
Finally, a series of dilactosyl diamides was synthesized (Scheme 3). Coupling of the lactosyl amine **17**, prepared by reduction of azide **11** as previously described,[3e,7c,10] with a variety of diacyl chlorides **18**–**21** and subsequent deacetylation gave **2** as well as **8**–**10**, completing the test panel (Fig. 1). In addition to compound characterization (please see Supplementary data) modeling for the bivalent ditriazoles and diamides, as described previously,[3e,7c] yielded inter-lactose (Glc C-1 to Glc C-1) distances ranging from 4.1 Å (**8**) to 19.6 Å (**7**).

These compounds were first tested in the solid-phase assay setup, which was performed as described previously.[11] In detail, the glycoprotein (ASF) was adsorbed to the surface of microtiter plate wells, and its three *N*-glycans were ligands for carbohydrate-dependent binding of the two labeled plant agglutinins and the galectins. The signal (optical density, OD) intensity, observed as a result of binding of the labeled lectin to the glycoprotein, depended on amount of glycoprotein coated and was saturable. The concentration of the lectins to be used in the experiments was hence optimized in each case to yield OD-readings in the linear range during the titrations. All compounds proved to be able to inhibit lectin binding to the glycans presented on the matrix to some degree. As a relative measure of the inhibitory potency of each compound, the concentration, which reduced the signal for bound lectin by 50% (the IC_{50}-value), was obtained from titration curves, as shown in Figure 3. Normalization of concentrations to lactose enabled to directly identify increases in inhibitory potency of the dilactosides relative to free lactose, which were obviously at work for CG-3 and compounds **1** and **4** (Fig. 3). Preliminary experimental runs for the ditriazoles (**1**, **6**, **7**) and diamides (**2**, **8**–**10**) revealed rather gradual changes within a rather narrow range, so we decided to focus the titrations on the representative compounds **1** and **2**, with inter-lactose distances of around 12.5 Å and 8.4 Å, respectively, and compare these with the glycocyclophane **3** and the more flexible analogue **4** where the inter-lactose distances would be greater. In several cases, the presence of the triazole was associated with an enhanced level of inhibition. For human orthologs, galectin-1 was rather insensitive to the various structural analogues, while susceptibility of galectin-3 to a triazole in the anomeric position has already emerged.[12] In our series, this factor appeared to be most pronounced for CG-3 (Table 1). In this special case, the monomeric compound **5** was between three and fourfold more potent than lactose. Although the triazole and/or glucuronic acid residue contributes to the binding, bivalency is clearly required to observe highest inhibitory activity against CG-3, even though the triazole and possibly also the glucuronic acid residue are contributing to the binding to CG-3. A tendency for increased binding was also seen when comparing CG-1A versus CG-1B/CG-2. The comparison between CG-3 and its truncated version (trCG-3) was of particular interest. Binding of trCG-3 to ASF was considerably less susceptible to inhibition with the tested dilactosides. This is because trCG-3 is less prone to form oligomers as it is a product of the proteolytic processing of CG-3, which leads to removal of the collagenase-sensitive stalk.

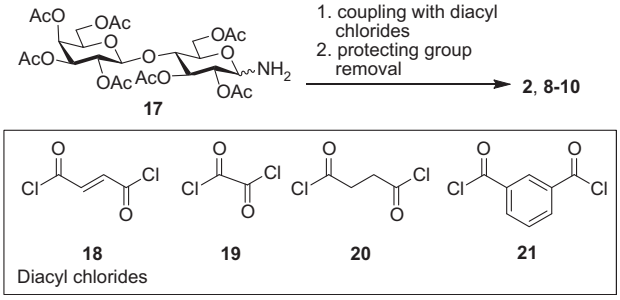
The relative flexibility, too, matters for compounds **3** and **4** and influences their binding affinities. The more rigid **3** was more potent for the plant agglutinins than its acyclic analogue **4**, albeit with only a slight increase relative to free lactose. It was also more potent for the tandem-repeat-type CG-8 and its N-terminal domain (Table 1). In contrast, CG-3 binding is clearly more sensitive for the more flexible **4**, whereas only minor differences between **3** and **4** occur among the proto-type proteins (Table 1). Thus, the different ratio of binding affinity of the glycocyclophane **3** and its more flexible analogue **4** appears to disclose disparities in lectin-site properties and/or reactivity to structural differences between



Scheme 1. Synthesis of **1**, **6** and **7**.



Scheme 2. Synthesis of **3** and **4**



Scheme 3. Synthesis of **2** and **8**–**10**.

3 and **4** for the tandem-repeat-type (bivalent) CG-8 when compared to the homodimeric proteins. In other words, this pair of synthetic compounds is a sensor to distinguish between the galectin subgroups depicted in Figure 2. The monomeric N-terminal domain of CG-8 (CG-8N) is rather equally well blocked by dilactosides, irrespective of the nature of the scaffold. In line with this result, proteolytic processing of CG-3, which abolishes oligomer formation via the collagen-like stalk, impairs this lectin's marked sensitivity to dilactosides (cf. CG-3 and trCG-3, Table 1). The acyclic compound **4** had highest relative potency of all the ligands for CG-3. Lectin binding to the surface-presented glycoprotein in this case was effectively impaired, with a reasonably high level of selectivity.

