DOI: 10.1002/cbic.200500165

Carbohydrate Array Analysis of Anti-Tn Antibodies and Lectins Reveals Unexpected Specificities: Implications for Diagnostic and Vaccine Development

Joseph C. Manimala,[a] Zhitao Li,[a] Amit Jain,[b] Sharanjeet VedBrat,[b] and Jeffrey C. Gildersleeve*[a]

The Tn antigen is a carbohydrate antigen expressed in most carcinomas, during embryogenesis, on pathogenic parasites, and on HIV. It has been evaluated extensively as a potential diagnostic marker and several Tn-based vaccines are in clinical trials. Based on discrepancies in the literature regarding Tn expression, we began to question whether antibodies and lectins used routinely to detect the Tn antigen were providing accurate information. To investigate this possibility, a carbohydrate microarray and a highly sensitive assay were developed and three frequently used Tn receptors (HBTn1, Bric111, and VVL-B4) were evaluated. Carbohydrate-array analysis revealed unexpected cross-reactivity with other human carbohydrate epitopes. VVL-B4 bound the Tn antigen, GalNAcα1-6Gal, and GalNAcα1-3Gal. Bric111 bound the Tn antigen, blood group A, GalNAcα1-6Gal, and GalNAcα1-3Gal. HBTn1 showed the best selectivity, but still displayed moderate binding to blood group A. Implications for the development of Tn-based diagnostics and vaccines are discussed.

Introduction

The Tn antigen,[1] a carbohydrate epitope composed of a GalNAc α-linked to a serine or threonine residue of a polypeptide, was first reported as a tumor-associated antigen about 30 years ago by Springer and has since been investigated extensively.[2,3] In addition to a broad range of carcinomas,[3,4] Tn expression has been reported during development/embryogenesis,[5–7] in pathogenic parasites,[8,9] on HIV-1,[10] and in a variety of rare human diseases such as Tn syndrome,[11] IgA Nephropathy,[12] Henoch–Schonlein purpura,[13] and Schindler–Kanzaki disease.[14,15] However, expression in normal adult tissue is rare. As a result, there has been significant interest in monitoring expression of the Tn antigen for diagnostic purposes as well as developing Tn-based vaccines for cancer and HIV.[3,4,16–18] In addition, a considerable effort has been made to understand the biological effects of Tn expression and its molecular mechanisms of action. Unfortunately, after decades of research and development, little is known about the biological roles of the Tn antigen.

Accurate detection of the Tn antigen is essential for both basic and applied research. However, monitoring the expression of carbohydrate antigens is not trivial. Few methods are available and most are extremely labor intensive. Therefore, expression is almost always monitored indirectly by measuring the binding of anti-carbohydrate antibodies and lectins. Detection of the Tn antigen is no exception. Of course, specific recognition is critical for detecting carbohydrate antigens with antibodies and lectins. If Tn receptors lack specificity, then other carbohydrate epitopes could be mistaken for the Tn antigen. While this potential problem could have enormous effects on basic science and medical applications, anti-Tn antibodies and lectins such as HBTn1, Bric111, and VVL-B4 have been used for years to monitor expression of the Tn antigen and were thought to be selective. For example, the VVL-B4 lectin readily distinguishes between the Tn antigen and the structurally related blood group A antigen and selectively binds carcinoma tissues over most normal adult tissues.[19,20] As a result of this perception, antibody and lectin binding has been taken as a reliable measure of Tn expression.

Although Tn receptors are regarded as standard tools, confusing and conflicting results are not uncommon. For example, Itzkowitz et al.[21] and Ching et al.[22] reported that the Tn antigen was expressed in normal pancreatic acinar cells, while Cao et al. found that it was not.[23] In addition, a number of studies indicated that the Tn antigen is expressed at an early stage of cancer progression,[24–27] while others concluded that it is

[a] *Dr. J. C. Manimala, Dr. Z. Li, Dr. J. C. Gildersleeve*
Laboratory of Medicinal Chemistry
Center for Cancer Research, NCI–Frederick
376 Boyles Street, Building 376, Room109, Frederick, MD 21702 (USA)
Fax: (+1) 301-846-6033
E-mail: gildersleevej@ncifcrf.gov

[b] *Dr. A. Jain, Dr. S. VedBrat*
KamTek Inc.
7858 Beechcraft Avenue, Gaithersburg, MD 20879 (USA)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

expressed at a late stage.[28–31] Some reports concluded that Tn expression is correlated with a poor prognosis,[28,32,33] while others found no correlation between Tn expression and prognosis.[34,35]

Although a variety of factors could contribute to these discrepancies, the inconsistent expression results led us to question whether binding studies with anti-Tn antibodies and lectins were providing accurate and reliable information. To test this, we needed to evaluate binding to a wide range of carbohydrate epitopes. Carbohydrate arrays have recently emerged as valuable tools for rapid and comprehensive analysis of carbohydrate–protein interactions.[36–44] Like the related DNA and protein microarrays, carbohydrate arrays possess many carbohydrate epitopes spotted onto a solid support, such as a glass slide. With a spot size of 100–300 μm, thousands of carbohydrates can be printed on each slide and only a minimal amount of sample is required. In addition, the microarray format facilitates high-throughput as well as thorough and direct comparisons of binding. As a result, carbohydrate arrays are ideal tools for probing the specificity of carbohydrate-binding proteins, identifying new carbohydrate-binding proteins, and developing diagnostic and therapeutic agents.

In this paper, the development of a carbohydrate microarray and the evaluation of three frequently used anti-Tn antibodies and lectins are described. The development of the array required selecting an appropriate format, synthesizing a variety of homogenous, structurally defined carbohydrate epitopes, and developing a highly sensitive assay to detect binding. The first-generation array contains 29 neoglycoconjugates and glycoproteins with a complete array printed in each well of a 16-well slide. The unique format permits analysis of many samples (e.g. dilution series, patient serum samples) in an economical manner and should prove especially useful for the development of diagnostics and vaccines. Carbohydrate array analysis of HBTn1, Bric111, and VVL-B4 revealed significant differences in specificity as well as cross-reactivity with some structurally related human carbohydrate epitopes. The results provide a reasonable explanation for the conflicting reports on Tn expression. Implications for the development of Tn-based diagnostics and vaccines are also discussed.

Results

Development of the carbohydrate array

Our initial objective was to determine if other carbohydrate epitopes could be mistaken for the Tn antigen. However, our group is also interested in using carbohydrate arrays for the development of cancer diagnostics, vaccines, and therapeutic agents. Since many of these objectives require evaluating large numbers of samples (e.g. patient serum samples) and/or conditions (e.g. series of dilutions), we wanted an array format that would permit the analysis of hundreds to thousands of samples in an economical fashion. Therefore, a slide containing 16 wells was chosen for the array. An entire array would be printed in each well, thus allowing 16 different samples or conditions to be examined on each slide. The spacing of the wells is comparable to two columns of a 96-well plate and is compatible with standard multichannel pipettors. While subdividing a slide into 16 sections reduces the total number of features that can be printed in each array, a significant amount of diversity can still be represented.

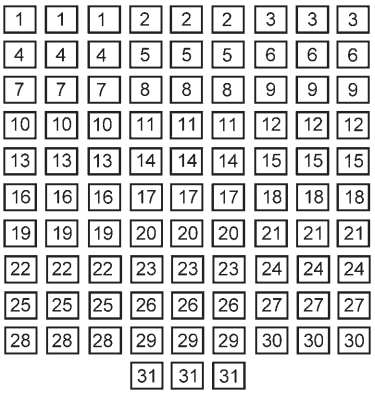
The nature of the carbohydrate epitopes was also an important consideration. Numerous papers have been published on printing proteins on glass microscope slides, and reliable procedures and conditions are known.[45] In addition, a variety of slides for printing proteins are commercially available. Therefore, we chose to print neoglycoconjugates and glycoproteins with the hope that existing slides, surface chemistry, and printing conditions would be suitable for printing carbohydrate-modified proteins. In addition to facilitating printing, neoglycoconjugates and glycoproteins display multiple copies of each epitope. Polyvalent presentation of the epitopes is critical, since most carbohydrate-binding antibodies and lectins achieve tight binding through polyvalent interactions (i.e. simultaneously binding two or more antigens at two or more binding sites). Moreover, specificity towards monovalent carbohydrates can be substantially different from the specificity for polyvalent carbohydrate ligands.[46,47] Since most biologically interesting recognition events and biochemical assays require high-affinity polyvalent binding, specificity should also be evaluated in the context of polyvalent binding. Whether or not the carbohydrate moieties would be accessible for binding under these conditions was of primary concern.

The next step involved choosing and obtaining carbohydrate epitopes for the array. The objective was to include a broad range of epitopes, with a subset being structurally related to the Tn antigen. Unfortunately, natural sources of carbohydrates—such as tissue samples, cells, and glycoproteins—display heterogeneous mixtures of carbohydrates, thus making analysis of the specificity complicated. Moreover, natural sources of specific carbohydrate epitopes of interest are frequently inaccessible. Therefore, chemical synthesis has become an important tool for obtaining structurally defined, homogeneous epitopes.

The specific glycoconjugates and glycoproteins used for the array are listed in Table 1. Two different sources of the Tn antigen were included: asialo-bovine submaxillary mucin (BSM) and Tn–bovine serum albumin (BSA). Asialo-BSM is a mucin obtained by desialylation of BSM. BSM and asialo-BSM predominantly express STn and Tn, respectively, in a natural context and have frequently been used to evaluate anti-Tn antibodies and lectins.[48] The synthesis of Tn–BSA, a neoglycoconjugate containing homogeneous, structurally defined Tn epitopes, is shown in Scheme 1. Acid **30** was coupled with linker **31**. After deprotection of the Fmoc group and acetylation of the N-terminal amine, the esters were hydrolyzed to produce fully deprotected acid **34**. Conjugation to BSA was effected by converting the acid to an *N*-hydroxysuccinimide (NHS) ester and then coupling with lysine residues on BSA to produce **11**. With a ratio of acid to BSA of 45:1, the resulting neoglycoconjugate possessed an average of 21 Tn epitopes per BSA, as determined by MALDI-MS.

In addition to the Tn antigen, a variety of Tn-related epitopes were included on the microarray. To identify epitopes

Table 1. Glycoconjugates and glycoproteins used in the array. Glycoconjugates and glycoproteins were printed in triplicate in each well. The location of each component is defined in the diagram.



Spot no.	Name (carbohydrate structures)	Spot no.	Name (carbohydrate structures)
1, 28	Cy3–BSA	16	LacNAc–BSA (Galβ1-4GlcNAc-)
2	BSA	17	TF-HAS (Galβ1-3GalNAc-)
3, 31	Cy5–BSA	18	mannotriose–BSA [Manα1-6[Manα1-3]Manα -]
4	KLH	19	3′Sialyl-LacNAc–BSA (Sialylα2-3Galβ1-4GlcNAc-)
5	oxKLH	20	Blood Group A–BSA [GalNAcα1-3(Fucα1-2)Galβ]
6	Glcβ–BSA	21	blood group B–BSA [Galα1-3(Fucα1-2)Galβ]
7	GalNAcα–BSA	22	blood group H–BSA [Fucα1-2Galβ]
8	Glcα–BSA	23	Le^x–BSA [Galβ1-4[Fucα1-3)GlcNAc]
9	Manα–BSA	24	Le^a–BSA [Galβ1-3[Fucα1-4)GlcNAc]
10	GlcNAcβ–BSA	25	Le^b–BSA [Fucα1-2Gal β 1-3-[Fucα1-4)GlcNAc]
11	Tn–BSA (GalNAcα1-Thr-)	26	SLe^x–BSA [Siaα2-3Galβ1-4-[Fucα1-3)GlcNAc]
12	α1-6–BSA (GalNAcα1-6Galβ-)	27	Le^y-HAS [Fucα1-2Gal β1-4-(Fucα1-3)GlcNAc]
13	A_{di}–BSA (GalNAcα1-3Galβ-)	28	BSM (STn, STF, Sia-GlcNAcβ1-3GalNAc)
14	GlcNAcα1-4Galβ–BSA	29	aBSM (Tn, TF, GlcNAcβ1-3GalNAc)
15	B_{di}–BSA Galα1-3Gal–BSA		

that might be mistaken for the Tn antigen, a database of over 7000 carbohydrates was searched by using three criteria.[49] First, only epitopes that are known to be expressed in humans were selected, since these have the potential to be present in samples of interest. Second, only epitopes that are found at the nonreducing terminus of oligosaccharide chains were chosen, since they are more likely to be accessible for binding. Third, epitopes with a high degree of structural similarity to the Tn antigen were given highest priority. Since the Tn antigen contains a terminal GalNAc α-linked to an amino acid, epitopes containing a terminal GalNAc with an α linkage were given highest priority; epitopes with a terminal GlcNAc with an α linkage or a terminal GalNAc with a β linkage were also considered. From this list, only the blood group A antigen was readily accessible in homogeneous form. Chemical synthesis was utilized to obtain additional Tn-like epitopes, such as **12**–**14**.

