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Determination of Carbohydrate-Binding Preferences of Human Galectins with Carbohydrate Microarrays

Tim Horlacher,[a, g] Matthias A. Oberli,[a, g] Daniel B. Werz,[a, b] Lenz Kröck,[a, g] Simone Bufali,[a] Rashmi Mishra,[c] Jens Sobek,[d] Kai Simons,[c] Mitsuomi Hirashima,[e] Toshiro Niki,[f] and Peter H. Seeberger *[a, g, h]

Galectins are a class of carbohydrate-binding proteins named for their galactose-binding preference and are involved in a host of processes ranging from homeostasis of organisms to immune responses. As a first step towards correlating the carbohydrate-binding preferences of the different galectins with their biological functions, we determined carbohydrate recognition fine-specificities of galectins with the aid of carbohydrate microarrays. A focused set of oligosaccharides considered relevant to galectins was prepared by chemical synthesis. Structure–activity relationships for galectin–sugar interactions were determined, and these helped in the establishment of redundant and specific galectin actions by comparison of binding preferences. Distinct glycosylations on the basic lactosyl motifs proved to be key to galectin binding regulation—and therefore galectin action—as either high-affinity ligands are produced or binding is blocked. High-affinity ligands such as the blood group antigens that presumably mediate particular functions were identified.

Introduction

Galectins are a large class of proteins that are characterized by a homologous carbohydrate recognition domain (CRD) of about 130 amino acids that generally binds to β-galactosides.[1–5] To date, fifteen mammalian galectins have been identified and putative homologues are found in all kingdoms, including fungi and plants.[6] As multifunctional proteins, galectins can act in several pathways both inside and outside the cell. Intracellular galectins are involved in mRNA splicing, regulation of the cell cycle, and apoptosis.[7] Sugar-mediated functions of galectins are localized extracellularly, because the corresponding ligands are not present inside the cell.[1] Secreted galectins modulate cell–cell, cell–matrix, and protein interactions through glycoprotein and glycolipid binding.[1,8] Galectin–glycoconjugate interactions on the cell surface can mediate signaling inside the cell.[1,9,10] In this way, galectins can induce apoptosis[11,12] and can also regulate cell adhesion,[13] growth,[10] and differentiation. Galectins are involved in modulation of the immune response,[14] homeostasis of organisms,[15] embryogenesis,[16] inflammation,[17] and tumorigenesis.[18,19]

Galectins are classed into prototype galectins that contain only one CRD (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) and into tandem repeat galectins that bear two linked CRDs (galectin-4, -6, -8, -9, and -12).[20] The two CRDs of a single tandem repeat galectin can differ in their sequences and carbohydrate affinities.[21,22] Galectin-3 is the only chimera-type galectin and has one CRD with a collagen-like N-terminal domain that oligomerizes galectin-3.[23] Prototype galectins dimerize depending on the concentration. Multivalent binding of galectins to sugar ligands strengthens carbohydrate interactions,[4] and resulting cross-linking of bound molecules by galectins might induce intracellular signaling.[24]

Extracellular galectin functions are generally mediated by galectin–glycan interactions. Galectin–carbohydrate interactions have therefore been studied extensively by hemagglutination assays,[25,26] frontal affinity chromatography (FAC),[21,27]

[a] Dr. T. Horlacher, Dr. M. A. Oberli, Dr. D. B. Werz, Dr. L. Kröck, Dr. S. Bufali, Prof. Dr. P. H. Seeberger
Federal Institute of Technology (ETH) Zürich
Wolfgang-Pauli-Strasse 10, 8093 Zürich (Switzerland)

[b] Dr. D. B. Werz
Present address: Institute for Organic and Biomolecular Chemistry
Georg-August-Universität Göttingen
Tammannstrasse 2, 37077 Göttingen (Germany)

[c] Dr. R. Mishra, Prof. Dr. K. Simons
Max-Planck-Institute for Cell Biology
Pfotenhauerstrasse 108, 01307 Dresden (Germany)

[d] Dr. J. Sobek
Functional Genomics Center Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)

[e] Prof. Dr. M. Hirashima
Departments of Immunology and Immunopathology
Faculty of Medicine, Kagawa University
1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793 (Japan)

[f] Dr. T. Niki
GalPharma Co., Ltd., FROM-Kagawa
2217-16 Hayashi-cho, Takamatsu, Kagawa 761-0301 (Japan)

[g] Dr. T. Horlacher, Dr. M. A. Oberli, Dr. L. Kröck, Prof. Dr. P. H. Seeberger
Present address: Max-Planck Institute of Colloids and Interfaces
Am Mühlenberg 1, 14476 Potsdam (Germany)
Fax: (+49) 331-567-9302
E-mail: peter.seeberger@mpikg.mpg.de

[h] Prof. Dr. P. H. Seeberger
Freie Universität Berlin
Arnimallee 22, 14195 Berlin (Germany)

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microarrays,[25,28] surface plasmon resonance (SPR),[25,29] isothermal titration calorimetry (ITC),[26] flow cytometry,[30] and ELISA.[31] These investigations provided a wealth of information relating to galectin preferences for distinct glycans and revealed the basic galectin-binding motif. Nonetheless, defining the various galectin functions and their molecular mode(s) of action is difficult and remains elusive in most processes because most galectins bind to the common carbohydrate recognition motifs lactose [Gal(β1–4)Glc] or LacNAc [Gal(β1–4)GlcNAc (called type 2 LacNAc) or Gal(β1–3)GlcNAc (called type 1 LacNAc)].[32] Such lactose- and LacNAc-containing carbohydrate ligands that mediate galectin function are present on most *N*- and *O*-glycans.[1,33] Various galectins exhibit overlapping actions as a result of similar glycan binding preferences that complicate the assessment of distinct galectin functions.[1,7,12,16] Single galectins typically bind to multiple glycan ligands and mediate several functions by different pathways.[1,3] Finally, many putative glycan ligands for galectins that have been identified during biochemical studies are ubiquitously expressed on cells.

