Lectin-Mediated Drug Targeting: Selection of Valency, Sugar Type (Gal/Lac), and Spacer Length for Cluster Glycosides as Parameters to Distinguish Ligand Binding to C-Type Asialoglycoprotein Receptors and Galectins

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Purpose. Common oligosaccharides of cellular glycoconjugates are ligands for more than one type of endogenous lectin. Overlapping specificities to β-galactosides of C-type lectins and galectins can reduce target selectivity of carbohydrate-ligand-dependent drug targeting. The purpose of this study is to explore distinct features of ligand presentation and structure for design of cluster glycosides to distinguish between asialoglycoprotein-specific (C-type) lectins and galectins.

Methods. Extent of binding of labeled sugar receptors to two types of matrix-immobilized (neo)glycoproteins and to cells was evaluated in the absence and presence of competitive inhibitors. This panel comprised synthetic mono-, bi-, and trivalent glycosides with two spacer lengths and galactose or lactose as ligand part.

Results. In contrast to C-type lectins of hepatocytes and macrophages, bi- and trivalent glycosides do not yield a notable glycoside cluster effect for galectins-1 and -3. Also, these Ca^{2+}-independent galactoside-binding proteins prefer to home in on lactose-bearing glycosides relative to galactose as ligand, while spacer length requirements were rather similar.

Conclusions. Trivalent cluster glycosides with Gal/GalNAc as ligand markedly distinguish between C-type lectins and galectins. Undesired side reactivities to galectins for C-type lectin drug delivery will thus be minimal.

KEY WORDS: asialoglycoprotein receptor; cluster glycoside; drug targeting; galectin; lectin; neoglycoprotein.

INTRODUCTION

The accurate intra- and intercellular routing of macromolecules sets an example for the development of carrier systems for targeted drug delivery. By copying and exploiting the natural postal-code-like molecular determinants cell-type-specific uptake of drugs can then be achieved. Besides peptide motifs the discovery of galactose/N-acetylgalactosamine-dependent hepatic clearance of serum glycoproteins has spurred to explore the applicability of oligosaccharides as targeting device (1–4). Fittingly, the calculation of the enormous information-storing capacity of glycan chains and the documentation of glycan functionality far beyond serum glycoprotein homeostasis underpin the concept of a sugar code (5,6). As attested by the classic example of the hepatic asialoglycoprotein receptor, suitable sugar determinants dock into binding sites of cellular lectins to complete a protein-carbohydrate recognition system (2,7–9). Having detected the presence of these activities, characterized their biochemical properties and assigned them to currently five distinct families, several important facts emerged from this work. Firstly, a defined carbohydrate epitope can well be accomodated in the binding site of more than one tissue lectin. Secondly, the modes of topological glycan presentation (i.e., branching, multivalency by bi- to pentaantennary N-glycans and patch establishment on mucin-like glycoproteins) have a strong influence on their affinity for lectins. A prominent example for binding promiscuity is given by the readily accessible β-galactoside termini of N-glycans. The intention of targeting to one lectin class can thus be compromised by undesired interference with cell functions of a second lectin family. In such a case, the dual functionality of the sugar epitope might be reduced by an adequate design of the glycan. To this end the presented study has been performed.

In addition to the hepatic asialoglycoprotein receptor several other C-type lectins, especially a macrophage receptor, bind β-galactosides (9). Together with C-type lectins this property is shared by the galectins. To limit the intra- and interfamily overlap of binding activity and thus the degree of unavoidable mistargeting to the highest possible extent it is essential to run a comparative analysis of ligand properties of substances designed for a distinct target. Since the hepatic lectin reacts with certain mono-, bi-, and trivalent galactose-exposing oligosaccharides (a numerical increase in valency) with a geometrical increase in affinity (10,11), the hepatic targeting system warrants to be evaluated for activity concerning other C-type lectins and galectins. Albeit less markedly, the increase of sugar density by branching also leads to a glycoside cluster effect for the related macrophage C-type lectin (12,13).