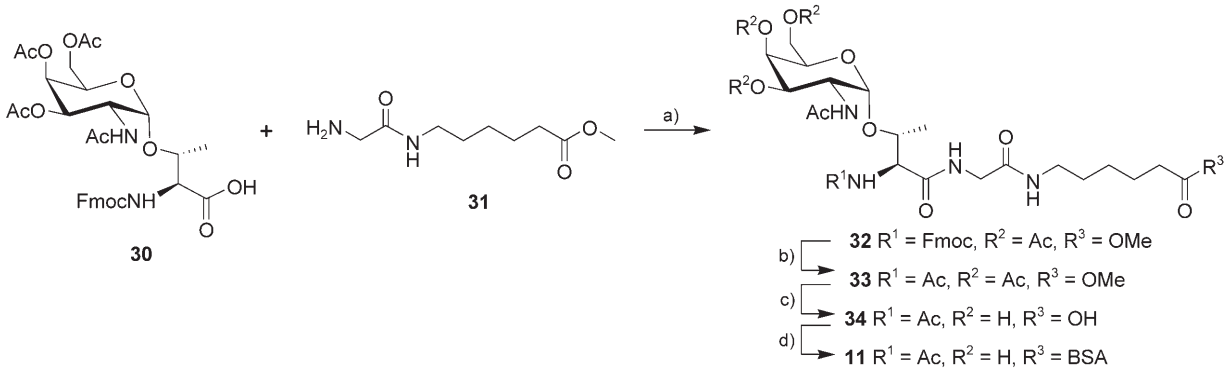
Epitopes **12**–**14** were assembled by using a common strategy (see Scheme 2). The key step for each was the formation of the glycosidic linkage. While the couplings of **35** with **36** and **47** with **48** proceeded stereoselectively under Koenigs–Knorr conditions, glycosylation of **42** with chloride **47** produced a 1:2 α/β mixture of disaccharides (**43**). After some experimentation, the stereoselectivity was improved to 10:1 α/β by using imidate **41** as the glycosyl donor. Next, a nine-atom flexible linker was installed by treating the thioglycosides with IBr[50] to produce the corresponding glycosyl bromides followed by addition of linker **53** under biphasic conditions to produce **38**, **44**, and **50**.[51] Following addition of the linker, the azide groups were reduced to amines by the Staudinger reaction and then acetylated to produce **39**, **45**, and **51**. Finally, hydrolysis of the ester protecting groups produced disaccharides **40**, **46**, and **52**. The corresponding acids were activated as NHS esters and then coupled to BSA to produce **12**–**14**. Each of the conjugates possessed approximately 20 carbohydrate epitopes per BSA.

To enhance diversity, a variety of other monosaccharide (see Supporting Information) and oligosaccharide conjugates such as blood groups A, B, and H, Le^x, Le^a, Le^b, and Le^y were included on the array (see Table 1). Overall, a total of 29 different components were utilized: two positive controls (Cy3– and Cy5–BSA), one negative control (BSA), five monosaccharide conjugates, seven disaccharide conjugates, ten trisaccharide or larger conjugates, and four glycoproteins.

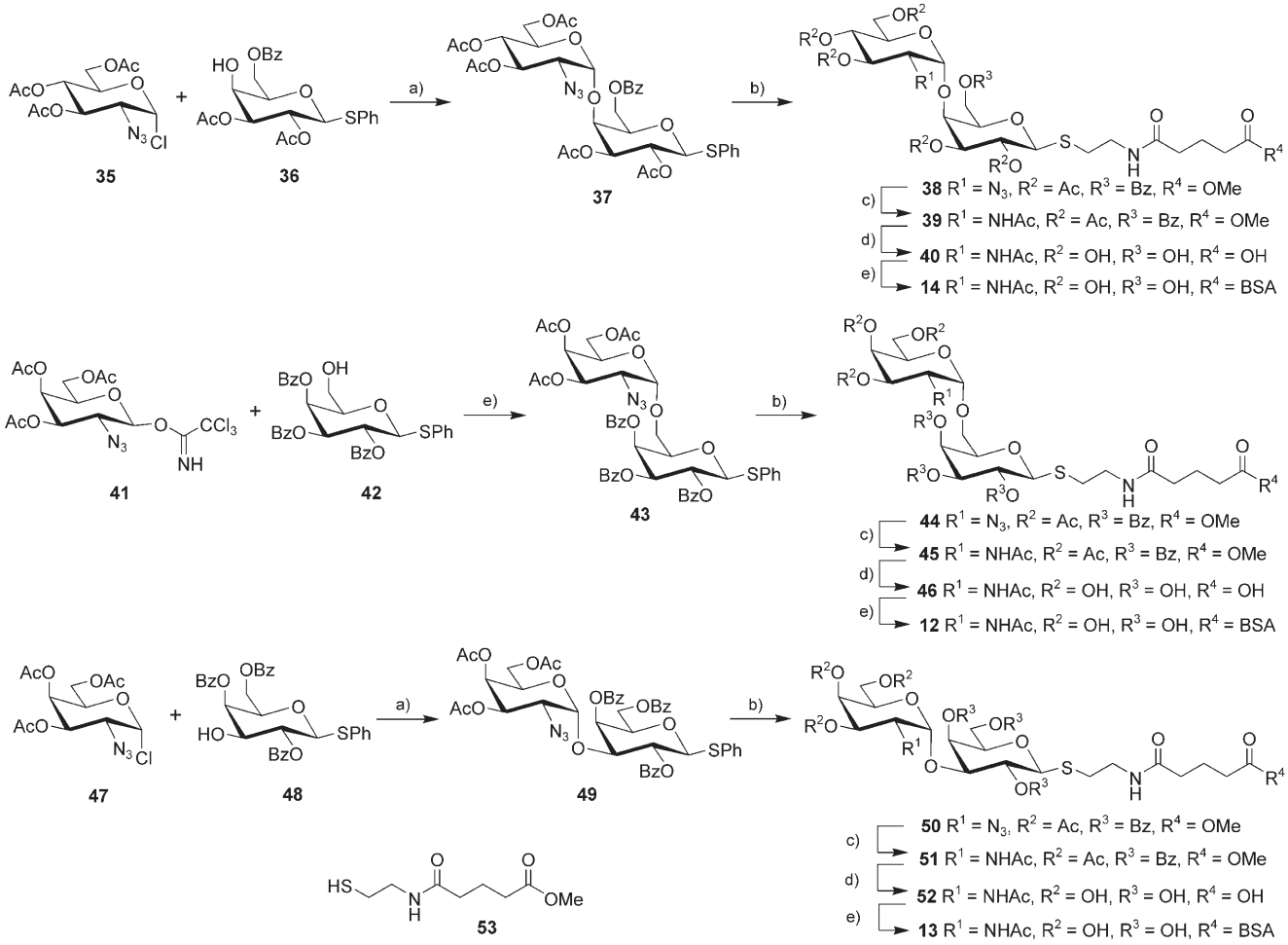
The next step in the development of the array involved printing the samples on the slides. Samples were printed from stock solutions at a concentration of 0.5 mg mL^{-1} in PBS containing 5% glycerol. A complete array was printed in each well of the Nunc ArrayCote16 glass slides by using a robotic arrayer fitted with quill pins. Each array contained a total of 93 features: each of the 29 samples printed in triplicate, along with an additional three spots each of Cy3- and Cy5-labeled BSA (31 sets of three spots, see Table 1). Spots were approximately 180 μm in size and highly reproducible under these conditions. As with protein and DNA arrays, the humidity was controlled to produce consistent spots and minimize evaporation of the stock solutions.

Evaluation of binding with the array

The next objective was to develop a highly sensitive, robust, and reproducible assay for evaluating lectin and antibody binding to the microarray. An ELISA/ELLA assay was adapted for the microarray format, and conditions were initially optimized for binding VVL-B4 to Tn–BSA. Binding could be detected under a variety of conditions, thus indicating that the carbohydrate epitopes are accessible under our printing conditions. To obtain high signal-to-noise ratios and minimal variation, however, a number of parameters, such as the blocking agent, the streptavidin–horseradish peroxidase (HRP) concentration, and the tyramide substrate concentration, were varied.



Scheme 1. Synthesis of Tn–BSA (**11**). Reaction conditions: a) PyBop, *N*-methylmorpholine, DMF (96%); b) pyrollidine, CH_2Cl_2, then Ac_2O, pyridine (76%); c) NaOH, MeOH (58%); d) EDC, NHS, then BSA.



Scheme 2. Synthesis of epitopes **12**–**14**. Reaction conditions: a) AgOTf, CH_2Cl_2, 2,6-di-*tert*-butyl-4-methylpyridine, −78 °C (72–86%); b) i. IBr, CH_2Cl_2, 0 °C (60–85%); ii. Bu_4NHSO_4, Na_2CO_3, **53**, EtOAc, H_2O (46–70%); c) Ph_3P, H_2O, THF, 50 °C, then Ac_2O, Et_3N, CH_2Cl_2 (67–76%); d) NaOH, MeOH (30–58%); e) TMS-OTf, Et_2O, 20 °C (76%); f) EDC, NHS, then BSA.

The optimal conditions were as follows. First, wells were incubated with 3% BSA to block nonspecific binding.[52] Next, wells were incubated with biotinylated-VVL-B4 at concentrations ranging from 3 to 2800 pM. Wells were washed with PBS and then incubated with 80 ng mL^{-1} of streptavidin-HRP for 1 h. To visualize binding and maximize sensitivity, the tyramide signal-amplification system was utilized.[53] Wells were incubated with 1 ng mL^{-1} of Cy5-labeled tyramide substrate (similar results were obtained with Cy3-labeled tyramide, data not shown) for 5 min, washed, and then dried. Slides were scanned by using a

GenePix microarray slide reader and analyzed by using GenePix Pro software to obtain relative fluorescent values (mean minus background) for each spot. An image of a representative well is shown in Figure 1. To detect antibody binding, goat anti-mouse Ig-HRP (2 μg mL^{-1}) was substituted for streptavidin-HRP.

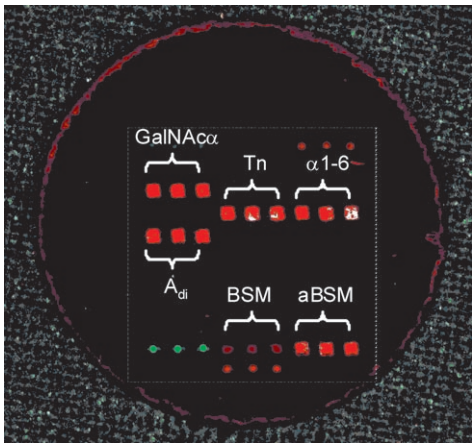


Figure 1. Assay results for a single well. An array containing 31 samples each spotted in triplicate was tested for VVL-B4 binding. First, the well was blocked with 3% BSA and then incubated with VVL-B4 (50 μL, 50 ng mL^{-1}) for 1 h. After being washed with PBS, the well was incubated with streptavidin-HRP (50 μL, 80 ng mL^{-1}) for 1 h. After being washed again with PBS, the well was incubated with Cy5-tyramide substrate (50 μL, 1 ng mL^{-1}) for 5 min. The well was washed with PBS, dried, and scanned by using a GenePix Microarray Scanner. A dashed line defines the borders of the printed area within the well. See Table 1 for array layout and abbreviations.

The results from our array and assay compare favorably with other arrays. One of the key issues is sensitivity. By using the above conditions, signal-to-noise ratios greater than 100 could be consistently obtained with high concentrations of lectin, and binding could easily be detected (signal-to-noise >5) with lectin concentrations as low as 15 pM. A second important consideration is reproducibility. Variation can arise from both the printing process and the assay. Under our conditions, spot-to-spot variation within a well and variation from well to well were both approximately 10%. With this level of consistency, twofold differences in binding can easily be detected. A third consideration is generality. We have successfully probed a wide range of lectins using this system (unpublished results). Given the price of slides and the wide range of conditions surveyed, printing and assay development would have been considerably more expensive with a single array per slide format.