In organisms, however, galectins generally bind only to few distinct—in most cases unknown—glycoconjugates that mediate galectin action.[34] Identification of these distinct glycoconjugates is essential for unraveling of galectin function. Differences in galectin binding specificity remain largely unclear and it is unknown what factors are responsible for the specificity. To understand how galectins generate specificity for glycan ligands is an essential first step, because specific glycan binding directs protein action to distinct glycoconjugates. Additional factors—including levels and localization of galectin expression, the underlying proteins that mediate galectin function, multivalency of binding, and competing glycan ligands—clearly contribute to galectin targeting to distinct glycoconjugates.[3]

Sugars next to the common galectin binding motif are likely to be most important in creating specificity for a distinct glycan ligand, because additional sugars or modifications at the reducing or nonreducing end on the basic lactose or LacNAc motif drastically affect galectin binding.[21,32] Binding of additional sugar moieties to extended binding sites can greatly increase binding, whereas steric hindrance can block binding to lactose or LacNAc motifs bearing inappropriate modifications.[21,25] In contrast to the common binding motifs, affinities for different substitution patterns vary among galectins.[21,25] Galectin preferences for glycan ligands therefore also separate galectin functions in organisms.

Here, we have systematically investigated the effects of distinct modifications and glycosylations of the common LacNAc motif on binding for the major human galectins with the aid of carbohydrate microarrays. Based on the binding preferences, it is possible to deduce which glycans the galectins are targeting in organisms. By analyzing and comparing a large number of galectins, we sought to identify common binding patterns that indicate general galectin features, as well as unique binding specificities that reveal individual functions.

The carbohydrate microarray approach enables efficient screening of many ligands in parallel under standardized conditions, thereby facilitating easy comparison of the results. In addition, the immobilized sugars on the array are presented in a fashion that mimics the natural presentation of oligosaccharides on the cell surface. The microarray method is particularly suitable for multivalent galectin binding. Microarrays minimize the amounts both of sugar and of protein required for binding experiments. Due to these characteristics carbohydrate microarrays are excellent tools for determination of carbohydrate binding specificities.[35] The utility of glycan arrays to examine galectin–carbohydrate interactions had previously been demonstrated by a comparative analysis of galectin-1, galectin-2, and galectin-3.[25]

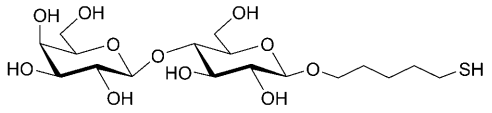
To measure and compare carbohydrate binding properties of the major human galectins (hGal-1, hGal-2, hGal-3, hGal-4, hGal-7, hGal-8, hGal-9), a carbohydrate microarray displaying a variety of synthetic β-galactosides that represent terminal, nonreducing end structures of *N*- and *O*-glycans, present on most glycoconjugates and glycolipids, was created. The focus of the selected ligands was on basic galectin carbohydrate binding motifs (**1**–**5**) and their modifications (**7**–**11**, **16**), rather than on complex glycan structures. This approach allows for the determination of modifications, particularly glycosylations, that alter galectin affinity for glycan ligands, resulting either in high-affinity ligands or in blocking of binding. Monosaccharides (**14**, **15**), sulfated galactosides (**12**, **13**), a rare digalactoside (**6**), and a mannoside control (**17**) completed the array. A straightforward labeling method was developed for efficient probing of proteins that are available in limited quantities.

With this focused galectin glycan array, glycan fine-specificities of human galectins were determined. The results explain how galectin specificity for distinct glycans is generated. High-affinity ligands that presumably mediate particular galectin functions were identified. Comparison of the galectin-binding preferences under standardized conditions helped in establishing general galectin binding modes, indicating how cells regulate their responsiveness toward galectin action. In addition, unique specificities that helped in dissection of galectin actions and aided the attribution of distinct galectin functions were observed.

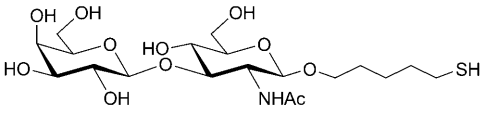
Results and Discussion

Chemical synthesis of carbohydrates used for microarray printing

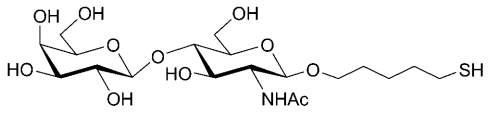
Chemical synthesis of carbohydrates provides access to usable amounts of pure oligosaccharides for biological investigations.[36] Improved protocols, modular approaches, and automated solid-phase synthesis[37] have greatly accelerated oligosaccharide assembly. A modular approach was used to synthesize the carbohydrates **1**–**17** that were utilized for the galectin binding array study. Recent bioinformatics investigations have shown that the occupied mammalian glycospace is much smaller than theoretically possible[38] and that a modular approach to access these mammalian oligosaccharides is possible. Key building blocks containing permanent (Bn, Piv) and temporary protecting groups (mainly Fmoc or Ac) to mask the



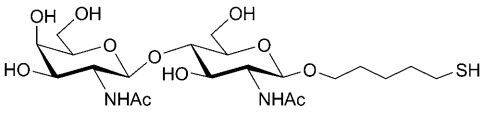
1 Gal(β1-4)Glcβ



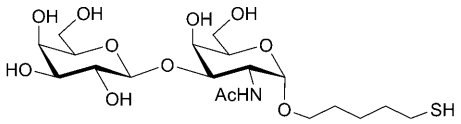
2 Gal(β1-3)GlcNAcβ



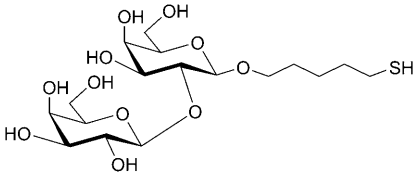
3 Gal(β1-4)GlcNAcβ



4 GalNAc(β1-4)GlcNAcβ



5 Gal(β1-3)GalNAcα



6 Gal(β1-2)Galβ

hydroxy groups were identified. The oligosaccharides were assembled in a linear fashion by solution-phase synthesis, after which global deprotection yielded the compounds ready for attachment to a microarray surface.