The other problem to be addressed concerns the mentioned family of galectins. They are involved in cell-cell/cell-matrix interactions and growth regulation (9,14). Although they are not endocytic receptors, blocking their binding sites in therapy can nonetheless elicit impairments in the mentioned respects. Concerning their reactivity to multivalent glycans it is known that a prototype galectin together with a galactoside-binding plant agglutinin can accept each of the three arms of a triantennary N-glycan without spatial restrictions (15). This aspect has so far not been adequately investigated in the evaluation of cluster glycosides. Thus, it is important to determine the binding properties of glycosides designed for C-type lectins in a standard assay system for representative galectins. To infer relative ligand capacity the synthetic mono-, bi-, and trivalent galactosides and lactosides were tested whether and to what extent they interfered with galectin binding to a surface-immobilized neoglycoprotein or asialofetuin and to cell surfaces. By including galectins-1 and -3 the typical organization forms to display one binding site per galectin molecule (galectin-3) or two carbohydrate recognition domains at opposing sides of a homodimeric molecule (galectin-1) can be examined. For further

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comparison a plant lectin with the same monosaccharide specificity and a lactoside-binding immunoglobulin G fraction from human serum were included. As aim of the study, the expectable ranges of reactivity of the synthetic glycosides for C-type lectins and galectins will be defined.

MATERIALS AND METHODS

Glycosides and Sugar Receptors

The active p-nitrophenyl esters of 3-(2,3,4,6-tetra-O-acetyl-1-thio-β-*D*-galactopyranosyl)-propionate or (2-(2-(2,3,4,6-tetra-O-acetyl-1-thio-β-*D*-galactopyranosyl)ethoxy)ethoxy)-acetic acid and its lactose derivative were used to prepare the bi- and trivalent glycosides with two spacer lengths by conjugation to the α- and ε-amino groups of *L*-lysine and *L*-lysyl-*L*-lysine, as described previously (16). The structures of the resulting panel of test substances is presented in Fig. 1, the glycosides harboring the shortened spacer length designated by addition of an S to the name of substance. Galectin-1 from bovine heart, recombinant murine galectin-3 from expression-vector (prCBP35s)-carrying *E. coli* JA221 cells, kindly provided by Dr. J. L. Wang (East Lansing, MI, USA), the galactoside-binding agglutinin from *Viscum album* L. (VAA) and the lactoside-specific immunoglobulin G fraction from human serum were purified with affinity chromatography on lactosylated Sepharose 4B obtained after divinyl sulfone activation as crucial step and then labeled by biotinylation under activity-preserving conditions, as outlined in detail previously (17,18).

Solid-Phase Assay

To determine the inhibitory potential of the monovalent sugar derivatives and the cluster glycosides, expressed as the concentration of the tested substance required for 50% inhibition of the reference OD_{490}-signal in the absence of the inhibitor (IC_{50}-value), a ligand-bearing matrix (lactosylated neoglycoprotein or asialofetuin to allow comparison of properties of clustered ligand presentation with 26–30 residues per carrier molecule vs those of a glycoprotein with three triantennary N-glycans) was established by marker adsorption to a plastic surface in microtiter plate wells, and the labeled sugar receptor was incubated under optimal equilibrium conditions for 60 min at 37°C (for concentrations see Table I and Table II) in the absence or presence of the inhibitor, as described (17,19). The extent of binding of labeled marker proteins was measured spectrophotometrically after generation of the dye reaction product by streptavidin-peroxidase (Sigma, Munich, Germany; used at a concentration of 0.5 μg/ml) and o-phenylenediamine (1 mg/ml)/H_2O_2 (1 μl/ml) as indicator substances. The range of sugar-inhibitable binding for each receptor type was determined with parallel controls using standard buffer with 0.1% carbohydrate-free bovine serum albumin to reduce protein-protein interaction and with a mixture of 75 mM lactose and 0.5 mg asialofetuin/ml as potent inhibitor of sugar receptor-carbohydrate interaction. The individual experimental series with at least duplicates were carried out independently for at least four times, as similarly performed in the assays with the test substances. The IC_{50}-value for each compound was obtained from the inhibition curves, as shown in Fig. 2.