Carbohydrate array analysis of VVL-B4, Bric111, and HBTn1

The first receptor to be evaluated was VVL-B4, a plant lectin isolated and characterized by Tollefsen.[19] It has been used for over 20 years to monitor the expression of the Tn antigen.[12,13,20,54–57] Based on 1) selective staining of carcinoma tissues, 2) excellent discrimination between the Tn antigen and the blood group A antigen, 3) a crystal structure, and 4) specificity studies with a limited number of glycopeptides and monosaccharides, VVL-B4 was thought to be highly selective for the Tn antigen.[19,58–62] Binding to carbohydrates on the array was evaluated by using the optimized conditions described above. The concentration of VVL-B4 was varied by carrying out a series of twofold dilutions. Graphs of the binding data are shown in Figure 2, and the minimum amount of VVL-B4 required to obtain a signal-to-noise ratio of at least 5 are listed in Table 2. In agreement with previous reports, VVL-B4 bound well to the Tn antigen (Tn–BSA and asialo-BSM) but not to blood group A.[63] However, strong signals were also observed for GalNAcα1-6Gal–BSA and GalNAcα1-3Gal–BSA; this demonstrates that the lectin does recognize other epitopes.

The next Tn receptor analyzed was Bric111, a monoclonal mouse IgG antibody raised against Tn erythrocytes in 1991.[64] Like VVL-B4, Bric111 selectively stains carcinoma tissues and is thought to be specific for the Tn antigen.[65–68] Binding to epitopes on the carbohydrate array was evaluated in a similar manner to that used for VVL-B4 (see Figure 2 and Table 2). However, goat anti-Ig-HRP was used as the secondary reagent rather than streptavidin-HRP. A wide range of epitopes such as Tn–BSA, GalNAcα1-6Gal–BSA, GalNAcα1-3Gal–BSA, blood group A, BSM, and asialo-BSM were all bound with similar affinity. The inability to discriminate between BSM and asialo-BSM is notable and consistent with previous reports that showed little difference between binding to glycophorin A and asialo-glycophorin A.[64] Based on this information, Bric111 does not appear to be a selective Tn receptor.

The third receptor analyzed was HBTn1, a monoclonal mouse IgM antibody raised against asialo-ovine submaxillary mucin (aOSM).[69] HBTn1 has been used extensively over the last ten years to monitor expression of the Tn antigen.[14,15,28,34,70–74] HBTn1 clearly demonstrated the best selectivity within our carbohydrate array (see Figure 2 and Table 2). Good binding to asialo-BSM was observed along with moderate binding to Tn–BSA. Unlike VVL-B4 and Bric111, little or no binding was observed to BSM.

Confirmation of the array results by ELISA/ELLA and SDS-PAGE

In cases in which positive signals were observed with the array, binding was also evaluated by standard ELISA/ELLA in a 96-well plate. Results were comparable (data not shown), but the array proved to be more sensitive. With biotinylated lectins, the array assay was approximately 10–20 times more sensitive; for antibodies, the array assay was about 2–4 times more sensitive. While giving similar results to an ELISA/ELLA, the array provided direct comparisons of binding to all carbohydrates under identical conditions and used significantly smaller amounts of carbohydrate samples and antibodies/lectins.

In samples of interest, such as patient tissue samples, Tn expression is typically detected by histochemical staining or staining of glycoprotein bands on a polyacrylamide gel. In these cases, one simply observes the presence or absence of staining and the intensity of staining. Therefore, the key issue is whether other epitopes bind well enough to produce a positive signal in the assay (e.g. withstand extensive washing). To examine this possibility, BSA conjugates were resolved by SDS-

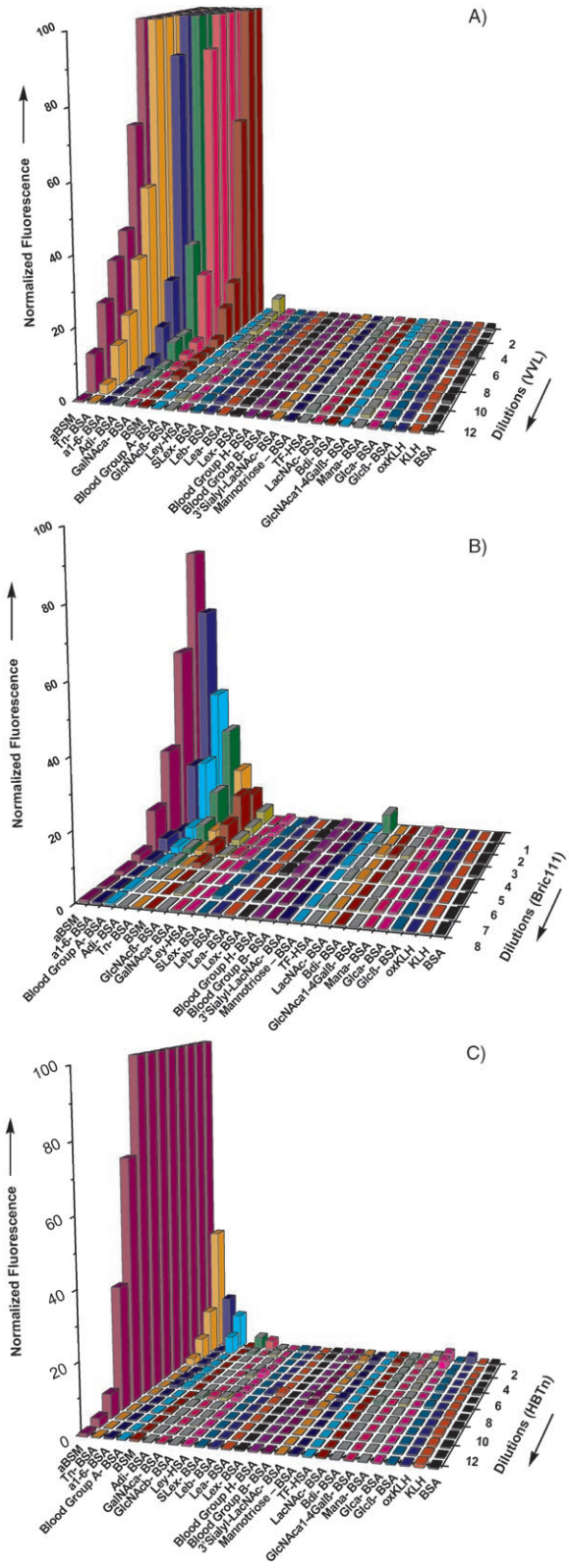


Figure 2. Carbohydrate-array binding profiles. For clarity, the epitopes are ordered from best (left) to worst (right). The fluorescence intensity was normalized by setting the saturation fluorescence at 100. The values at different dilutions were then taken as a percentage of saturation fluorescence. Normalized fluorescence intensities for each of the epitopes in the array for a series of dilutions of A) VVL, B) Bric111, and C) HBTn. Color codes for epitopes discussed in the text: red = aBSM, olive = Tn–BSA, green = α1-6–BSA, orange = A_{di}–BSA, and wine = blood group A, purple = BSM.

Table 2. Binding profiles for VVL-B_4, Bric111, and HBTn1.

Glycoconjugates	Detection limit [pM][a] [VVL]	[HBTn]	[Bric111]
GalNAc-α–BSA	120	6000	NB[b]
Tn–BSA	29	810	3000
α1-6–BSA	120	6000	2000
A_{di}–BSA	120	3000	3000
blood group A–BSA	NB[b]	3000	2000
BSM	230	NB[b]	2000
aBSM	15	8.1	830

[a] The lowest receptor concentration to give a signal to noise ratio of at least 5:1. [b] no binding at the highest concentration tested: VVL = 3 nM (0.4 μg mL^{-1}), HBTn = 6 nM (1:50 dilution), and Bric111 = 13 nM (1:50 dilution).

PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and then probed with Tn receptors (see Figure 3). With VVL-B4, strong positive signals/bands were observed for GalNAcα1-6Gal–BSA and GalNAcα1-3Gal–BSA. With Bric111, positive signals could be detected for blood group A, GalNAcα1-6Gal–BSA, and GalNAcα1-3Gal–BSA. Even HBTn1, which showed the best selectivity within our panel of carbohydrate epitopes, could produce a weak positive signal with blood group A. These results are consistent with the results of the carbohydrate-array assay. Moreover, the results show that a positive signal with these receptors is not a reliable measure of Tn expression; the signal could be due to expression of the Tn antigen, expression of a cross-reacting epitope, or expression of a combination of epitopes.[75]

Discussion

The Tn antigen is a tumor-associated carbohydrate antigen that has been studied extensively over the last 40 years. In addition to understanding the biological role(s) of Tn, there has been considerable effort to develop Tn-based diagnostics and vaccines for cancer. Naturally, accurate and reliable information on the expression of the Tn antigen is critical for these studies. The vast majority of expression information has been obtained by probing the binding of anti-Tn antibodies and lectins. Since this approach is an indirect measure of Tn expression, selective recognition is crucial. However, selective recognition of carbohydrate epitopes is challenging, especially for small epitopes such as the Tn antigen. Moreover, conflicting reports regarding Tn expression are not uncommon. Therefore, we began to question whether anti-Tn antibodies and lectins were providing accurate information.

The primary concern was whether other carbohydrate epitopes could be recognized and mistaken for the Tn antigen by antibodies and lectins. Unfortunately, this possibility could not be ruled out based on existing information on specificity. The anti-Tn antibodies and lectins that have become standard tools were chosen primarily for their ability to selectively recognize cancer cells/tissue over normal cells/tissues. Cells and tissues, however, express large mixtures of carbohydrate epitopes. Moreover, many epitopes have not yet been characterized. Therefore, it can be extremely difficult to determine which car-

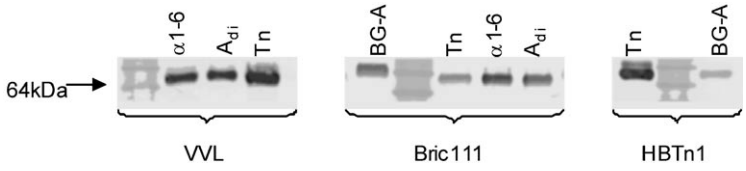


Figure 3. SDS-PAGE/blot binding analysis. SDS-PAGE was carried out in 10% TG polyacrylamide gels. For VVL-B_4, 50 ng of BSA conjugate were loaded into each lane; for Bric111 and HBTn1, 500 ng of BSA-conjugate were loaded into each lane. Proteins were electrophoretically transferred to PVDF membranes, blocked with 5% BSA/PBS, and then incubated with biotinylated VVL-B4 (2 $\mu g\,mL^{-1}$), Bric111 (diluted 1:500, 2 $\mu g\,mL^{-1}$), or HBTn1 (diluted 1:500, 1 $\mu g\,mL^{-1}$) in 3% BSA/PBS followed by streptavidin–alkaline phosphatase (diluted 1:2000, for blots with VVL-B4) or goat *anti*-mouse Ig–alkaline phosphatase (diluted 1:1000, for blots with Bric111 or HBTn1) in 3% BSA/PBS for 1 h. Blots were washed and then incubated with BCIP/NBT (1 $mg\,mL^{-1}$) for 20 min. See Table 1 for abbreviations.

bohydrate structure or structures are responsible for a positive signal. In addition to tissue staining, homogeneous glycopeptides, monosaccharides, and/or oligosaccharides have also been examined. While this approach provides information on specific structures, there an enormous number of carbohydrate epitopes are expressed in humans. However, only a tiny fraction of them have ever been tested for binding, primarily due to difficulties in obtaining homogeneous carbohydrates. Moreover, the specific epitopes examined were generally chosen because they were accessible rather than for more relevant reasons such as structural similarity.

The design and development of a carbohydrate microarray and assay are described in this paper. The array contained a variety of carbohydrates, such as tumor antigens, blood group antigens, and Lewis antigens, as well as a number of Tn-related epitopes. Results from the array were consistent with other biochemical assays such as ELISA/ELLA and SDS-PAGE, thus indicating that the carbohydrate epitopes are accessible for binding and presented in a comparable manner. Due to the high sensitivity of the assay and microarray format, only tiny amounts of each carbohydrate epitope and the lectin/antibody-containing samples were required.

The array was utilized to determine if widely used Tn receptors, such as HBTn1, Bric111, and VVL-B4, can bind other carbohydrate epitopes. Consistent with previous results, all three showed excellent selectivity for the Tn antigen over epitopes that do not contain a terminal GalNAc. However, carbohydrate array analysis as well as ELISA and Western blot analysis, did reveal cross-reactivity with several other carbohydrate epitopes, such as GalNAcα1-3Gal, GalNAcα1-6Gal, and blood group A. Like the Tn antigen, each of these epitopes has a terminal GalNAc residue with an α linkage. However, these proteins cannot simply be described as GalNAc-binding receptors. For example, VVL-B4 did not bind at all to the blood group A antigen. It is important to note that each of the cross-reactive carbohydrate epitopes has been isolated from human sources. The blood group A antigen is expressed in many locations in individuals with blood type A. GalNAcα1-3Gal and GalNAcα1-6Gal are expressed on mucins of the human colon,[76] but very little is known about these epitopes. Since all three epitopes are expressed in humans, each could contribute to the overall signal in a histochemical stain or blot for a tissue sample of interest. *In fact, a strong positive signal could be produced in the complete absence of Tn expression.* Therefore, conclusions based on binding data alone may be entirely inaccurate. Promiscuous binding might be an especially important consideration when weak-to-moderate signals are observed.