Carbohydrate microarray fabrication and binding experiments

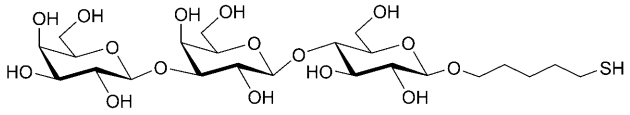
Carbohydrates were printed onto maleimide-functionalized microarrays at four concentrations (2 mM, 0.4 mM, 80 μM, and 16 μM) in replicates of ten as described in the Experimental Section. The use of an automated printing robot guaranteed deposition of equal amounts of carbohydrate solutions on the slide. Equal surface coupling of the different carbohydrates was tested by time-of-flight secondary ion mass spectrometry analysis, indicating comparable amounts of immobilized carbohydrates on the arrays.[39]

Galectins were incubated on the slide at concentrations of 10 $\mu g\,mL^{-1}$ (50 $\mu g\,mL^{-1}$ for hGal-7) in PBS and binding was measured by detection of fluorescence intensity as described in the Experimental Section. Fluorescence intensities were derived as the means over the spot areas, thereby compensating for inhomogeneous spot morphology. Normalized averages of ten replicate spots for each carbohydrate and galectin were obtained from at least three independent experiments.

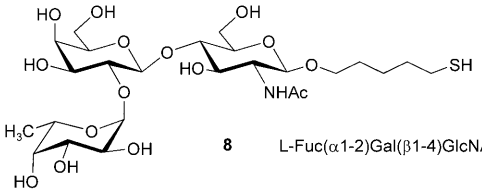
In order to determine differences in binding to the different glycans directly for the various galectins, we used galectin concentrations (10 $\mu g\,mL^{-1}$, corresponding to approximately 0.5 μM per CRD) at which glycan binding was not saturated.[25] In-depth galectin dose–response studies were not carried out, because of the large number and limited amounts of the investigated proteins. Nonsaturated binding was verified in this study by analyzing dose-dependent galectin binding with respect to the different printed glycan concentrations (representative graphs for galectin-1 and galectin-3 are shown in Figure S1 in the Supporting Information). All galectins bound to the immobilized glycans in a dose-dependent manner. For most glycans and galectins, binding was not saturated even at the highest printed glycan concentrations, whereas binding of galectin-1, galectin-2, and galectin-4 displayed saturated glycan binding for some carbohydrates at the highest printed glycan concentration (2 mM), but not at lower concentrations. Here we have only considered binding at nonsaturated conditions (that is, to the highest possible nonsaturated glycan concentration) in cases in which differences in binding directly relate to different binding strengths. The normalized binding specificities (Table 1, Figure 1, and Table S1 in the Supporting Information) established substantial differences in binding strength relating to glycans and galectins.

Lectin binding controls and carbohydrate microarray validation

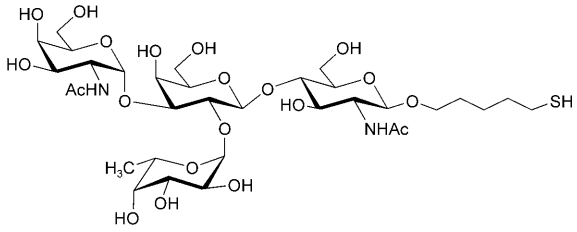
The carbohydrate microarray was analyzed by incubation with control lectins of known binding specificities (carbohydrates **1**–**18**, Table 2). As would be expected, *Dolichos biflorus* agglutinin (DBA) bound GalNAc-terminated saccharides (**4**, **9**), *Canavalia ensiformis* agglutinin (ConA) bound mannose-containing saccharides (**11**, **17**), *Ulex europaeus* agglutinin (UEA) bound fucose-containing oligosaccharides (**8**, **9**, **10**, **11**), and *Triticum vulgaris* agglutinin (WGA) bound (β1–4)-linked GlcNAc-containing sugars. *Viscum album* agglutinin (VAA) bound β-galactose-terminated glycans, galactose-containing oligosaccharides slightly less well, and 6′-*O*-sulfated galactose (**13**) best. All control lectins bound to the arrayed compounds consistently with known binding preferences and validated the microarray binding assay.[40]



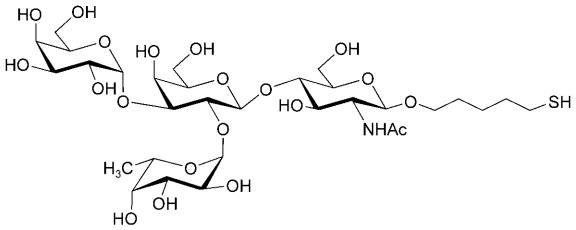
7 Gal(β1-3)Gal(β1-4)Glcβ



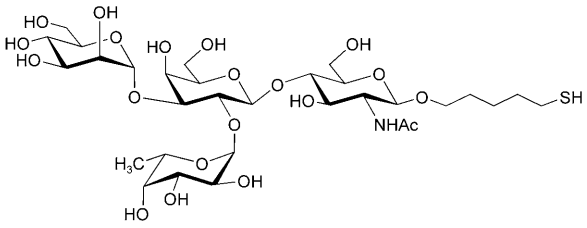
8 L-Fuc(α1-2)Gal(β1-4)GlcNAcβ (H-antigen)



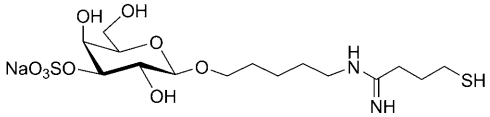
9 L-Fuc(α1-2)[GalNAc(α1-3)]Gal(β1-4)GlcNAcβ (A-antigen)



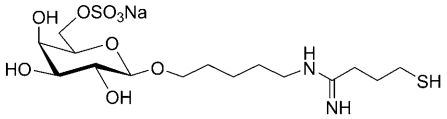
10 L-Fuc(α1-2)[Gal(α1-3)]Gal(β1-4)GlcNAcβ (B-antigen)



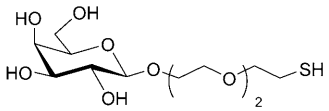
11 L-Fuc(α1-2)[Man(α1-3)]Gal(β1-4)GlcNAcβ