In order to establish whether the solid-phase assay has predictive value for a compounds' ability to inhibit the binding of a lectin to cells we next performed such bioassays. On cell surfaces, a natural panel of high-affinity ligands for lectins is presented in the physiologically relevant mode. Experimentally, the labeled lectin, in the absence or presence of inhibitors, was incubated with aliquots of cell suspensions from the same passage, in order to avoid glycophenotype changes upon prolonged periods in culture, and the extent of binding was quantitated by cytofluorometry, as reported previously when testing aglyconic extensions of lactose and dilactosides with human galectins.[3e,12]

The binding data obtained by cytofluorometry is documented as the percentage of positive cells and the mean fluorescence intensity for each experiment shown in Figure 4; the two determined variables will range between two controls, the first obtained in

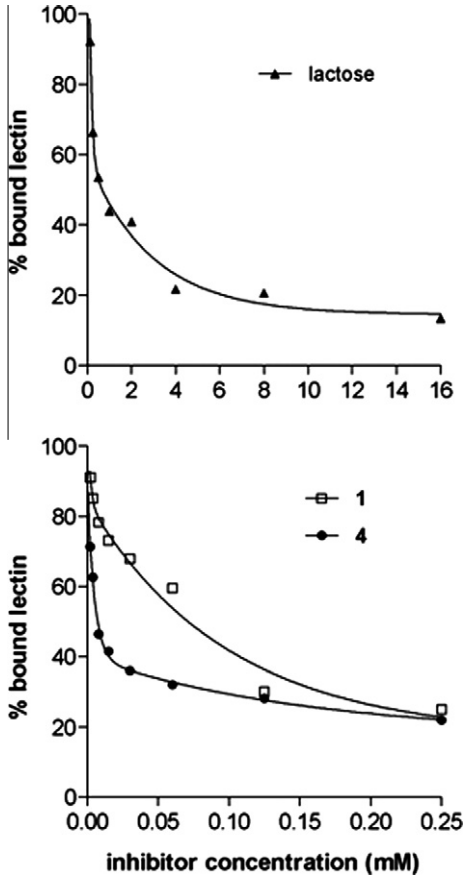


Figure 3. Titration curves for extent of binding of biotinylated CG-3 (1 μg/ml) to surface-immobilized glycoprotein (ASF) in the presence of increasing amounts of lactose (top) and compounds **1** and **4** (bottom), respectively. For compilation of IC_{50}-values, please see Table 1.

Table 1
IC_{50}-values of four bivalent lactosides and free lactose (Lac) for blocking binding of biotinylated lectins to surface-immobilized ASF (in mM)[a]

Lectin inhibitor	VAA (1.5 μg/ml)	ECA (0.2 μg/ml)	CG-1A (8 μg/ml)	CG-1B (3 μg/ml)	CG-2 (4 μg/ml)	CG-3 (1 μg/ml)	trCG-3 (1.5 μg/ml)	CG-8S (0.75 μg/ml)	CG-8L (0.75 μg/ml)	CG-8N (15 μg/ml)
1	0.13 (4.6)	0.6 (1.8)	0.22 (2.3)	1.5 (2.7)	1.2 (5.0)	0.07 (15.7)	3.1 (1.5)	0.7 (2.6)	1.8 (2.8)	0.5 (3.2)
2	0.55 (1.1)	1.2 (0.9)	0.36 (1.4)	1.3 (3.1)	1.4 (4.3)	0.6 (1.8)	4.2 (1.1)	2.5 (0.7)	4.5 (1.1)	0.8 (2.0)
3	0.24 (2.5)	0.8 (1.4)	1.6 (0.3)	0.6 (6.7)	0.7 (8.6)	0.08 (13.8)	5.3 (0.8)	0.4 (4.5)	0.6 (8.3)	0.3 (5.3)
4	1.3 (0.5)	1.3 (0.8)	1.4 (0.5)	0.46 (8.7)	0.65 (9.2)	0.014 (78.6)	5.0 (0.9)	3 (0.6)	2.4 (2.1)	0.4 (4.0)
Lactose	0.6 (1)	1.1 (1)	0.5 (1)	4 (1)	6 (1)	1.1 (1)	4.5 (1)	1.8 (1)	5 (1)	1.6 (1)

[a] For structures of **1**–**4**, see Figure 1; assays at a constant amount of 0.5 μg ASF used for coating of microtiter plate wells were routinely done in triplicates for up to five independent series with standard deviations not exceeding 12.4%. The lectin concentration is given in each case. Numbers in brackets denote the inhibitory potency relative to free lactose. Concentration values are normalized to lactose in all cases.