Differences in specificity and "off target" binding revealed by carbohydrate-array analysis can provide reasonable explanations for inconsistent information on Tn expression in the literature. For example, previous reports that the Tn antigen is expressed in normal pancreatic acinar cells utilized the VVL-B4 lectin to detect Tn expression.[21,22] Alternatively, evidence that the Tn antigen is not expressed in normal pancreatic acinar cells was obtained by using two monoclonal antibodies (TEC-02 and 12A8-C7-F5) to detect Tn expression.[23] Promiscuous binding by VVL-B4 could account for the discrepancy. If, for example, the GalNAcα1-6Gal epitope were expressed on these cells, the VVL-B4 lectin could stain positive while the antibodies stain negative.

The results with the carbohydrate array also have important implications for developing Tn-based diagnostics and vaccines. Correlations between Tn expression and cancer stage, aggressiveness, and prognosis are frequently studied. Due to conflicting reports, it is not clear whether the Tn antigen is expressed at an early or late stage of disease development. However, this information is vital for diagnostic development. Interestingly, many of the reports that concluded that the Tn antigen is expressed at an early stage utilized the VVL-B4 lectin for Tn detection; none used HBTn1. In contrast, reports that the Tn antigen is expressed at a late stage primarily used HBTn1. Therefore, differences in specificity could provide a reasonable explanation for these inconsistencies. Moreover, the correlation suggests that a cross-reactive epitope, rather than the Tn antigen, is being detected by VVL-B4 at an early stage of disease development. If so, the cross-reactive epitope(s) might prove more useful for early detection and diagnosis of cancer. In any event, differences in specificity and off-target binding should be considered when developing diagnostics. Accurate information is also critical for the proper implementation of Tn-based vaccines, since they are likely to be more effective in patients with Tn-positive tumors.

Finally, the carbohydrate microarray described in this paper could be useful for a wide range of other applications. The high sensitivity of the assay should permit detection of even trace amounts of carbohydrate-binding toxins, pathogens, and proteins. In addition, the unique multiwell format is well-suited to analysis of hundreds to thousands of clinical samples in an economical fashion. As such, the array should be especially advantageous for the development of diagnostics and vaccines.

Experimental Section

Neoglycoconjugates and glycoproteins for the array: Blood group A–BSA, blood group B–BSA, blood group H–BSA, Le^x–BSA, Le^a–BSA, Le^b–BSA, Le^y–HSA, SLe^x–BSA, Galβ1-4GlcNAc–BSA, Galα1-3Gal–BSA, mannotriose–BSA, and Siaα2-3Galβ1-4GlcNAc–BSA were

purchased from Dextra Labs (Reading, UK). Galβ1-3GalNAc-HAS was purchased from Isosep (Tullinge, Sweden). KLH (keyhole limpet hemocyanin), BSA, and BSM were purchased from Sigma. Cy3- and Cy5-labeled BSA were used as positive controls, while unmodified BSA was used as the negative control.

General synthetic methods. Dimethylformamide (DMF), *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC), and NHS were purchased from Aldrich. PyBOP was purchased from EMD Bioscience (San Diego, CA, USA). NMR spectra were recorded on a Unity Inova 400 Fourier transform NMR spectrometer. Proton chemical shifts are reported in parts per million downfield from tetramethylsilane (TMS) unless otherwise noted. Carbon chemical shifts are reported in parts per million downfield from TMS with $CDCl_3$ as an internal reference unless otherwise noted.

Synthesis of the Tn antigen (11). Compound **30** (100 mg, 0.149 mmol)[77,78] was dissolved in DMF (1 mL), and *N*-methylmorpholine (50 μL, 0.448 mmol) and PyBOP (94 mg, 0.180 mmol) were added. After 5 min, linker **31** (35 mg, 0.173)[79] was dissolved in DMF (0.5 mL) and then added to the reaction mixture. The solution was stirred for about 3 h, then diluted in H_2O and extracted with ethyl acetate. The organic phase was then washed with HCl (1 M), sat. $NaHCO_3$, and brine. The aqueous phases were re-extracted with ethyl acetate. The organic phases were combined, dried over Na_2SO_4, decanted, and concentrated in vacuo. The product was purified by flash chromatography (50% acetone/dichloromethane) to afford **32** (125 mg, 96% yield). $R_f = 0.30$ (50% acetone/dichloromethane); ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 7.80$ (d, $J = 7.6$ Hz, 2H), 7.68 (m, 2H), 7.40 (app t, $J = 7.4$ Hz, 2H), 7.32 (m, 2H), 5.41 (d, $J =$ 2.8 Hz, 1H; GalNAc H4), 5.12 (dd, $J = 2.8$, 11.4 Hz, 1H; GalNAc H3), 5.00 (d, $J = 3.6$ Hz, 1H; GalNAc H1), 4.63 (dd, $J = 6.3$, 10.6 Hz, 1H), 4.44 (dd, $J = 6.3$, 10.6 Hz, 1H), 4.39 (dd, $J = 3.6$, 11.4 Hz, 1H; GalNAc H2), 4.33 (app t, $J = 6.5$ Hz, 2H), 4.24 (m, 2H), 4.10 (m, 2H), 3.89 (d, $J = 16.4$ Hz, 1H; Gly), 3.79 (d, $J = 16.4$ Hz, 1H; Gly), 3.15 (m, 2H), 2.28 (t, $J = 7.4$ Hz, 2H), 2.13 (s, 3H), 2.05 (s, 3H), 1.94 (s, 3H), 1.91 (s, 3H), 1.58 (m, 2H), 1.46 (m, 2H), 1.30 (m, 2H), 1.23 (d, $J = 6.3$ Hz, 3H); ^{13}C NMR (CD_3OD, 100 MHz): $\delta =$ 175.8, 173.6, 172.8, 172.2, 172.1, 172.0, 171.1, 159.1, 145.4, 145.2, 142.8, 128.9, 128.3, 126.3, 126.1, 121.3, 121.1, 100.7, 78.0, 70.1, 68.9, 68.4, 67.9, 63.3, 60.7, 52.1, 49.0, 43.2, 40.3, 34.7, 30.0, 27.5, 25.7, 23.0, 20.8, 20.7, 20.6, 18.9; HRFAB-MS calcd for $C_{42}H_{55}N_4O_{15}$: 855.3664 $[M+H]^+$; found 855.3679.

The Fmoc group was removed, and the free amine was acetylated. Briefly, **32** (125 mg, 0.146) was dissolved in dichloromethane (3 mL), and pyrollidine (200 μL) was added. After 2 h, toluene (10 mL) was added, and the solvent was removed in vacuo. The crude amine was dissolved in dichloromethane (3 mL), and pyridine (71 μL, 0.88 mmol) and acetic anhydride (41 μL, 0.44 mmol) were added. After 2 h, toluene (10 mL) was added, and the solvent was removed in vacuo. The product was purified by flash chromatography (75% acetone/dichloromethane + 0.5% acetic acid) to afford **33** (75 mg, 76% yield). $R_f = 0.35$ (75% acetone/dichloromethane + 0.5% acetic acid); ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 5.41$ (br d, $J = 2.0$ Hz, 1H; GalNAc H4), 5.18 (dd, $J = 3.3$, 11.3 Hz, 1H; GalNAc H3), 5.05 (d, $J = 3.7$ Hz, 1H; GalNAc H1), 4.56 (d, $J = 2.5$ Hz, 1H), 4.38 (m, 3H), 4.10 (m, 2H; GalNAc H6R + S), 3.88 (d, $J =$ 16.7 Hz, 1H; Gly), 3.82 (d, $J = 16.7$ Hz, 1H; Gly), 3.65 (s, 3H), 3.17 (t, $J = 7.2$ Hz, 2H), 2.34 (t, $J = 7.4$ Hz, 2H), 2.15 (s, 3H), 2.11 (s, 3H), 2.02 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H), 1.63 (m, 2H), 1.50 (m, 2H), 1.35 (m, 2H), 1.29 (d, $J = 6.5$ Hz, 3H); HRFAB-MS calcd for $C_{29}H_{47}N_4O_{14}$: 675.3089 $[M+H]^+$; found 675.3100.

Next, the *O*-acetyl groups and methyl ester were deprotected. Briefly, **33** (24 mg, 0.036 mmol) was dissolved in methanol (0.5 mL), and NaOH (aq) (1 M, 200 μL) was added. After 2 h, Amberlyst acidic resin was added to neutralize the reaction, the reaction mixture was filtered, and the filtrate was concentrated in vacuo. The product was purified by flash chromatography (10%, then 15%, then 20% methanol/dichloromethane + 0.5% acetic acid) on diol-derivatized silica (Silicycle) to afford **34** (11 mg, 58% yield). $R_f = 0.20$ (30% methanol/dichloromethane + 0.5% acetic acid, TLC on silica plates); ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 4.89$ (d, $J = 3.9$ Hz, 1H; GalNAc H1), 4.48 (d, $J = 2.7$ Hz, 1H; Thr Hα), 4.24 (dq, $J = 2.7$, 6.3 Hz, 1H; Thr Hβ), 4.19 (dd, $J = 3.9$, 11.0 Hz, 1, 1H; GalNAc H2), 3.88 (br dd, $J = 5.9$, 6.3 Hz, 1H; GalNAc H5), 3.84 (br d, $J = 3.2$ Hz, 1H; GalNAc H4), 3.82 (s, 2H; Gly Hα), 3.74 (dd, $J = 3.2$, 11.0 Hz, 1H; GalNAc H3), 3.67 (m, 2H; GalNAc H6); 3.16 (m, 2H), 2.25 (t, $J =$ 7.3 Hz, 2H), 2.06 (s, 3H), 1.98 (s, 3H), 1.58 (tt, $J = 7.3$, 7.5 Hz, 2H), 1.49 (tt, $J = 7.3$, 7.5 Hz, 2H), 1.33 (m, 3H), 1.25 (d, $J = 6.3$ Hz, 3H; Thr Hγ); ^{13}C NMR (CD_3OD, 100 MHz): $\delta =$ 174.3, 174.0, 172.8, 171.2, 100.7, 77.1, 73.0, 70.4, 70.3, 62.8, 58.8, 51.7, 43.4, 40.4, 35.1, 30.1, 27.5, 25.8, 23.1, 22.6, 19.0; HRFAB-MS calcd for $C_{22}H_{39}N_4O_{11}$: 535.2615 $[M+H]^+$; found 535.2610.

Finally, compound **34** was coupled to BSA. Briefly, **34** was dissolved in DMF/H_2O (50:50) to a final concentration of 150 mM. EDC was dissolved in DMF/H_2O (50:50) to a final concentration of 300 mM. NHS was dissolved in DMF to a final concentration of 300 mM. The NHS ester was preformed by combining the carbohydrate, EDC, and NHS in an Eppendorf tube at a ratio of 2:1:1 and allowing the mixture to stand at room temperature with occasional gentle mixing. After 50 min, the reaction mixture was added to a solution of BSA in aqueous borate buffer (10 mM sodium borate, 90 mM NaCl, pH 8.0) that had been precooled to 4 °C to give a final carbohydrate/BSA ratio of approximately 45:1. After 15 min, the solution was warmed to room temperature, then allowed to stand for 1 h. BSA-conjugate **11** was then dialyzed extensively against water (SpectraPor 7, MWCO = 10000). Analysis by MALDI-TOF MS gave an average mass for BSA of 66431 and an average mass for Tn–BSA (**11**) of 77365; these correspond to an average of 21 epitopes per BSA (each epitope adds 516 to the molecular weight).

Synthesis of GlcNAcα1–4Gal–BSA (14). Phenyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (2.39 g, 5.3 mmol)[80] was dissolved in acetic acid (80%), and the mixture was heated at 70 °C for 3 h. The solvent was evaporated, and the residue was azeotroped with toluene. The residue was then dissolved in dichloromethane (50 mL) and cooled to −20 °C. Triethylamine (5 mL) was added followed by benzoyl chloride (600 μL, 1 equiv). The solution was kept at −20 °C for 1.5 h, then diluted with dichloromethane. The solution was washed with 1 N HCl, sat. $NaHCO_3$, and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by column chromatography (ethyl acetate/hexanes 1:1) to give phenyl 2,3-di-*O*-acetyl-6-*O*-benzoyl-1-thio-β-D-galactopyranoside **36** as white powder (1.77 g, 72% yield). $R_f = 0.2$ (50% ethyl acetate/hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 8.04–8.02 (m, 2H), 7.62–7.58 (m, 1H), 7.51–7.44 (m, 4H), 7.26–7.16 (m, 3H), 5.32 (t, $J = 10.0$ Hz, 1H), 5.04 (dd, $J = 10.0$, 3.2 Hz, 1H), 4.74 (d, $J = 10.0$ Hz, 1H), 4.65–4.53 (m, 2H), 4.16 (d, $J =$ 3.2 Hz, 1H), 3.98–3.95 (m, 1H), 2.098 (s, 3H), 2.096 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 170.2, 169.8, 166.5, 133.6, 132.9, 132.4, 130.0, 129.7, 129.1, 128.7, 128.2, 86.8, 76.3, 74.4, 67.73, 67.70, 63.3, 21.04, 21.02.