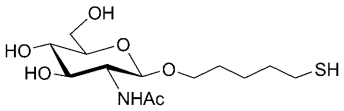
12 Gal-3-OSO_3Na



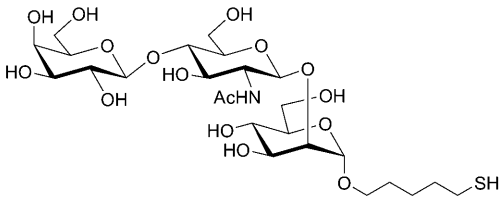
13 Gal-6-OSO_3Na



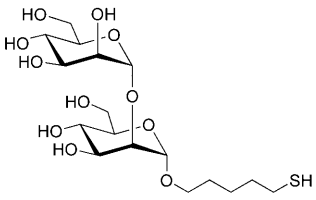
14 Galβ



15 GlcNAcβ



16 Gal(β1-4)-GlcNAc(β1-2)Manα



17 Man(α1-2)Manα

PBS buffer

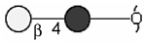
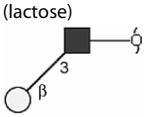
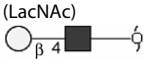
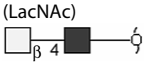
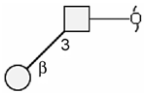
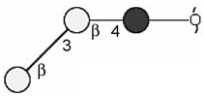
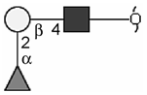
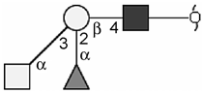
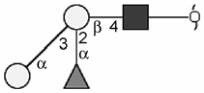
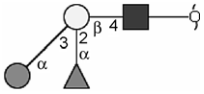
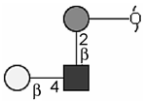
18

To confirm sugar specific binding by the galectins on the microarray, lactose was co-incubated in parallel in order to inhibit binding to the immobilized galactosides. Addition of lactose abolished or greatly decreased galectin binding to the arrays, whereas co-incubation with maltose (to rule out osmotic effects) did not decrease binding. A representative picture of human galectin-9-ND microarray binding and inhibition is shown in Figure 2. Heat-denatured galectins did not bind to arrayed compounds.

Human galectin-1

Galectin-1 bound weakly in relation to other galectins (Table 1), though it bound well to all lactose-containing com-

Table 1. Human galectin binding preferences.[a]

	Carbohydrate	hGal-1	hGal-2	hGal-3	hGal-4	hGal-7	hGal-8	hGal-9	hGal-9-ND
1	 (lactose)	++	++	++	++	+	++	++	++
2	 (LacNAc)	+	++	+	++	–	+	+	(+)
3	 (LacNAc)	+	+	+	(+)	–	(+)	(+)	(+)
4		+	+	+	(+)	–	+	+	+
5		+	++	+	++	–	+	+	(+)
6		+	++	–	(+)	–	(+)	(+)	–
7		++	++	++	+++	++	+++	++	+++
8	 (H)	++	++	+++	+	-	+	++	+
9	 (A)	+	+++	+++	++	++	++	+++	+++
10	 (B)	(+)	+	+++	+++	++	+++	+++	+++
11		+	++	+++	+++	++	+++	+++	+++
12	 (Gal-3-SO_3)	+	++	–	–	–	–	–	–
13	 (Gal-6-SO_3)	–	–	–	–	–	–	–	–
14	 (Gal)	+	++	–	–	–	(+)	(+)	–
15	 (GlcNAc)	+	++	–	–	–	–	–	–
16		++	++	++	(+)	–	(+)	++	++
17		–	–	–	–	–	–	–	–
18	PBS	–	–	–	–	–	–	–	–

[a] Mean values were normalized to lactose (**1**) binding; the symbols represent relative binding in correlation to lactose (**1**, *x*-fold) as: $+++ \geq 1.6 \leq ++ \geq 0.6 \leq + \geq 0.15 \leq (+) \geq 0.03 \leq -$; common symbolic representation of glycans is described in the Supporting Information.

pounds (**1**, **7**). Interestingly, galectin-1 bound better to lactose than to the other disaccharides tested, including β1-3- and β1-4-linked LacNAc (**2**, **3**). Binding was enhanced when LacNAc was coupled to mannose at the reducing end (**16**). Incorporation of fucose slightly improved binding (**8**), whereas the addition of further residues at the 3′-hydroxyl decreased binding to LacNAc (**9**, **10**, **11**). In comparison to other galectins, galectin-1 also showed binding, albeit weak, to sugar **6** and to the monosaccharides galactose (**14**), *N*-acetylglucosamine (**15**), and 3′-*O*-sulfated galactose (**12**).

Human galectin-2

Galectin-2 bound to most β-galactosides arrayed, but binding was generally weak in relation to other galectins. Galectin-2 bound well to *O*-linked core 1 (**5**) and to type 1 LacNAc (**2**), showing type 1 preferences. Addition of fucose to LacNAc (**8**) increased binding, whereas the 3′-*O*-glycosylations had little effect on binding affinity. In case of the blood group B saccharide (**10**), the affinity was even slightly decreased. In comparison to other galectins, galectin-2 bound well to Gal(β1-2)Gal (**6**) and to the monosaccharides galactose (**14**), *N*-acetylglucosamine (**15**), and 3′-*O*-sulfated galactose (**12**).

Human galectin-3

Galectin-3 bound well to the arrayed sugars. It recognized lactose-containing glycans (**1**, **7**) well, and also the modified LacNAc saccharides **8**, **9**, **10**, **11**, and **16**. Binding to lactose was stronger than to other disaccharides. Addition of fucose to LacNAc (**8**) strongly increased binding affinity, which was further strengthened by 3′-*O*-glycosylation (**9**, **10**, and **11**). LacNAc coupled to mannose (**16**) bound

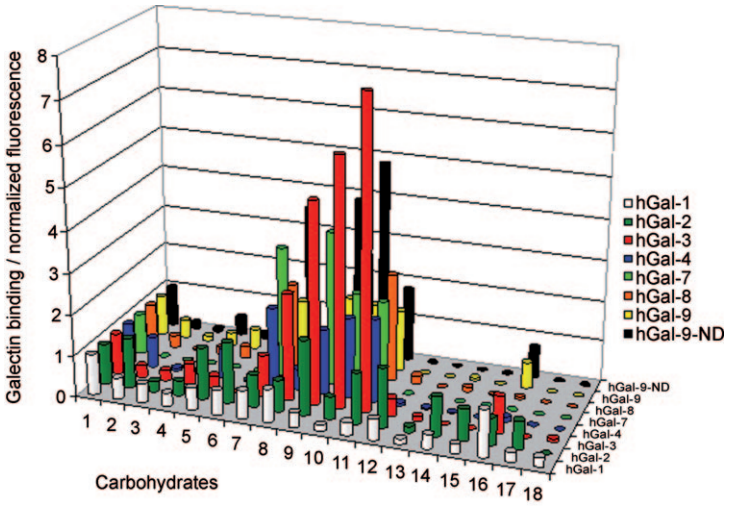


Figure 1. Overview of the human galectin binding preferences. Carbohydrate microarrays were incubated with 10 μg mL^{-1} (50 μg mL^{-1} in the case of hGal-7) biotinylated human galectins and binding was detected with the aid of Cy3-labeled streptavidin. Slides were scanned and the fluorescence intensities were evaluated. The means of the normalized fluorescence intensities of the spots from independent experiments are given.