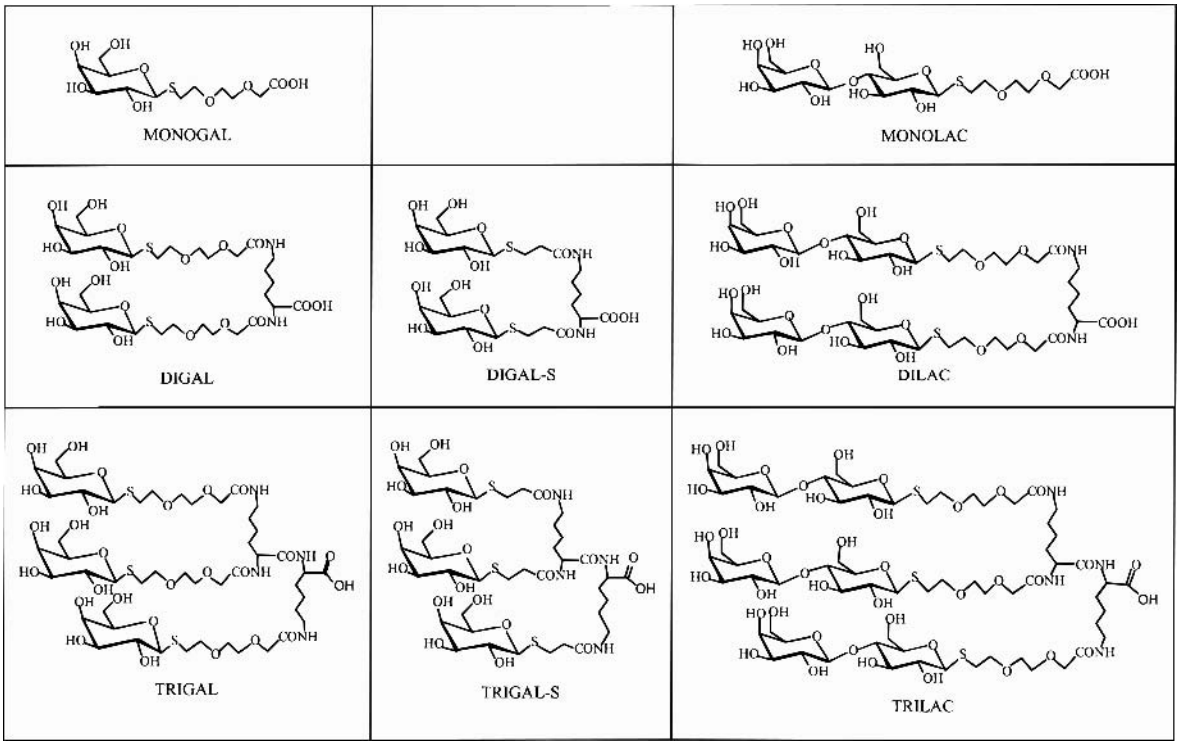

Fig. 1. Structures of the mono-, bi-, and trivalent glycoside derivatives with two lengths of the spacer (DIGAL/LAC, TRIGAL/LAC vs DIGAL/TRIGAL-S).

Table I. Determination of the IC_{50}-values and the Relative Inhibitory Capacity of the Synthetic Derivatives and Free Sugars in a Solid-Phase Assay with Surface-Immobilized Lactosylated Bovine Serum Albumin (Lac-BSA (thio.))