A mixture of glycosyl chloride **35** (467 mg, 1.33 mmol, 1.2 equiv).[81] acceptor **36** (520 mg, 1.11 mmol, 1 equiv), and di-*tert*-butylmethylpyridine (273 mg, 1.33 mmol, 1.2 equiv) was dried under vacuum for 1 h, then dissolved in dichloromethane. Molecular sieves were added, and the mixture was stirred and cooled to −78 °C. AgOTf

(570 mg, 2.22 mmol, 2 equiv) was added to the reaction mixture, and the mixture was allowed to warm to 0 °C over 2.5 h. The reaction was quenched with $NaHCO_3$, and the mixture was filtered to remove the molecular sieves. The filtrate was extracted with dichloromethane (3×). The organic layers were combined and washed with brine. The solvent was removed, and the residue was purified by column chromatography to give phenyl (3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-1-thio-β-D-galactopyranoside (**37**) as a white solid (750 mg, 86% yield). R_f = 0.50 (ethyl acetate/hexanes 1:1); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.04 (m, 2H), 7.60 (m, 1H), 7.54 (m, 2H), 7.46 (t, *J* = 8.0 Hz, 2H), 7.32 (m, 1H), 7.24 (m, 1H), 7.17 (m, 1H), 5.34 (dd, *J* = 10.4, 9.6 Hz, 1H), 5.13 (t, *J* = 10.0 Hz, 1H), 5.06 (dd, *J* = 10.4, 9.2 Hz, 1H), 4.92 (dd, *J* = 10.4, 2.8 Hz, 1H), 4.81 (d, *J* = 3.6 Hz, 1H), 4,74 (dd, *J* = 11.2, 6.4 Hz, 1H), 4.72 (d, *J* = 9.6 Hz, 1H), 4.63 (dd, *J* = 11.2, 6.4 Hz, 1H), 4.29–4.24 (m, 2H), 4.09 (dd, *J* = 12.8, 2.4 Hz, 1H), 4.05, (t, *J* = 11.6 Hz, 1H), 3.95 (m, 1H), 3.68 (dd, *J* = 10.4, 3.6 Hz, 1H), 2.11 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 170.5, 169.8, 169.7, 169.0, 166.0, 133.4, 133.3, 131.0, 129.6, 129.0, 128.9, 128.5, 128.4, 128.2, 125.3, 99.9, 85.5, 78.0, 76.1, 74.1, 71.9, 68.4, 68.0, 66.7, 62.8, 62.6, 61.0, 21.0, 20.7, 20.7; HRMS, calcd for $C_{35}H_{39}N_3O_{15}NaS$: 796.1994 $[M+Na]^+$; found: 796.2030.

Disaccharide **37** (391 mg, 0.5 mmol, 1 equiv) was dissolved in dichloromethane and cooled to 0 °C. A solution of IBr (1 M) in DCM (0.55 mL, 1.1 equiv) was added to the mixture dropwise. The reaction was kept at 0 °C for 30 min and then quenched with 10% aqueous $Na_2S_2O_3$. The crude product was purified by column chromatography (30% ethyl acetate in hexanes) to give (3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-α-D-galactopyranosyl bromide as a white solid (253 mg, 67% yield). R_f = 0.6, (ethyl acetate/hexanes 1:1); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.03 (m, 2H), 7.60 (m, 1H), 7.47 (t, *J* = 8.0 Hz, 2H), 6.74 (d, *J* = 4.0 Hz, 1H), 5.47 (dd, *J* = 10.4, 9.2 Hz, 1H), 5.35 (dd, *J* = 10.8, 2.4 Hz, 1H), 5.16 (dd, *J* = 11.2, 4.0 Hz, 1H), 5.10 (t, *J* = 9.2 Hz, 1H), 4.94 (d, *J* = 4.4 Hz, 1H), 4.71 (m, 1H), 4.56 (m, 2H), 4.41 (d, *J* = 2.8 Hz, 1H), 4.34–4.26 (m, 2H), 4.10 (m, 1H), 3.65 (dd, *J* = 10.8, 3.6 Hz, 1H), 2.13, 2.12, 2.10, 2.07, 2.06(5 s, 3H each); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 170.5, 170.1, 169.8, 169.7, 169.6, 165.8, 133.6, 129.9, 129.5, 128.7, 99.7, 88.9, 76.6, 72.9, 71.4, 68.7, 68.4, 67.8, 62.2, 61.9, 61.5, 21.2, 20.94, 20.86, 20.82; HRMS calcd for $C_{29}H_{34}N_3O_{15}NaBr$: 766.1066 $[M+Na]^+$; found: 766.1073.

Linker **53** (50 mg, 0.24 mmol, 3 equiv)[82] was dissolved in 10% aqueous Na_2CO_3 (0.5 mL), and tetrabutylammonium hydrogensulfate (27 mg, 0.08 mmol, 1 equiv) was added. A solution of glycosyl bromide (60 mg, 0.08 mmol, 1 equiv) in ethyl acetate (0.5 mL) was added to this mixture. The mixture was stirred at room temperature until all glycosyl bromide was gone, as judged by TLC. The mixture was extracted by ethyl acetate, and the crude product was purified by column chromatography (50% ethyl acetate in hexanes) to give methyl *N*-{2-[(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosylsulfanyl]ethyl}glutamate **38** as a white solid (51 mg, 73% yield). R_f = 0.5 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.99 (m, 2H), 7.58 (m, 1H), 7.45 (t, *J* = 8.0 Hz, 2H), 6.43 (t, *J* = 4.2 Hz, 1H), 5.45 (dd, *J* = 10.4, 9.6 Hz, 1H), 5.34 (t, *J* = 10.0 Hz, 1H), 5.12 (t, *J* = 9.3 Hz, 1H), 4.79 (dd, *J* = 10.4, 2.8 Hz, 1H), 4.91 (d, *J* = 3.6 Hz, 1H), 4.62–4.65 (m, 2H), 4.50 (d, *J* = 10.0 Hz, 1H), 4.31–4.28 (m, 2H), 4.25 (d, *J* = 2.4 Hz, 1H), 4.14 (m, 1H), 4.01 (t, *J* = 6.4 Hz, 1H), 3.70 (dd, *J* = 10.4, 3.2 Hz, 1H), 3.64 (s, 3H), 3.62–3.54 (m, 1H), 3.43–3.34 (m, 1H), 2.92–2.84 (m, 1H), 2.80–2.72 (m, 1H), 2.36 (t, *J* = 7.2 Hz, 2H), 2.24 (t, *J* = 7.2 Hz, 2H), 2.10, 2.08, 2.07, 2.06, 2.05 (5 s, 3H each), 1,94 (pent, *J* = 7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 173.8, 172.5, 170.7, 170.5, 170.4, 170.0, 169.8, 166.1, 133.7, 129.8, 129.5, 128.8, 99.8, 83.4, 77.4, 76.4, 73.8, 71.6, 68.9, 68.2, 66.8, 62.6, 62.4, 61.4, 51.7, 39.9, 35.4, 33.4, 29.9, 29.1, 21.2, 21.0, 20.93, 20.86; HRMS calcd for $C_{37}H_{48}N_4O_{18}NaS$: 891.2577 $[M+Na]^+$; found: 891.2576.

A mixture of compound **38** (51 mg, 0.059 mmol, 1 equiv) and Ph_3P (31 mg, 0.12 mmol, 2 equiv) in THF (2 mL, contains water) was stirred at 50 °C for 7 h. The solvent was then evaporated, and the residue was azeotroped with toluene. The residue was dissolved in dichloromethane (1 mL), and acetic anhydride (8 μL, 3 equiv) and triethyl amine (24 μL, 4 equiv) were added. The mixture was stirred at room temperature overnight. The reaction was quenched with 1 N HCl, and the crude product was purified by column chromatography (70% ethyl acetate in hexanes) to give methyl *N*-{2-[(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosylsulfanyl]ethyl}glutamate **39** as white solid (37 mg, 73%). R_f = 0.50 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.98 (m, 2H), 7.58 (m, 1H), 7.45 (t, *J* = 8.0 Hz, 2H), 7.09 (d, *J* = 8.4 Hz, 1H), 6.16 (m, 1H), 5.39 (t, *J* = 10.0 Hz, 1H), 5.31 (dd, *J* = 11.2, 9.2 Hz, 1H), 5.19 (t, *J* = 9.6 Hz, 1H), 5.05–5.01 (m, 2H), 4.73 (dd, *J* = 11.2, 6.8 Hz, 1H), 4.50 (d, *J* = 9.6 Hz, 1H), 4.43–4.37 (m, 1H), 4.32–4.22 (m, 2H), 4.18 (d, *J* = 2.4 Hz, 1H), 4.10 (dd, *J* = 13.2, 3.2 Hz, 1H), 4.01 (t, *J* = 7.8 Hz, 1H), 3.65 (s, 3H), 3.60–3.48 (m, 2H), 3.10–3.02 (m, 1H), 2.70–2.62 (m, 1H), 2.34 (t, *J* = 7.6 Hz, 2H), 2.25- 2.21 (m, 2H), 2.08 (s, 9H), 2.06 (s, 3H), 2.03 (s, 6H), 1.91 (pent, *J* = 7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 173.7, 172.5, 171.4, 171.1, 170.9, 170.1, 169.9, 169.7, 166.1, 133.7, 129.9, 129.2, 128.8, 99.9, 83.1, 76.6, 76.2, 73.6, 70.4, 68.5, 68.4, 66.9, 61.9, 61.5, 52.6, 51.8, 40.0, 35.5, 33.2, 28.8, 22.9, 21.2, 20.94, 20.92, 20.87; HRMS: calcd for $C_{39}H_{52}N_2O_{19}NaS$ 907.2777; found: 907.2729.

A mixture of compound **39** (43 mg, 0.05 mmol, 1 equiv) and NaOMe (cat. amount) in MeOH (2 mL) was stirred at room temperature for 2 h until TLC showed that all starting materials had been consumed. The reaction mixture was neutralized by ion-exchange resin (Amberlite 15), then filtered, and the filtrate was concentrated. The residue was dissolved in water/MeCN (1:1) and extracted with hexanes until all methyl benzoate was removed. The aqueous phase was concentrated, the residue was dissolved in 1 M NaOH (1 mL), and the mixture was stirred at room temperature overnight. The solution was neutralized by ion-exchange resin (Amberlite 15) and filtered. The filtrate was concentrated and purified by reversed-phase HPLC or reversed-phase column chromatography to give *N*-{2-[(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-β-D-galactopyranosylsulfanyl]ethyl}glutaric acid **40** as a white powder (11 mg, 41% yield): ^{1}H NMR (CD_3OD, 400 MHz): δ = 4.92 (d, *J* = 3.6 Hz, 1H), 4.46–4.40 (m, 1H), 4.21–4.16 (m, 1H), 4.00 (s, 1H), 3.93 (dd, *J* = 10.8, 3.6 Hz, 1H), 3.82 (dd, *J* = 11.6, 2.4 Hz, 1H), 3.76–3.38 (m, 10H), 2.96–2.88 (m, 1H), 2.80–2.72 (m, 1H), 2.35 (t, *J* = 7.6 Hz, 2H), 2.27 (t, *J* = 7.6 Hz, 2H), 2.04 (s, 3H), 1.90 (pent, *J* = 7.6 Hz, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ = 182.0, 176.2, 173.8, 100.6, 87.9, 80.8, 79.3, 76.1, 74.0, 72.3, 72.2, 71.5, 62.5, 61.4, 55.9, 41.3, 38.3, 37.0, 30.5, 24.1, 22.8; HRMS: calcd for $C_{21}H_{36}N_2O_{13}NaS$ 579.1830; found: 579.1817.

Compound **40** was coupled to BSA (following the procedure for conjugate **1**) to produce GlcNAc α1–4Gal–BSA (**14**). Analysis by MALDI-TOF MS gave an average mass of 77171, corresponding to an average of 20 epitopes per BSA (each epitope adds 536 to the molecular weight).