Table 2. Control lectin binding to the microarrays.[a]

	DBA[b]	WGA[b]	UEA[b]	ConA[b]	VAA[b]
1	–	–	–	(–)	++
2	–	(+)	–	(–)	++
3	–	+	–	–	++
4	+	++	–	(–)	++
5	–	(+)	–	(–)	++
6	–	–	–	(–)	+++
7	–	–	–	(–)	+++
8	–	+	+++	(–)	+
9	+++	+++	+	–	+
10	–	+	(+)	–	++
11	–	+	+	++	+
12	–	(–)	–	–	(+)
13	–	(–)	–	–	+++
14	–	–	–	(–)	++
17	–	–	–	+++	–
18	–	–	–	–	–

[a] +++: very strong binding, ++: strong binding; +: fair binding; (+): detectable binding; (–): background binding; –: no signal. [b] DBA: *Dolichos biflorus* agglutinin. ConA: *Canavalia ensiformis* agglutinin. UEA: *Ulex europaeus* agglutinin. WGA: *Triticum vulgaris* agglutinin. VAA: *Viscum album* agglutinin.

much more tightly than LacNAc (**3**) alone, demonstrating the importance of the vicinal sugar for galectin-3 binding. Monosaccharides were generally not recognized, whereas the H-antigen and its glycosylated derivatives were the highest-affinity ligands.

Human galectin-4

Galectin-4 bound well to sugars **1**, **2**, **5**, **7**, **9**, **10**, and **11**, revealing a slight type 1 preference. Galectin-4 also bound comparably well to core 1 (**5**). 3′-*O*-Galactosylation of lactose (**7**) strengthened binding. A reducing-end mannose had no effect on the affinity for LacNAc, and 2′-*O*-fucosylation of LacNAc improved binding only marginally. Further 3′-*O*-glycosylation of the H-antigen (**9**, **10**, and **11**) greatly enhanced binding, similarly to the galactosylation of lactose (**7**). 3′-*O*-Glycosylation thus generally improved galectin-4 binding; this demonstrates the impact of these modifications on glycan binding of this protein.

Human galectin-7

Galectin-7 was the weakest glycan-binding protein tested. Binding was only detected when higher protein concentrations (50 μg mL^{-1}) were incubated on the array. Galectin-7 bound weakly to some lactose-containing sugars (**1**, **7**) and to the 3′-*O*-glycosylated fucosylated sugars **9**, **10**, and **11**.

Human galectin-8

Galectin-8 bound well to most immobilized oligosaccharides, but not to monosaccharides (Figure 3). The protein recognized lactose (**1**), whereas 3′-*O*-galactosylation (**7**) further strengthened binding. 2′-*O*-Fucosylation (**8**) enhanced binding to LacNAc and 3′-*O*-mannosylation (**11**) or 3′-*O*-galactosylation (**10**) drastically increased the affinity, whereas addition of *N*-acetylgalactosamine (**9**) had a minor effect, revealing some blood group preference. A reducing-end mannose (**16**) did not alter binding strength.

Human galectin-9 N-terminal domain and full-length protein

The binding patterns of the full-length human galectin-9 and of just the human galectin-9 N-terminal domain (Figure 2) were investigated. The full-length protein and the N-terminal domain displayed very similar binding patterns. The only differences were slightly improved binding of the full-length protein to type 1 LacNAc (**2**), to disaccharide **6**, and to the H-antigen (**8**).

Apart from the monosaccharides, galectin-9 bound well to most galactosides. The lactose-containing sugars **1** and **7** were recognized well. Fucosylation (**8**) improved binding to LacNAc. 3′-*O*-Glycosylations strongly enhanced binding for all tested carbohydrates, especially for the glycosylated H-antigens (**9**, **10**, and **11**), which proved to be the best binders. Binding to LacNAc was strongly improved when LacNAc was coupled to a reducing-end mannose (**16**).

Galectin binding preference comparison

Comparison of the galectin binding preferences (Table 1) allows identification of common motifs and unique specificity. Despite similarities, each galectin revealed a distinct binding pattern. Galectin-1, for instance, is the only galectin that bound weakly to the blood group A antigen (**9**). Even galectins with widely overlapping binding patterns differed significantly