	matrix	0.05 μg Lac-BSA(thio.)		1 μg Lac-BSA(thio.)		0.25 μg Lac-BSA(thio.)		0.05 μg Lac-BSA(thio.)	
	probe	VAA (1.5 μg/ml)		galectin-1 (10 μg/ml)		galectin-3 (5 μg/ml)		IgG($\alpha^-\beta^+$) (1 μg/ml)	
Type of inhibitor	lac content/ molecule	IC_{50} (mM)	Relative potency	IC_{50} (mM)	Relative potency	IC_{50} (mM)	Relative potency	IC_{50} (mM)	Relative potency
D-Galactose	1	2.5	0.4	40	0.03	60	0.07	100	0.03
Lactose	1	1	1	1.1	1	0.5	1	3	1
MONOGAL	1	10	0.1 (0.1)	12 mM = 26% inhibition	<0.1 (<0.1)	no inhibition up to 12 mM	<0.04 (<0.04)	no inhibition up to 12 mM	<0.25 (<0.25)
DIGAL	2	0.20	5 (2.5)	1.3	0.8 (0.4)	no inhibition up to 5 mM	<0.1 (<0.05)	no inhibition up to 5 mM	<0.6 (<0.3)
DIGAL-S	2	0.78	1.3 (0.65)	no inhibition up to 6 mM	<0.18 (<0.06)	no inhibition up to 6 mM	<0.08 (<0.04)	no inhibition up to 3 mM	<1 (<0.33)
TRIGAL	3	0.05	20 (6.7)	1.6 mM = 40% inhibition	<0.69 (<0.23)	no inhibition up to 1.6 mM	<0.3 (<0.1)	no inhibition up to 1.6 mM	<1.9 (<0.63)
TRIGAL-S	3	0.78	1.3 (0.43)	no inhibition up to 4 mM	<0.25 (<0.08)	no inhibition up to 4 mM	<0.13 (<0.04)	no inhibition up to 4 mM	<0.75 (<0.25)
MONOLAC	1	0.79	1.3 (1.3)	2	0.55 (0.55)	no inhibition up to 8 mM	<0.06 (<0.06)	no inhibition up to 8 mM	<0.4 (<0.4)
DILAC	2	0.36	2.8 (1.4)	3.6 mM = 14% inhibition	<0.31 (<0.16)	1.4	0.36 (0.18)	no inhibition up to 2 mM	<1.5 (<0.75)
TRILAC	3	0.12	8.3 (2.8)	0.32	3.4 (1.13)	0.44	1.1 (0.37)	0.17	17.6 (5.9)

Note: The numbers in parentheses express the relative potency of each lactose unit in the multivalent inhibitor compared to carrier-free lactose; the standard deviations in each experimental series for plotting the inhibition curve did not exceed 12.5%.

Table II. Determination of the IC_{50}-values and the Relative Inhibitory Capacity of the Synthetic Derivatives and Free Sugars in a Solid-phase Assay with Surface-Immobilized Asialofetuin (ASF)

	matrix	1 μg ASF		1 μg ASF		1 μg ASF	
	probe	VAA (1.5 μg/ml)		galectin-1 (10 μg/ml)		galectin-3 (5 μg/ml)	
Type of inhibitor	lac content/ molecule	IC_{50} (mM)	Relative potency	IC_{50} (mM)	Relative potency	IC_{50} (mM)	Relative potency
D-Galactose	1	5	0.6	70	0.06	55	0.02
Lactose	1	3	1	4	1	1	1
MONOGAL	1	2.6	1.2 (1.2)	no inhibition up to 12 mM	<0.3 (<0.3)	no inhibition up to 12 mM	<0.08 (<0.08)
DIGAL	2	1	3 (1.5)	no inhibition up to 5 mM	<0.8 (<0.4)	no inhibition up to 5 mM	<0.2 (<0.1)
DIGAL-S	2	6.2 mM = 44% inhibition	<2.1 (<1.1)	no inhibition up to 6 mM	<0.7 (<0.35)	no inhibition up to 6 mM	<0.17 (<0.08)
TRIGAL	3	0.02	125 (42)	no inhibition up to 1.6 mM	<2.4 (<0.8)	no inhibition up to 1.6 mM	<0.6 (<0.2)
TRIGAL-S	3	1	3 (1)	no inhibition up to 4 mM	<1 (<0.33)	no inhibition up to 4 mM	<0.25 (<0.08)
MONOLAC	1	8	0.38 (0.4)	no inhibition up to 8 mM	<0.5 (<0.5)	no inhibition up to 8 mM	<0.13 (<0.13)
DILAC	2	1.8 mM = 44% inhibition	<0.6 (<0.3)	no inhibition up to 3.6 mM	<1.0 (<0.5)	1.8	0.6 (0.3)
TRILAC	3	0.17	17.6 (5.9)	1.2 mM = 35% inhibition	<3.3 (<1.11)	1.2	0.83 (0.3)

Note: The numbers in parentheses express the relative potency of each lactose unit in the multivalent inhibitor compared to carrier-free lactose; the standard deviations in each experimental series for plotting the inhibition curves did not exceed 12.5%.