GalNAcα1–6Gal (12). A mixture of donor **41** (95 mg, 0.2 mmol, 1 equiv)[83] and acceptor **42** (117 mg, 0.2 mmol, 1 equiv)[84] was dried under vacuum for 1 h and then dissolved in diethyl ether. Molecular sieves were added, and the solution was cooled to

−20 °C. TMS-OTf (0.02 mmol, 0.1 equiv) was added, and the mixture was stirred at −20 °C for 0.5 h. The reaction was quenched by adding $NaHCO_3$. The crude product was purified by column chromatography (ethyl acetate/hexanes 1:1) to give phenyl (3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-1-thio-β-D-galactopyranoside **43** as white solid (136 mg, 76% yield). R_f = 0.3 (ethyl acetate/hexane 1:2); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.99–7.96 (m, 2H), 7.93–7.90 (m, 2H), 7.76–7.74 (m, 2H), 7.64–7.60 (m, 1H), 7.56–7.51 (m, 3H), 7.49–7.44 (m, 2H), 7.43–7.37 (m, 6H), 7.22 (t, *J* = 8.4 Hz, 2H), 5.93 (d, *J* = 2.8 Hz, 1H), 5.78 (t, *J* = 10.0 Hz, 1H), 5.60 (dd, *J* = 8.8, 3.2 Hz, 1H), 5.38 (dd, *J* = 3.2, 0.8 Hz, 1H), 5.35 (dd, *J* = 11.2, 3.6 Hz, 1H), 5.13 (d, *J* = 10.0 Hz, 1H), 4.95 (d, *J* = 3.2 Hz, 1H), 4.32–4.27 (m, 2H), 4.04 (d, *J* = 7.2 Hz, 2H), 3.95 (dd, *J* = 10.4, 7.2 Hz, 1H), 3.73 (dd, *J* = 10.4, 4.0 Hz, 1H), 3.67 (dd, *J* = 11.2, 3.6 Hz, 1H), 2.13, 2.08, 1.95 (3 s, 3H each); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 170.3, 170.0, 169.6, 165.4, 165.4, 165.2, 133.6, 133.4, 133.2, 132.9, 131.9, 130.0, 129.8, 129.7, 129.2, 129.0, 128.8, 128.7, 128.6, 128.4, 128.2, 98.1, 85.4, 76.4, 73.0, 68.9, 68.2, 67.8, 67.7, 67.5, 66.9,61.6, 57.5, 20.7, 20.59, 20.58; HRMS: calcd for $C_{45}H_{43}N_3O_{15}NaS$: 920.2307 [*M*+Na]$^+$; found: 920.2294.

Compound **43** was treated with IBr (see conversion of **37** to **38**) to produce (3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-(1→6)-2,3,4-*O*-benzoyl-α-D-galactopyranosyl bromide (85% yield): R_f = 0.6, (50% ethyl acetate/hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.09–8.06 (m, 2H), 8.02–8.00 (m, 2H), 7.80–7.76 (m, 2H), 7.66–7.63 (m, 1H), 7.54–7.49 (m, 3H), 7.46–7.38 (m, 3H), 7.27–7.23 (m, 2H), 6.96 (d, *J* = 4.0 Hz, 1H), 6.05–6.01 (m, 2H), 5.68–5.63 (m, 1H), 5.47 (d, *J* = 2.4 Hz, 1H), 5.33 (dd, *J* = 11.2, 3.2 Hz, 1H), 4.95 (d, *J* = 3.6 Hz, 1H), 4.82–4.79 (m, 1H), 4.28 (t, *J* = 7.2 Hz, 1H), 4.13–4.07 (m, 2H), 3.95–3.90 (m, 1H), 3.82–3.79 (m, 1H), 3.68 (dd, *J* = 11.2, 3.6 Hz, 1H), 2.13, 2.05, 2.01 (3 s, 3H each); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 170.6, 170.2, 169.8, 165.8, 165.5, 165.4, 134.03, 133.97, 133.5, 130.2, 130.1, 129.9, 129.00, 128.96, 128.7, 128.5, 98.4, 88.2, 72.8, 69.1, 68.8, 68.7, 68.4, 67.7, 67.2, 66.9, 61.8, 57.5, 20.83, 20.79; HRMS, calcd for $C_{39}H_{38}N_3O_{15}NaBr$: 890.1379 [*M*+Na]$^+$; found: 890.1357.

Coupling with linker **53** (see conversion of **37** to **38**) produced methyl *N*-{2-[(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-β-D-galactopyranosylsulfanyl]ethyl}-glutamate **44** (70% yield). R_f = 0.4 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.07–8.05 (m, 2H), 7.97–7.94 (m, 2H), 7.77–7.74 (m, 2H), 7.65–7.63 (m, 1H), 7.54–7.48 (m, 3H), 7.43–7.37 (m, 3H), 7.27–7.21 (m, 2H), 6.37–6.34 (m, 1H), 5.92 (d, *J* = 3.2 Hz, 1H), 5.79 (t, *J* = 9.6 Hz, 1H), 5.62 (dd, *J* = 9.6, 3.2 Hz, 1H), 5.43 (dd, *J* = 3.2, 1.2 Hz, 1H), 5.36 (dd, *J* = 11.2, 3.2 Hz, 1H), 4.99 (d, *J* = 3.6 Hz, 1H), 4.92 (d, *J* = 10.0 Hz, 1H), 4.33–4.25 (m, 2H), 4.12–4.02 (m, 2H), 3.92 (dd, *J* = 10.8, 8.0 Hz, 1H), 3.71–3.65 (m, 2H), 3.64 (s, 3H), 3.58–3.48 (m, 2H), 3.04–2.97 (m, 1H), 2.89–2.82 (m, 1H), 2.34 (t, *J* = 7.2 Hz, 2H), 2.21 (t, *J* = 7.2 Hz, 2H), 2.12, 2.07, 1.92 (3 s, 3H each), 1.94 (pent, *J* = 7.2 Hz, 2H).^{13}C NMR δ 173.7, 172.5, 170.6, 170.3, 170.1, 165.7, 165.6, 165.5, 133.9, 133.6, 133.5, 130.1, 129.9, 129.8, 129.1, 129.0, 128.9, 128.8, 128.6, 128.4, 98.2, 84.5, 76.7, 72.7, 69.1, 68.5, 68.3, 67.6, 67.5, 67.2, 67.7, 57.5, 51.6, 39.5, 35.3, 33.3, 30.6, 20.9, 20.8, 20.7, 20.6; HRMS, calcd for $C_{47}H_{52}N_4O_{18}NaS$: 1015.2890 [*M*+Na]$^+$; found: 1015.2839.

Conversion of the azide to *N*-acetyl (see conversion of **38** to **39**) produced methyl *N*-{2-[(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O* -benzoyl-β-D-galactopyranosyl-sulfanyl]ethyl}glutamate **45** (76% yield). R_f = 0.4 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.05–8.03 (m, 2H), 7.98–7.95 (m, 2H), 7.76–7.73 (m, 2H), 7.67–7.63 (m, 1H), 7.56–7.49 (m, 3H), 7.45–7.38 (m, 3H), 7.24–7.21 (m, 2H), 6.22 (d, *J* = 10.0 Hz, 1H), 6.16 (t, *J* = 6.0 Hz, 1H), 5.99 (dd, *J* = 3.2, 0.8 Hz, 1H), 5.80 (t, *J* = 10.0 Hz, 1H), 5.68 (dd, *J* = 10.0, 3.6 Hz, 1H), 5.41 (d, *J* = 2.0 Hz, 1H), 5.08 (dd, *J* = 11.6, 3.2 Hz, 1H), 4.92 (d, *J* = 10.4 Hz, 1H), 4.80 (d, *J* = 3.6 Hz, 1H), 4.65–4.59 (m, 1H), 4.24 (t, *J* = 6.8 Hz, 1H), 4.20 (t, *J* = 7.2 Hz, 1H), 4.13- 4.06 (m, 2H), 3.96 (dd, *J* = 10.0, 6.8 Hz, 1H), 3.69–3.64 (m, 1H), 3.65 (s, 3H), 3.55–3.45 (m, 2H), 3.04–2.98 (m, 1H), 2.89–2.83 (m, 1H), 2.37–2.33 (m, 2H), 2.21–2.17 (m, 2H), 2.15, 2.00, 1.98, 1.97 (4 s, 3H each), 1.92 (pent, *J* = 7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 173.6, 172.5, 170.9, 170.8, 170.6, 170.5, 166.0, 165.7, 165.4, 134.1, 133.7, 133.5, 130.0, 129.97, 129.8, 129.1, 129.0, 128.8, 128.7, 128.6, 128.5, 98.2, 84.9, 75.8, 72.5, 68.6, 68.5, 67.3, 67.0, 65.3, 61.9, 51.7, 47.1, 39.5, 35.4, 33.2, 30.8, 23.2, 20.88, 20.85, 20.75. HRMS, calcd for $C_{49}H_{56}N_2O_{19}NaS$: 1031.3090 [*M*+Na]$^+$; found: 1031.3082.

Global deprotection (following the procedure for conversion of **39** to **40**) produced compound **46** (30% yield): ^{1}H NMR (CD_3OD, 400 MHz): δ = 4.39 (d, *J* = 8.8 Hz, 1H; Gal H1), 4.26 (dd, *J* = 8.1, 3.4 Hz, 1H), 3.91–3.73 (m, 8H), 3.62–3.78 (m, 4H), 3.41–3.35 (m, 1H), 2.87–2.72 (m, 2H), 2.31 (t, *J* = 7.6 Hz, 2H), 2.27 (t, *J* = 7.6 Hz, 2H), 2.02 (s, 3H; acetyl H), 1.90 (pent, *J* = 7.6 Hz, 2H). ^{13}C NMR (CD_3OD, 100 MHz): δ = 176.9, 175.5, 174.1, 99.2, 87.9, 78.6, 76.2, 72.6, 71.5, 70.8, 70.4, 69.8, 68.4, 62.9, 51.6, 41.2, 36.2, 34.2, 30.8, 22.9, 22.3; HRMS, calcd for $C_{21}H_{36}N_2O_{13}NaS$: 579.1830 [*M*+Na]$^+$; found: 579.1817.

Compound **46** was coupled to BSA (following the procedure for conjugate **1**) to produce GalNAc α1-6Gal–BSA (**12**). Analysis by MALDI-TOF MS gave an average mass of 78434, corresponding to an average of 22 epitopes per BSA (each epitope adds 536 to the molecular weight).

GalNAcα1-3Gal (13). Phenyl 2,6-di-*O*-benzyol-3,4-*O*-isopropylidene-1-thio-β-D-galactopyranoside (700 mg, 1.34 mmol)[85] was dissolved in 90% acetic acid (6 mL). The mixture was stirred at 90 °C for 3 h. The solvent was removed, and the residue was azeotroped with toluene. Dichloromethane (2 mL), trimethyl orthobenzoate (300 μL), and (1*S*)-(+)-10-camphorsulfonic acid (cat. amount) were added, and the mixture was stirred at room temperature for 30 min to produce the orthoester.[86] The solvent was removed, and the residue was azeotroped twice with toluene. The residue was dissolved in 90% acetic acid (4 mL), then stirred at room temperature for 30 min and concentrated. The crude product was purified by column chromatography (40% ethyl acetate in hexanes) to give phenyl 2,4,6-tri-*O*-benzoyl-1-thio-β-D-galactopyranoside **48** as white solid (650 mg, 83% yield). R_f = 0.2 (ethyl acetate/hexanes 1:3); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.12–8.09 (m, 2H), 8.05–8.03 (m, 2H), 7.98–7.96 (m, 2H), 7.65–7.43 (m, 11H), 7.34–7.32 (m, 1H), 7.25–7.21 (m, 2H), 5.79 (d, *J* = 2.8 Hz, 1H), 5.30 (t, *J* = 9.6 Hz, 1H), 4.95 (d, *J* = 9.6 Hz), 4.58 (dd, *J* = 11.6, 7.2 Hz, 1H), 4.47 (dd, *J* = 11.6, 7.2 Hz, 1H), 4.24–4.21 (m, 1H), 4.19–4.14 (m, 1H), 2.74 (d, *J* = 6.4 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 167.0, 166.31, 166.26, 134.0, 133.8, 133.5, 131.5, 130.3, 130.2, 130.0, 129.7, 129.5, 129.12, 129.05, 128.8, 128.7, 128.6, 128.5, 85.6, 75.5, 73.2, 71.8, 70.9, 63.1.