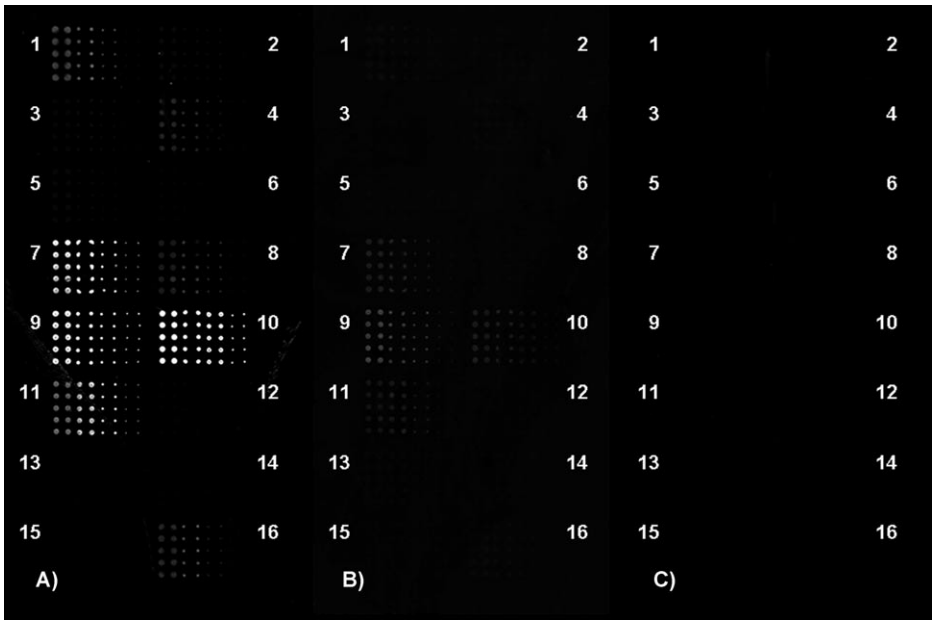


Figure 2. Binding of human galectin-9 *N*-terminal domain (ND) to microarrays. Carbohydrates were printed in replicates of ten at four different dilutions. A) Binding of human galectin-9-ND to the microarray. B) Inhibition of human galectin-9-ND binding by lactose. C) Binding of denatured human galectin-9-ND.

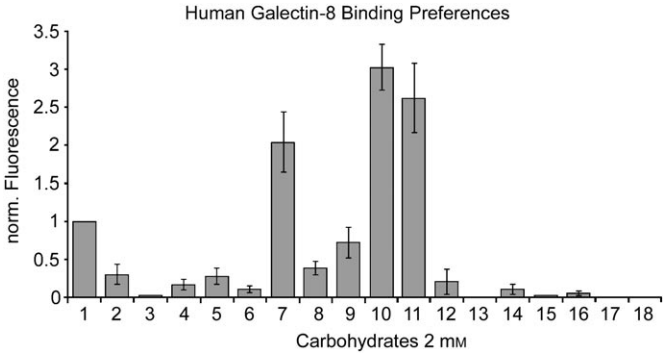


Figure 3. Human galectin-8 binding preferences as an example for microarray data. Carbohydrate microarrays were incubated with biotinylated human galectin-8 (10 $\mu g\,mL^{-1}$) and binding was detected with use of Cy3-labeled streptavidin. Slides were scanned and the fluorescence intensities were evaluated. The means of the normalized fluorescence intensities of 2 mM carbohydrate spots from three different experiments are given. Error bars indicate standard error.

for at least some sugar preferences. Nonetheless, common binding motifs were observed, because some glycans were bound well by most galectins. For example, all galectins bound well to the lactose-containing carbohydrates **1** and **7**. With the exception of galectin-2 and galectin-4, which bound well to saccharides **2** and **5**, galectins bound weakly to type 1 (**2**) and type 2 (**3**) LacNAc, LacDiNAc (**4**), and core 1 (**5**); binding to sugars **3** and **4** in particular was generally mediocre to weak. Galectins, particularly galectin-2, galectin-4, galectin-8, and galectin-9, often bound better to (β1-3)-linked (type 1) LacNAc (**2**) than to (β1-4)-linked (type 2) LacNAc (**3**). Of the sulfated sugars, 3′-*O*-sulfated galactose was recognized only by galectin-1 and galectin-2, but galactose was bound equally well by these galectins. In contrast, 6′-*O*-sulfation generally abolished galectin binding. This finding ruled out nonspecific interactions with the 3′-*O*-sulfated galactose based purely on ionic interactions, whereas VAA binding demonstrates the presence and accessibility of **13**.

Galectin ligands comparison

Comparison of the apparent relative binding strengths of different glycan ligands (Table 1 and Figure 1) provides an understanding of how binding affinity is affected by distinct glycosylations on the basic binding motifs. Most glycosylations drastically altered galectin affinity towards a ligand (Figure 4). Type 2 LacNAc (**3**) is bound weakly by all galectins, but the trisaccharide **16**, in which LacNAc is coupled to mannose at the reducing end, was recognized well by galectin-1, galectin-3, and galectin-9. Addition of sugar residues at the 3′-hydroxy moiety generally improved binding of tandem repeat galectins and of galectin-3. 3′-*O*-Galactosylation of lactose, resulting in the trisaccharide **7**, often improved binding in relation to lactose (**1**) and never decreased it. 2′-*O*-Fucosylated LacNAc (**8**) strongly increased binding of galectin-3 and modestly enhanced binding of the other galectins investigated. The 3′-*O*-modifications of the H-antigen decreased galectin-1 binding, but strongly enhanced the affinity towards the tandem-repeat galectins and galectin-3. Glycosylations appear to be most important for modification of galectin binding to glycan ligands.

The synthetic galactoside microarray and the quick biotinylation method enabled the fast determination of galectin sugar-binding preferences of the major human galectins hGal-1 through hGal-9. As discussed below, the results explain how galectin specificity for glycans is generated and indicate high-affinity ligands that presumably mediate specific interactions in organisms.

According to the binding model,[2] galectins possess five sugar binding sites, termed A to E. Sites C and D are occupied by lactose or LacNAc, and the interactions of specific amino acids with the sugars are conserved, thus generating the basic binding motif. The other sites (A, B or E) can accommodate further residues of an oligosaccharide chain and the fits of these moieties affect binding affinity positively or negatively. In agreement with the model, the galectins that were tested bound lactose or LacNAc, but did not interact with dimannoside **17** above background, thereby confirming the basic binding motif. Binding of disaccharides was comparatively weak

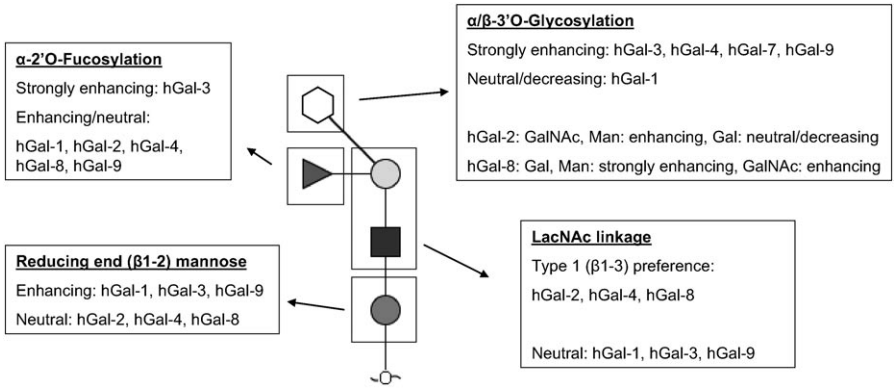


Figure 4. Influence of glycosylations on galectin binding.