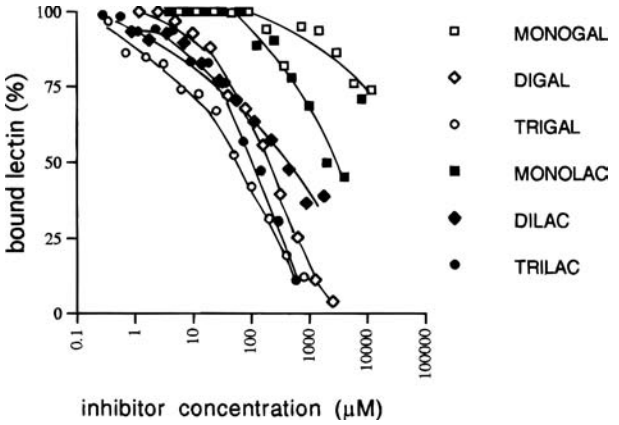

Fig. 2. Inhibition curves of binding of biotinylated *Viscum album* L. agglutinin to surface-immobilized lactosylated neoglycoprotein using the mono-, bi-, and trivalent galactose and lactose derivatives as competitive inhibitor.

Flow Cytofluorometric Analysis

Cells of the human colon adenocarcinoma line SW480, obtained from the American Type Culture Collection (Rockville, MD, USA), and the human B-lymphoblastoid line Croco II, established and maintained as described (20), were carefully washed with Dulbecco's phosphate-buffered saline solution containing 0.1% carbohydrate-free bovine serum albumin to remove any interfering glycoproteins from the serum of the culture medium and then incubated for 30 min at 4°C with the labeled lectins in the absence or presence of inhibitors (4×10^5 cells per assay). Extent of sugar-dependent cell binding was assessed by automated flow cytofluorometric measurements in a FACScan instrument (Becton-Dickinson, Heidelberg, Germany) equipped with the software CONSORT 30 by using the fluorescent marker streptavidin-R-phycoerythrin (1:40 dilution; Sigma, Munich, Germany), as described (18). The relative potency of each test substance was calculated on the basis of the mean signal intensity changes relative to reference measurements without an inhibitor and in the presence of the potent mixture to suppress protein-carbohydrate interaction.

RESULTS

Inhibition of Lectin-(Neo)glycoprotein Binding

The versatile synthetic approach yielded mono-, bi- and triantennary glycosides with 1-thio-β-derivatives of D-galactose (Gal) and lactose (Lac) as ligands. To introduce variations in the sets of interpyranose distances two lengths of the spacer arms were deliberately established allowing sets of distances of either 14, 15 and 24 Å for TRIGAL-S or 22, 25 and 32 Å for TRIGAL (Fig. 1; 16). In order to preclude any influence of adsorption to plastic on the structure of the lectins, protein scaffolds with the glycoligands were coated onto the surface of wells of standard microtiter plates, and the receptors were kept in a native state in solution. Indeed, these assay conditions mimic the situation after therapeutic glycoside administration and the known secretion of galectins *in vitro* and *in vivo* (9,18). As ligands, two modes of sugar presentation were tested, i.e., a synthetic neoglycoprotein with glycosidic maxiclusters and a glycoprotein with three primarily triantennary N-glycans (miniclusters). Since crosslinking of receptor-ligand complexes would effectively reduce the concentrations of both reactants in solution, attention was given to adjust their concentrations to a range where no precipitation could be observed. Under these conditions, the inhibitory potency of the test panel could readily be assessed, as exemplarily illustrated by the inhibition curves of VAA binding to the neoglycoprotein (Fig. 2). Commonly, these curves provide the IC_{50}-values as a measure of relative inhibitory capacity. In those cases with rather weak impairment of lectin binding, the actual percentage of inhibition ($<50\%$) at a certain concentration is given in the data compilations.