Following the procedure used to couple **35** and **36**, glycosyl chloride **47**[87] and acceptor **48** were coupled to produce phenyl (3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-(1→3)-2,4,6-tri-*O*-benzoyl-1-thio-β-D-galactopyranoside **49** (72% yield). R_f = 0.3 (ethyl acetate/hexane 1:2); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.13–8.11 (m, 2H), 8.06–8.04 (m, 2H), 7.98–7.96 (m, 2H), 7.62–7.59 (m, 5H), 7.49–7.44 (m, 6H), 7.38–7.34 (m, 1H), 7.28–7.24 (m, 2H), 5.94 (d, *J* = 2.8 Hz, 1H), 5.59 (t, *J* = 9.6 Hz, 1H), 5.30 (d, *J* = 3.6 Hz, 1H), 4.96 (d, *J* = 9.6 Hz, 1H), 4.90–4.85 (m, 2H), 4.61 (dd, *J* = 11.6, 7.2 Hz, 1H), 4.45 (dd, *J* = 11.6, 7.2 Hz, 1H), 4.25–4.21 (m, 2H), 3.81–3.78 (m, 3H), 3.57 (dd, *J* = 10.4, 3.2 Hz, 1H), 2.03, 1.91, 1.90 (3 s, 3H each); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 170.1, 169.9, 169.3, 166.3, 166.0, 164.9, 94.0, 85.7, 75.1, 74.8, 68.8, 68.3, 67.1, 66.9, 65.3, 62.8, 61.3,

57.1, 20.8, 20.7, 20.6; HRMS: calcd for $C_{45}H_{43}N_3O_{15}NaS$: 920.2307 $[M+Na]^+$; found: 920.2349.

Compound **49** was treated with IBr (see conversion of **37** to **38**) to produce (3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-(1→3)-2,4,6-*O*-benzoyl-α-D-galactopyranosyl bromide (78% yield). R_f=0.6 (ethyl acetate/hexanes 1:1); ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 8.16–8.13 (m, 2H), 8.10–8.07 (m, 2H), 8.05–8.02 (m, 2H), 7.64–7.56 (m, 3H), 7.51–7.43 (m, 6H), 6.93 (d, J=4.0 Hz, 1H), 6.09 (dd, J=2.4, 1.2 Hz, 1H), 5.57 (dd, J=10.0, 4.0 Hz, 1H), 5.39 (d, J=3.6 Hz, 1H), 4.98 (dd, J=2.8, 1.2 Hz, 1H), 4.89 (dd, J=11.2, 3.6 Hz, 1H), 4.77 (t, J=6.8 Hz, 1H), 4.69 (dd, J=10.4, 3.6 Hz, 1H), 4.60 (dd, J=11.6, 6.8 Hz, 1H), 4.46 (dd, J=11.2, 5.6 Hz, 1H), 4.18–4.15 (m, 1H), 4.04–3.94 (m, 2H), 3.65 (dd, J=10.8, 3.6 Hz, 1H), 2.09, 2.08, 1.88 (3 s, 3H each); ^{13}C NMR ($CDCl_3$, 100 MHz): δ=170.5, 170.0, 169.4, 166.2, 166.0, 165.3, 134.2, 133.9, 133.6, 130.2, 130.0, 129.5, 128.96, 128.92, 128.87, 128.82, 128.7, 93.8, 89.4, 72.1, 70.1, 69.1, 68.3, 67.5, 67.4, 65.2, 62.3, 62.0, 57.3, 21.0, 20.7, 20.6; HRMS, calcd for $C_{39}H_{38}N_3O_{15}NaBr$: 890.1379 $[M+Na]^+$; found: 890.1356.

Coupling with the linker **53** (see conversion of **37** to **38**) produced methyl *N*-{2-[(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-(1→3)-2,4,6-tri-*O* -benzoyl-β-D-galactopyranosylsulfanyl]ethyl}-glutamate **50** (59% yield). R_f=0.4 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz): δ=8.16–8.14 (m, 2H), 8.08–8.06 (m, 2H), 8.03–8.01 (m, 2H), 7.65–7.56 (m, 3H), 7.53–7.43 (m, 6H), 6.12 (t, J=6.0 Hz, 1H), 5.99 (d, J=2.4 Hz, 1H), 5.68 (t, J=10.0 Hz, 1H), 5.31 (d, J=2.7 Hz, 1H), 4.90–4.85 (m, 2H), 4.79 (d, J=10.0 Hz, 1H), 4.63 (dd, J=11.6, 6.8 Hz, 1H), 4.42 (dd, J=11.6, 5.6 Hz, 1H), 4.26–4.21 (m, 2H), 3.89–3.80 (m, 3H), 3.63 (s, 3H), 3.60 (dd, J=10.4, 3.6 Hz, 1H), 3.57–3.51 (m, 2H), 3.08–3.00 (m, 1H), 2.88–2.81 (m, 1H), 2.31 (t, J=7.6 Hz, 2H), 2.17 (t, J=7.2 Hz, 2H), 2.03, 1.94, 1.90 (3 s, 3H each), 1.89 (pent, J=7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ=173.7, 172.4, 170.2, 169.9, 169.3, 166.3, 166.1, 165.2, 133.9, 133.8, 133.6, 130.1, 130.0, 129.9, 129.8, 129.5, 129.1, 129.0, 128.8, 128.7, 94.2, 83.9, 75.6, 74.5, 70.8, 68.9, 68.2, 67.0, 66.9, 65.6, 62.7, 61.2, 57.1, 51.7, 39.0, 35.4, 33.2, 30.5, 29.8, 20.9, 20.8, 20.6, 20.5; HRMS, calcd for $C_{47}H_{52}N_4O_{18}NaS$: 1015.2890 $[M+Na]^+$; found: 1015.2898.

Conversion of the azide to *N*-acetyl (see conversion of **38** to **39**) produced methyl *N*-{2-[(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-(1→3)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-sulfanyl]ethyl}glutamate **51** (67% yield). R_f=0.4 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz): δ=8.18–8.16 (m, 2H), 8.05–8.03 (m, 2H), 8.00–7.98 (m, 2H), 7.70–7.66 (m, 1H), 7.63–7.55 (m, 4H), 7.50–7.42 (m, 4H), 6.08–6.03 (m, 2H), 5.85 (d, J=3.2 Hz, 1H), 5.62 (t, J=10.0 Hz, 1H), 5.14 (d, J=3.6 Hz, 1H), 4.91 (dd, J=3.2, 1.2 Hz, 1H), 4.77–4.73 (m, 2H), 4.66 (dd, J=11.6, 6.8 Hz, 1H), 4.54–4.78 (m, 1H), 4.36 (dd, J=11.2, 6.4 Hz, 1H), 4.24–4.18 (m, 2H), 3.91–3.88 (m, 1H), 3.76 (dd, J=11.2, 8.4 Hz, 1H), 3.63 (s, 3H), 3.62–3.51 (m, 3H), 3.07–3.00 (m, 1H), 2.87–2.80 (m, 1H), 2.32 (t, J=7.2 Hz, 2H), 2.17 (t, J=7.2 Hz, 2H), 2.02, 1.93, 1.89, 1.57 (4s, 3H each), 1.90 (pent, J=7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ=173.7, 172.4, 170.5, 170.24, 170.22, 170.1, 166.3, 166.1, 165.7, 134.4, 134.0, 133.7, 130.4, 130.0, 129.9, 129.3, 129.14, 129.06, 128.9, 128.69, 128.66, 98.1, 84.1, 77.9, 75.3, 69.4, 67.6, 67.4, 67.3, 66.8, 62.2, 60.7, 51.7, 47.4, 39.1, 35.4, 33.2, 30.5, 22.7, 20.9, 29.77, 20.74; HRMS calcd for $C_{49}H_{56}N_2O_{19}NaS$: 1031.3090 $[M+Na]^+$; found: 1031.3048.

Global deprotection (following the procedure for conversion of **39** to **40**) produced compound **52** (50% yield). ^{1}H NMR (D_2O, 400 MHz): δ=4.98 (d, J=4.0 Hz, 1H; GalNAc H1), 4.43 (d, J=10.0 Hz, 1H; Gal H1), 4.13–4.07 (m, 2H), 4.03 (d, J=3.2 Hz, 1H), 3.92–3.8 (m, 2H), 3.66–3.52 (m, 7H), 3.35 (t, J=8.1 Hz, 2H), 2.86–2.80 (m, 1H), 2.77- 2.71 (m, 1H), 2.18–2.10 (m, 4H), 1.93 (s, 3H acetyl H), 1.72 (pent, J=7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ= 179.4, 176.2, 174.7, 93.8, 85.7, 78.9, 78.0, 70.9, 68.4, 68.2, 67.6, 65.0, 61.2, 60.9, 49.7, 39.4, 35.0, 34.1, 29.4, 22.0, 21.2; HRMS calcd for $C_{21}H_{36}N_2O_{13}NaS$: 579.1830 $[M+Na]^+$; found: 579.1858.

Compound **52** was coupled to BSA by following the procedure for conjugate **1** to produce GalNAc α1-3Gal–BSA (**13**). Analysis by MALDI-TOF MS gave an average mass of 77849, corresponding to an average of 21 epitopes per BSA (each epitope adds 536 to the molecular weight).

Mucins: BSM was purchased from Sigma. Asialo-BSM was prepared by removing sialic acid residues as reported previously.[58] BSM contains approximately 70% carbohydrate by weight (as compared to about 15% for the BSA conjugates). About 45–50% of the carbohydrate is either NeuAcα2-6Tn or NeuGcα2–Tn. About 35% of the carbohydrate is either NeuAcα2-6GlcNAcβ1–GalNAc or NeuGcα2-6GlcNAcβ1-3GalNAc. A number of other minor components such as Le^y, blood group A, Sialyl-TF, and blood group H are also present.[88]

Array printing: The array was printed on epoxy Nunc ArrayCote 16-well slides (Nalge Nunc International, Rochester, NY, USA). All the glycoconjugate samples were printed at a concentration of 0.5 mg mL^{-1} with 5% glycerol in PBS buffer (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, and 150 mM NaCl, pH 7.2). Samples were dispensed in specially designed polypropylene 384-well plates with conical well profiles for maximum sample. Arrays were printed with Virtex Chipwriter TM Pro technology based on super-precision robotics and microfluidic systems in HEPA filtered clean air and humidity-controlled chambers (Bio-Rad). SMP6B quill pins were used to obtain ~150–180 μm spots. Due to the large capacity of the pins and viscous nature of the glycerol-containing samples, the dip time for sample pick up was set at 55 s. Samples were blotted twice and then printed in triplicate with a dwell time of 0.2 s. Pins were washed five times prior to printing the next sample. Once printed, the slides were air dried for 24 h, then stored at −20 °C until used.

Evaluation of binding with the array: Each well of the slide was incubated in blocking buffer (100 μL of 3% BSA in PBS) for an hour. The wells were then incubated with biotinylated VVL-B4 (Vector Labs, Burlingame, CA, USA), Bric111 (Accurate Chemical, Westbury, NY, USA), or HBTn1 (Dako Cytomation, Carpenteria, CA, USA) at a range of concentrations (serial dilutions) in binding buffer I (50 μL, 0.3% BSA in PBS). After 1 h of incubation with the receptors, the wells were washed with PBS (3× 100 μL per well). The wells that were treated with biotinylated VVL-B4 were then incubated with streptavidin-HRP (50 μL at 80 ng mL^{-1}, Southern Biotech, Birmingham, AL, USA) in binding buffer II (3% BSA in PBS) for 1 h. The wells that were treated with HBTn1 or Bric111 were incubated with Goat anti-mouse Ig-HRP conjugate (50 μL at 2 μg mL^{-1}, Southern Biotech) in binding buffer II. After 1 h of incubation with the HRP conjugates, the slides were washed with PBS (7× 100 μL per well). The HRP-bound wells were then incubated with the Cyanin 5-labeled tyramide substrate (50 μL, 1 ng mL^{-1}, PerkinElmer Life and Analytical Sciences, Inc.). After 5 min of signal amplification, the slides were washed with PBS (3×), and the 16-well chamber was removed from the slides. The slides were then incubated in PBS buffer for an additional 5 min, spun in an Eppendorf centrifuge 5810R (400 *g*) for 5 min, and dried.

The slides were then scanned by using a GenePix scanner (GenePix 4000B Microarray Scanner, Molecular Devices Corporation, Union City, CA). The fluorescence was measured at a photomultiplicator level (PMT) of 600 at the emission wavelength of the fluorophore.

The fluorescence was quantified by using GenePix Pro 6.0 with a GenePix Array List (GAL) file. The mean values minus the background (typically about 200) for each of the three spots of a particular sample were averaged, and average values greater than 1000 (five times the background) were defined as a positive signal. The average adjusted fluorescence was normalized to a percentage scale, with 100 being the saturation fluorescence (65000). The normalized fluorescence was then plotted against the lectin or antibody concentration.