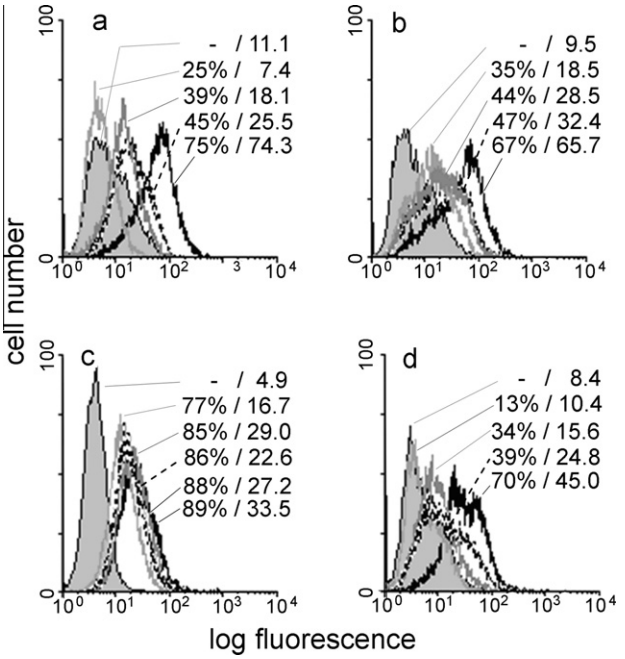


Figure 4. Cell surface staining (percentage of positive cells/mean fluorescence intensity) by labeled lectins and the impact of presence of inhibitors. Binding curves for CG-3 (a) and trCG-3 (b) at 5 μg/ml in the absence of inhibitor (100%-value) and (listed from bottom to top) 1 mM lactose, 1 mM **2** and 0.25 mM **1** (a, gray area: 0%-value, background control in the absence of lectin) or lactose, **2** and **1** at 2 mM using CHO cells (b). Low level of sensitivity was recorded for ECA (2 μg/ml) and human SW480 colon adenocarcinoma cells for **2**, **3**, lactose and **1** at 0.2 mM (c), whereas marked differences were present for interfering of binding of CG-8S (5 μg/ml) to CHO cells by **2**, lactose and **1** tested at 1 mM (d). Aliquots of the same batch were analyzed in triplicates in at least three independent series with standard deviations not exceeding 11.3% after normalization of the data.

the absence of lectin (0%-value; gray area in each panel of Fig. 4) and the second obtained in the presence of the lectin without addition of inhibitor (100%-value; black line in each panel of Fig. 4). Blocking lectin binding by an inhibitor will reduce the 100%-value according to its potency. The results of these assays, when carried out with the compounds, were consistently in agreement with the results obtained from the solid-phase binding assays; this is exemplarily documented for the two forms of CG-3 (Fig. 4a, b), the leguminous lectin and its low level of sensitivity (Fig. 4c) as well as for CG-8 (Fig. 4d). At the same time, the experiments extend the data basis for differences in binding affinity toward the tested lectins to cell surfaces as the assay platform and hereby afford a determination of the potential of the compounds to reduce lectin binding to cell surfaces.

Overall, the combined data shown in this communication leads to the conclusion that bivalency contributes to inhibitory activity. Kinetic aspects of binding affecting on/off rates to increase affinity, as reported for mucin loading,[13] may be a reasonable explanation also in this context, whereas simultaneous reactivity (intramolecular binding) with extended sites, as discussed for selectins,[14] appears less likely, despite binding of histo-blood group tetrasaccharides, digalactosides or poly-*N*-acetyllactosamine repeats presenting two galactose units to the extended contact site of chicken and human galectins.[15] In general, density of ligand presentation appears to be a key factor for galectins when targeting distinct counter-receptors from the glycome complexity, at the level of *N*-glycan branching and presentation in microdomains, also seen between CG-1A and CG-1B when testing polyvalent glycoproteins.[15a,16] The example of 'non-spanning' bivalent ligands and the cholera toxin B—pentamer teaches the lesson that transient, non-specific binding can also have a significant bearing on affinity.[17] Dilactosides with a triazole at the anomeric carbon appeared more favorable than those with an amide in most cases. Screening in two assay types with increasing biorelevance were in agreement and revealed notable differences in binding within the set of galectins, separating the two natural forms of the chimera-type galectin as well as the tandem-repeat-type protein and the non-covalently associated homodimers.

In summary, the compounds described herein block glycoprotein and cell binding to various lectins, with differences in potency and selectivity in some cases. This provides a basis for further research with a view to accomplish selectivity enhancement. Exploration of the impact of (i) substitutions in the sugar headgroup, with guidelines provided by chemical mapping, glycan testing, crystallographic analysis and molecular modeling[15,16,18] and (ii) increasing valency would both be of interest in this regard.

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Supplementary data

Supplementary data (experimental details for synthesis of the compounds and analytical data) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.010.

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