and most galectins generally bind more tightly to lactose than to LacNAc. Whereas stronger lactose binding has been reported for some galectins, such as galectin-8,[22] stronger binding of galectin-1, galectin-2, and galectin-3 to lactose than to LacNAc contrasts with previous findings and is presumably assay-specific, as is commonly observed for glycan analysis and microarray experiments.[21,25,41]

The binding preferences of galectins established here emphasize that distinct glycosylations on the basic lactose or LacNAc motifs are extremely important for targeted galectin binding, because sugar residues adjacent to basic motifs alter galectin affinity most drastically (Figure 4). In particular, 3′-*O*-glycosylations of LacNAc, including 3′-*O*-β galactosidation of lactose as well as (α−)blood group glycosylations and α-mannosylation, strongly enhanced binding of most galectins. 2′-*O*-Fucosylation also strengthens binding of most galectins, especially of galectin-3. Such sugar residues occupy the galectin B-site and interact with amino acids to increase the affinity. The specificity of the B-site for distinct sugar moieties proves to be a major determinant in generating galectin specificity for glycans and thus for the glycoconjugates that mediate galectin action. Interestingly, 3′-*O*-mannosylated LacNAc (**11**), a glycan that has not yet been described in nature, is generally recognized as well as blood group antigens (**9**, **10**). The B-site thus seems to be less stringent than the C- and D-sites with regard to sugar specificity. However, glycosylations of the basic motifs can also block binding, when the sugar does not fit into the binding (B) site. Blood group glycosylations diminish galectin-1 binding, for instance.

Little is known about the molecular basis for specific B-site binding. An extended binding site was postulated for galectin-3 on the basis of a crystal structure that identified amino acids, particularly Arg-144, that might interact with 3′-*O*-linked sugar moieties.[42] Binding of 3′-*N*-substituted LacNAc analogues to the galectin-3 extended B-site was confirmed by crystallography.[43] It was speculated that galectin-1 does not bind to 3′-*O*-glycosylated lactosides, because the Arg-144 homologous amino acid is mutated in galectin-1 and another bulky amino acid (valine instead of alanine) might block access to the extended binding site in question.[42] Most, but not all, human galectins that bound 3′-*O*-glycosylated glycans contain an arginine at the position that is homologous to galectin-3 Arg-144, whereas homologous bulky amino acids as in galectin-1 are present in several galectins that bound well to 3′-*O*-glycosylated glycans (Figure S2 in the Supporting Information). A comprehensive analysis based on crystal structures of the fungal galectin CGL-2 from *Coprinopsis cinerea* with different substituted lactosides identified several further amino acids that interact with sugars in the B-site.[44] For the bound A blood group antigen, most glycan–amino acid interactions, including binding to fucose as well as galactosamine present in the A blood group antigen, were observed, thereby explaining better binding than solely 3′-*O*-glycosylated or 2′-*O*-fucosylated glycans.[44] However, the amino acids in question are not strictly conserved in all galectins that bind well to blood group antigens (Figure S2 in the Supporting Information). Accordingly, the arginine homologous residue that mediates B-site sugar binding in galectin-3 is altered to threonine in CGL-2. The binding site requirements for binding extended glycans thus appear to be more complex than those for binding to the basic lactosyl motif.

Generally, glycosylations 4′-*O*- and 6′-*O*- linked to galactose and 3′-*O*-linked to (*N*-acetyl)glucosamine of Lac(NAc) abolish binding, because these hydroxy groups interact with amino acids of the galectins and are essential for binding.[21,25,44] These findings explain why the 3′-*O*-sulfated galactose (**12**) binds better than the 6′-*O*-sulfated galactose (**13**) on the array. However, binding of galectins to the arrayed sulfated galactoses (**12**, **13**) was generally weak, presumably due to the presentation as sulfated monosaccharides, indicating a requirement of additional sugar moieties for high-affinity binding. Some galectins—galectin-4, for example—might also recognize moieties preceding the reducing end, including the lipid part of glycolipids, because 3′-*O*-sulfated galactosides, including sulfatides, are important motifs in some high-affinity galectin ligands, particularly on glycosphingolipids.[45]

The sugar preceding LacNAc at the reducing end also modulates galectin binding (Figure 4), because this residue can bind to the E-site. Galectin-1, galectin-3, and galectin-9 bind more strongly to LacNAc when coupled to mannose (**16**) than to directly immobilized LacNAc (**3**). With other galectins, this modification has no effect. The sugar at the reducing end of LacNAc might reflect the type of glycan to which the common terminal LacNAc structure is attached. Enhanced binding to such galactosides might thus specify the respective galectin activity for certain types of glycoconjugates. Mannose preceding LacNAc, as in the case of the trisaccharide **16**, is mainly found on *N*-glycans and dystroglycans. Galectin-1, galectin-3, and galectin-9 presumably interact preferentially with these types of glycans.

Identification of these exact binding preferences provides the basis for understanding of targeted galectin action. Basic galectin-binding motifs, particularly LacNAc, are common terminal structures that can be found on many glycoconjugates and glycolipids, but galectins interact only moderately with these motifs. As discussed above, sugar moieties attached to these basic binding motifs drastically alter galectin affinity (Figure 4), ranging from the prevention of binding to the generation of high-affinity ligands that presumably mediate specific galectin functions. The results indicate two major ways in which the adjacent residue on the basic binding motif might direct and regulate galectin action. Firstly, the E-site preference for the reducing-end sugar of LacNAc might dictate the types of glycans that galectins bind, as described above. Secondly, high-affinity ligands are generated or masked through the action of distinct glycosyltransferases on the basic structure. Expression of distinct glycosyltransferases or glycosidases regulates the presence of distinct glycans and is thus an important way to modulate the susceptibility of a cell to galectin action. In some cases, glycan expression can be directly correlated to galectin function.[12,46] *N*-Acetylglucosaminyltransferase V (GNTV) adds a GlcNAc residue to a specific *N*-glycan precursor to construct complex type *N*-glycans with high-affinity poly-LacNAc galectin binding sites. GNTV regulates, at least partially, antigen recognition by T cells through the synthesis of binding sites that mediate galectin-3 function.[47] Expression of glycosyltransferases and glycan-modifying enzymes can also block galectin action. A previously susceptible cell line became resistant to galectin-1-induced apoptosis through the overexpression of the sulfotransferase ST6Gal I, which adds sulfates to galactose.[48]

The binding preferences determined in this study (Table 1) reveal galectin specificity for glycan ligands. These insights facilitate the identification and attribution of galectin-responsive cells expressing such glycan ligands. Future determination of the glycoconjugates and glycoproteins that mediate galectin function is essential for unraveling of the largely unknown modes of galectin action.