As reference point to judge the inhibitory capacity of the synthetic products lactose was tested. Its efficiency to invariably interfere with lectin binding also assured that no protein-protein interactions were involved in signal generation. For the tetrameric plant lectin with its two high-affinity binding sites an increase of inhibitory capacity with increasing valency was observed for both surface-immobilized (neo)glycoproteins (Table I, Table II). The trivalent galactoside reached a level of 42-fold enhancement, emphasizing in principle the concern due to overlapping reactivities. Also, the effect clearly depended on the length of the spacer, the propionate in the activated ester being less favorable than the incorporation of a 2-(2-(2-iodoethoxy)ethoxy)ethanoic acid (Fig. 1; Table I, Table II). VAA binding was sensitive to the choice of the ligand section, galactose yielding results surpassing those of lactose (Table I, Table II). If galectins exhibited similar properites, *in vivo* problems by side effects could be anticipated.

In the case of the mammalian galectins, it is firstly remarkable in view of the comparison to C-type lectins that galactose is a fairly weak inhibitor (Table I, Table II). The next attribute tested is the valency. Importantly, cluster design did not improve this result, and even the Lac-bearing derivatives showed notable, albeit not impressive potency only for the trivalent species (Table I, Table II). A similar result was obtained by testing a polyclonal immunoglobulin G fraction from human serum, another potential source of side effects *in vivo* (Table I). Thus, binding of these endogenous lectins to model matrices with Gal-terminated ligands is not effectively reduced by the synthetic derivatives. To address the question whether this conclusion will also be reached when a physiological ligand profile on a cell surface and not on the surface of a plastic well is presented, we extended our analysis to a cell line and flow cytofluorometry.

Inhibition of Lectin-Cell Surface Binding

Binding of labeled lectins to human colon adenocarcinoma cells (SW480) was sensitive to the presence of haptenic sugar in the incubation medium. As shown for the plant lectin and galectin-1, addition of lactose and synthetic derivatives reduced lectin binding (Fig. 3). Running experimental series similar to the solid-phase assays with cells, no marked cluster effect could be observed for the lactosides and VAA binding to SW480 (Fig. 3a, b) and also Croco II cells (not shown). Moreover, the increase in valency is only weakly reflected in the relative potency of the synthetic lactose-bearing products to reduce galectin binding (Fig. 3c, d). This factor did not exceed a 2.9-fold enhancement, corroborating the data sets with artificial glycan matrices.

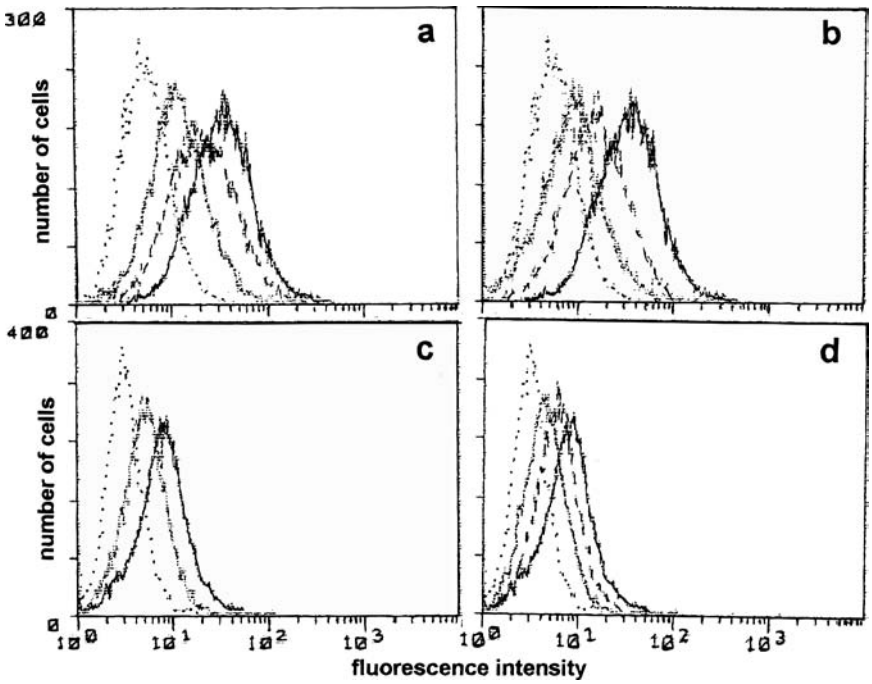

Fig. 3. Semilogarithmic representation of the binding of biotinylated *Viscum album* L. agglutinin (a, b) and mammalian galectin-1 (c, d) to cells of the colon adenocarcinoma line SW480 in the absence of any inhibitor (—) and in the presence of 0.6 and 5 mM lactose (---, ·····) or 0.4 and 1.8 mM DILAC (---, ·····) in panels a and b, or 1.25 and 2.5 mM lactose (---, ·····) or 0.3 and 0.6 mM TRILAC (---, ·····) in panels c and d. The control of the staining behavior of the cell population exposed to the fluorescent marker streptavidin/R-phycoerythrin only (=0%) is included (···).

DISCUSSION

The abundance and spatial accessibility of β-galactosides renders it likely that such determinants are docking points for more than one class of endogenous lectins. It is thus not only important to tailor ligand properties with exclusive consideration of the target lectin in focus. Additionally, the extent of interference of the applied glycoside system with other lectins is to be examined carefully *in vitro* to preclude clinical side effects. Indeed, it has even been surmised that the development of the hepatic clearance system for asialoglycoproteins protects the body from harm by serum glycoproteins after desialylation, which might otherwise block galectins (8). Matching the topology of carbohydrate recognition domains in the hetero-oligomeric hepatic C-type lectin trivalent galactosides have proven to be capable ligand configurations for this receptor facilitating delivery of a variety of payloads (3,10–13,21–23). Our results with a plant agglutinin as model underscore the potential for concern that the trivalent galactoside is also a suitable homing device for other sugar receptors. Since galectins lack a transmembrane domain, our assay system with common representatives of this lectin family in solution is designed to mimic the physiological situation after their secretion. Several instructive lessons emerge from our experiments:

1. Similar to C-type lectins the length of the aglyconic spacer can significantly modulate ligand performance.

2. The presence of galactose as sugar ligand is inferior relative to its extension by a glucose residue for galectins in contrast to the C-type lectins.

3. Branching from the chosen core (*L*-lysine and *L*-lysyl-*L*-lysine) does not generate a remarkable glycoside cluster effect for galectins. The way in which topological ligand presentation impinges on inhibitory efficiency under identical conditions is illustrated by the 185-fold relative potency of a tetravalent lactose-containing starburst glycodendrimer in the case of galectin-1 (19). For glycodendrimers binding studies with rat hepatic membranes point to a reduced discriminatory power relative to the cluster glycosides on this scaffold (24). To also account for a potential influence of the ligand presentation on the inhibitory properties of the glycosides (10,25,26), two different glycoproteins (a neoglycoprotein with a variety of intergalactose distances and a glycoprotein with three triantennary N-glycans) had been tested as matrix. The comparable results in these assays and also in the cell-binding experiments exclude an impact of this factor on the given conclusions.

4. Similar to galectins the binding of the immunoglobulin G fraction was primarily sensitive to the lactose-containing triglycoside. An indication for a significant side effect by the trigalactoside on this receptor system could not be delineated.

General strategies to reduce overlap of ligand reactivity by lectins include tinkering with functional groups by conversion to deoxy or O-methyl derivatives or to exploit the recently discovered differential conformer selection (27). In the studied case, the preference of C-type lectins for trivalent galactosides renders an appropriate selectivity amenable without further refinements. In view of an anti-adhesion therapy by blocking galectins (14) the data with lactose as ligand do not encourage

further exploitation of this type of scaffold. Also, N-acetylgalactosamine is known to be even less potent than galactose in inhibition assays for human galectin-1 (25). Efforts in this area can thus focus on ways to reduce intrafamily misrouting for C-type macrophage and hepatic asialoglycoprotein receptors (12,13). Here, presentation of trivalent N-acetylgalactosamine, facile synthetic procedures for this product being available (28,29), may be helpful, although the human macrophage C-type lectin also is a Tn-specific receptor (30). Binding to galectins with this monosaccharide is not expectable so that testing of a panel of spatial configurations to define discriminatory ligand presentation can confidently focus on the related C-type lectins.

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