Blood group antigens were recognized particularly well by all galectins except galectin-1, consistently with previous studies.[25] Blood group antigens are expressed on all major glycoconjugates of hematopoietic lines and epithelial cells.[1,49] Several galectins are expressed in epithelial cells and colocalize with blood group antigens.[50] Furthermore, galectins bind to glycoproteins that carry ABH antigens.[51] Because of their strong binding it seems likely that blood group antigens are important ligands for galectins, but their particular function and regulation of galectin function remains to be investigated. Blood-group-related functions in the immune response have been proposed for galectin-2 and galectin-3,[25] and might also apply for further galectins that bind blood group antigens tightly (hGal-4, hGal-8, hGal-9). Recently, it has been shown that galectin-4 and galectin-8 are capable of killing bacteria expressing blood group antigens.[52] The findings support the biological importance of the observations made with the aid of the glycan microarrays reported here. The biological role of galectin binding to blood group antigens will be a prominent target for future investigations.

Interestingly, galectin-2 and galectin-8 showed differences in blood group A and blood group B preference, with galectin-2 binding better to the A-tetrasaccharide and galectin-8 preferring the B-tetrasaccharide. The tandem-repeat galectins bound only weakly to the H-antigen, but strongly to the A- and B-tetrasaccharides. It is not known whether humans of different blood types exhibit differential phenotypes due to altered galectin susceptibility. The expression of A- and B-antigens is lost in many types of cancer; this results in the enhanced presence of the H-antigen.[53] Several galectins are expressed differently in many tumors and often correlate with tumor malignancy and progression.[19] A link between galectin function and blood group antigen expression in tumorigenesis remains elusive.

Conclusion

A focused synthetic carbohydrate microarray of putative galectin ligands was prepared in order to determine galectin binding preferences. Comparisons of the binding patterns allowed galectin functions to be dissected. High-affinity galectin ligands were identified and underscore the notion that glycosylation of common core structures is important for the regulation of galectin function. This galectin carbohydrate microarray constitutes a valuable tool for rapid analysis and comparison of the growing number of galectins.

Experimental Section

Proteins: Human galectin-1 to galectin-8 were purchased from R&D Systems (Abingdon, UK) and biotinylated control lectins and chemicals were purchased from Sigma–Aldrich. Full-length human galectin-9 and the hGal-9 N-terminal domain were purified as described.[54]

Synthesis of the mono- and oligosaccharides: A modular approach was used for the chemical synthesis of carbohydrates **1**–**17**. Key building blocks bearing permanent (Bn, Piv) and temporary protecting groups (mainly Fmoc or Ac) for the hydroxy functionalities were identified. Linear solution-phase synthesis, installation of the reactive thiol moiety at the linker, and global deprotection by Birch reduction furnished the desired oligosaccharides (for detailed synthetic protocols and analytical data, see the Supporting Information).

Fabrication of carbohydrate microarrays: Carbohydrate microarrays were produced as described previously.[55] In brief, carbohydrate compounds were diluted to four concentrations (2 mM, 400 μM, 80 μM, and 16 μM) in PBS buffer with one molar equivalent tris(2-carboxyethyl)phosphine (TCEP). Maleimide-functionalized microarrays were produced by submerging amine-coated slides (GAPS II slides, Corning) in 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (2 mM) in DMF with diisopropylethylamine (2.5% *v/v*) for 24 h at room temperature. Slides were washed three times with water and three times with ethanol, centrifuged to dryness, and stored under argon until spotting. Diluted and reduced compounds were printed onto the functionalized microarray slides at 1 nL per spot by an automatic piezoelectric arraying robot (Scienion, Berlin, Germany). For completion of the immobilization reaction, printed slides were stored for 24 h in a humified chamber.

Galectin biotinylation: Galectins (0.5 mg mL^{-1}) in PBS were incubated with biotin 3-sulfo-*N*-hydroxysuccinimide ester (2.5 mM) first for 30 min at room temperature and then for 1 h at 4 °C. Unreacted biotin 3-sulfo-*N*-hydroxysuccinimide ester was quenched by the addition of glycine in PBS (100 mM , 1 vol equiv) and incubation for 30 min at room temperature.

Binding experiments: Microarray slides were washed three times with water. Unreacted maleimide was quenched by submerging the slides in β-mercaptoethanol (0.1%, *v/v*) in PBS for 1 h at room temperature. Slides were washed three times with water and with ethanol, centrifuged to dryness, and blocked with BSA (2.5%, *w/v*) in PBS for 1 h at room temperature. Blocked slides were washed twice with PBS, centrifuged, and incubated with biotinylated galectins or control lectins (each 10 μg mL^{-1}) in PBS with BSA (1%, *w/v*) and Tween-20 (0.1%, *v/v*) for 1 h at room temperature. For inhibition, lactose (100 mM) was added to the incubation solution. Incubated slides were washed with PBS, centrifuged, and overlaid with Cy3-streptavidin (10 μg mL^{-1}) in PBS with BSA (1%, *w/v*) and Tween-20 (0.1%, *v/v*) for 1 h at room temperature. Slides were washed twice with PBS and centrifuged to dryness. For detection, slides were scanned with a fluorescence microarray scanner (Tecan, Maennedorf, Switzerland). Spot intensities were evaluated by use of Genespotter software (MicroDiscovery, Berlin, Germany).

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Keywords: carbohydrate chemistry · carbohydrate microarrays · carbohydrates · galectins · glycan binding · lectins

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