Evaluation of binding by SDS-PAGE: SDS-PAGE was carried out in 10% TG polyacrylamide gels. FBSA conjugate was loaded in each lane (50 ng for blots with VVL-B_4, 500 ng for Bric111 and HBTn1). Proteins were electrophoretically transferred to PVDF membranes. Membranes were incubated with 5% BSA/PBS for 2 h to block nonspecific binding. Membranes were then incubated with biotinylated VVL-B_4 (2 $\mu g\,mL^{-1}$), Bric111 (diluted 1:500, 2 $\mu g\,mL^{-1}$), or HBTn1 (diluted 1:500, 1 $\mu g\,mL^{-1}$) in 3% BSA/PBS. Blots were washed with PBS (4×) and then incubated with streptavidin–alkaline phosphatase (diluted 1:2000, for blots with VVL-B4) or goat anti-mouse Ig–alkaline phosphatase (diluted 1:1000, for blots with antibodies) in 3% BSA/PBS for 1 h. Blots were washed with PBS (10×) and ddH_2O (1×), and then incubated with BCIP/NBT (1 $mg\,mL^{-1}$) for 10–20 min. Blots were washed with ddH_2O (3×), dried, and then scanned.

Acknowledgements

We thank Jack Simpson (Protein Chemistry Laboratory, NCI–Frederick) for MALDI-TOF MS analysis of the BSA conjugates. In addition, we thank Dr. David Munroe and Dr. Lisa Gangi (Laboratory of Molecular Technology, SAIC–Frederick) for assistance in scanning the arrays and for many helpful discussions. We thank the U.C. Riverside Mass Spectroscopy Facility for high-resolution mass spectral analysis. We thank Dr. Terry Burke and Dr. Joseph Barchi for critical review of this manuscript. None of the authors has any financial interest in or conflict of interest with the material covered in this paper. This research was supported by the Intramural Research Program of the NIH, NCI.

Keywords: carbohydrates · glycoconjugates · glycosylation · microarrays · Tn antigen

[1] J. Dausset, J. Moullec, J. Bernard, *Blood* **1959**, *14*, 1079.
[2] G. F. Springer, *Science* **1984**, *224*, 1198.
[3] G. F. Springer, *J. Mol. Med.* **1997**, *75*, 594.
[4] P. Desai, *Transfus. Med. Rev.* **2000**, *14*, 312.
[5] M. Grosso, E. Vitarelli, G. Giuffre, G. Tuccari, G. Barresi, *Eur. J. Histochem.* **2000**, *44*, 359.
[6] K. Akita, S. Fushiki, T. Fujimoto, M. Inoue, K. Oguri, M. Okayama, I. Yamashina, H. Nakada, *J. Neurosci. Res.* **2001**, *65*, 595.
[7] Y. Zhang, H. Iwasaki, H. Wang, T. Kudo, T. B. Kalka, T. Hennet, T. Kubota, L. Cheng, N. Inaba, M. Gotoh, A. Togayachi, J. Guo, H. Hisatomi, K. Nakajima, S. Nishihara, M. Nakamura, J. D. Marth, H. Narimatsu, *J. Biol. Chem.* **2003**, *278*, 573.
[8] C. Casaravilla, T. Freire, R. Malgor, A. Medeiros, E. Osinaga, C. Carmona, *J. Parasitol.* **2003**, *89*, 709.
[9] T. Freire, C. Casaravilla, C. Carmona, E. Osinaga, *Int. J. Parasitol.* **2003**, *33*, 47.
[10] J. E. S. Hansen, C. Nielsen, M. Arendrup, S. Olofsson, L. Mathiesen, J. O. Nielsen, H. Clausen, *J. Virol.* **1991**, *65*, 6461.
[11] E. G. Berger, *Biochim. Biophys. Acta* **1999**, *1455*, 255.
[12] A. C. Allen, E. M. Bailey, J. Barratt, K. S. Buck, J. Feehally, *J. Am. Soc. Nephrol.* **1999**, *10*, 1763.
[13] A. Allen, F. Willis, T. Beattie, J. Feehally, *Nephrol. Dial. Transplant.* **1998**, *13*, 930.
[14] A. Kanda, S. Tsuyama, F. Murata, K. Kodama, Y. Hirabayashi, T. Kanzaki, *J. Dermatol. Sci.* **2002**, *29*, 42.
[15] H. Sakuraba, F. Matsuzawa, S. Aikawa, H. Doi, M. Kotani, H. Nakada, T. Fukushige, T. Kanzaki, *J. Hum. Genet.* **2004**, *49*, 1.
[16] S. F. Slovin, G. Ragupathi, C. Musselli, K. Olkiewicz, D. Verbel, S. D. Kuduk, J. B. Schwarz, D. Sames, S. J. Danishefsky, P. O. Livingston, H. I. Scher, *J. Clin. Oncol.* **2003**, *21*, 4292.
[17] G. Ragupathi, D. M. Coltart, L. J. Williams, F. Koide, E. Kagan, J. Allen, C. Harris, P. W. Glunz, P. O. Livingston, S. J. Danishefsky, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13699.
[18] E. Kagan, G. Ragupathi, S. S. Yi, C. A. Reis, J. Gildersleeve, D. Kahne, H. Clausen, S. J. Danishefsky, P. O. Livingston, *Cancer Immunol. Immunother.* **2005**, *54*, 424.
[19] S. Tollefsen, R. Kornfeld, *J. Biol. Chem.* **1983**, *258*, 5172.
[20] D. Avichezer, R. Arnon, *FEBS Lett.* **1996**, *395*, 103.
[21] S. Itzkowitz, T. Kjeldsen, A. Friera, S. Hakomori, U. S. Yan, Y. S. Kim, *Gastroenterology* **1991**, *100*, 1691.
[22] C. K. Ching, S. W. Holmes, G. K. Holmes, R. G. Long, *Pancreas* **1994**, *9*, 698.
[23] Y. Cao, P. Stosiek, G. F. Springer, U. Karsten, *Histochem. Cell Biol.* **1996**, *106*, 197.
[24] A. Babino, P. Oppezzo, S. Bianco, N. Barrios, H. Navarrete, E. Osinaga, *Int. J. Cancer* **2000**, *86*, 753.
[25] M. Inoue, S. M. Ton, H. Ogawa, O. Tanizawa, *Am. J. Clin. Pathol.* **1991**, *96*, 711.
[26] S. H. Itzkowitz, E. J. Bloom, T. S. Lau, Y. S. Kim, *Gut* **1992**, *33*, 518.
[27] G. Konska, J. Guillot, M. de Latour, Y. Fonck, *Int. J. Oncol.* **1998**, *12*, 361.
[28] C. Carrilho, M. Cantel, P. Gouveia, L. David, *Virchows Arch.* **2000**, *437*, 173.
[29] S. Hamada, H. Furumoto, M. Kamada, T. Hirao, T. Aono, *Cancer Lett.* **1993**, *74*, 167.
[30] K. Terasawa, H. Furumoto, M. Kamada, T. Aono, *Cancer Res.* **1996**, *56*, 2229.
[31] M. Ghazizadeh, H. Ogawa, Y. Sasaki, T. Araki, K. Aihara, *Hum. Pathol.* **1997**, *28*, 960.
[32] B. L. Wang, G. F. Springer, S. C. Carlstedt, *J. Histochem. Cytochem.* **1997**, *45*, 1393.
[33] J. Kanitakis, I. al-Rifai, M. Faure, A. Claudy, *J. Clin. Pathol.* **1998**, *51*, 588.
[34] B. Davidson, W. H. Gotlieb, G. Ben-Baruch, J. Kopolovic, I. Goldberg, J. M. Nesland, A. Berner, A. Bjamer, M. Bryne, *Gynecol. Oncol.* **2000**, *77*, 35.
[35] M. H. Therkildsen, L. J. Andersen, M. Christensen, H. S. Hansen, T. Schiodt, E. Dabelsteen, *Oral Oncol.* **1998**, *34*, 44.
[36] D. Wang, S. Liu, B. J. Trummer, C. Deng, A. Wang, *Nat. Biotechnol.* **2002**, *20*, 275.
[37] S. Fukui, T. Feizi, C. Galustian, A. M. Lawson, W. Chai, *Nat. Biotechnol.* **2002**, *20*, 1011.
[38] B. Houseman, M. Mrksich, *Chem. Biol.* **2002**, *9*, 443.
[39] K. R. Love, P. H. Seeberger, *Angew. Chem.* **2002**, *114*, 3733; *Angew. Chem. Int. Ed.* **2002**, *41*, 3583.
[40] T. Feizi, F. Fazio, W. Chai, C.-H. Wong, *Curr. Opin. Struct. Biol.* **2003**, *13*, 637–645.
[41] M. Schwarz, L. Spector, A. Gargir, A. Shtevi, M. Gortler, R. T. Altstock, A. A. Dukler, N. Dotam, *Glycobiology* **2003**, *13*, 749.
[42] O. Blixt, S. Head, T. Mondala, C. Scanlan, M. E. Huflejt, R. Alvarez, M. C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D. J. Stevens, J. J. Skehel, I. van Die, D. R. Burton, I. A. Wilson, R. Cummings, N. Bovin, C.-H. Wong, J. C. Paulson, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17033.
[43] I. Shin, J. W. Cho, D. W. Boo, *Comb. Chem. High Throughput Screening* **2004**, *7*, 565.
[44] S. Angeloni, J. L. Ridet, N. Kusy, H. Gao, F. Crevoisier, S. Guinchard, S. Kochhar, H. Sigrist, N. Sprenger, *Glycobiology* **2005**, *15*, 31.
[45] M. F. Templin, D. Stoll, J. M. Schwenk, O. Potz, S. Kramer, T. O. Joos, *Proteomics* **2003**, *3*, 2155.
[46] K. H. Mortel, R. V. Weatherman, L. L. Kiessling, *J. Am. Chem. Soc.* **1996**, *118*, 2297.
[47] R. Liang, J. Loebach, N. Horan, M. Ge, C. Thompson, L. Yan, D. Kahne, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10554.

[48] Dozens of minor components such as blood group A, blood group H, Sialyl-TF, and LeY are also present, see Chai, W. Hounsell, W. F. Cashmore, G. C. Rosankiewicz, J. R. Bauer, C. J. Feeney, J. Feizi, T. Lawson, *Eur. J. Biochem.* **1992**, *203*, 257 – 268.
[49] The Glycominds Ltd. carbohydrate data base was searched via the Consortium for Functional Genomics web site.
[50] K. P. R. Kartha, P. Cura, M. Aloui, S. K. Readman, T. J. Rutherford, R. A. Field, *Tetrahedron: Asymmetry* **2000**, *11*, 581.
[51] X. M. Zhu, K. Pachamuthu, R. R. Schmidt, *J. Org. Chem.* **2003**, *68*, 5641.
[52] Poly(vinylpyrrolidone) and Tween 20 were also tested as blocking agents, but the best results were obtained with BSA.
[53] M. N. Bobrow, T. D. Harris, K. J. Shaughnessy, G. J. Litt, *J. Immunol. Methods* **1989**, *125*, 279.
[54] G. Konska, C. Vissac, K. Zagla, F. Chezet, M.-P. Vasson, D. Bernard-Gallon, J. Guillot, *Int. J. Oncol.* **2002**, *21*, 1009.
[55] P. T. Martin, J. R. Sanes, *Neuron* **1995**, *14*, 743.
[56] T. Freire, A. Medeiros, C. A. Reis, F. X. Real, E. Osinaga, *Oncol. Rep.* **2003**, *10*, 1577.
[57] D. Cai, J. Y. Leem, J. P. Greenfield, P. Wang, B. S. Kim, R. Wang, K. O. Lopes, S.-H. Kim, H. Zheng, P. Greengard, S. S. Sisodia, G. Thinakaran, H. Xu, *J. Biol. Chem.* **2003**, *278*, 3446.
[58] A. M. Wu, *FEBS Lett.* **2004**, *562*, 51.
[59] E. Osinaga, S. Bay, D. Tello, A. Babino, O. Pritsch, K. Assemat, D. Cantacuzene, H. Nakada, P. Alzari, *FEBS Lett.* **2000**, *469*, 24.
[60] K. D. Puri, B. Gopalakrishnan, A. Surolia, *FEBS Lett.* **1992**, *312*, 208.
[61] A. Babino, D. Tello, A. Rojas, S. Bay, E. Osinaga, P. M. Alzari, *FEBS Lett.* **2003**, *536*, 106.
[62] VVL-B4 binds both mono- and clustered-Tn antigen; see E. Osinaga, S. Bay, D. Tello, A. Babino, O. Pritsch, K. Assemat, D. Cantacuzene, H. Nakada, P. Alzari, *FEBS Lett.* **2000**, *469*, 24 – 28.
[63] The lack of signal for blood group A indicates that the VVL-B4 sample used for the assays is not contaminated with VVL-A4 or VVL-A2B2.
[64] M. King, S. Parsons, A. Wu, N. Jones, *Transfusion* **1991**, *31*, 142.
[65] K. Hempel, W. Deubel, R. Lorenz, C. Reiners, *Mut. Res./Fundam. Mol. Mech. Mut.* **2003**, *525*, 29.
[66] M. King, A. Chan, R. Roe, B. Warren, A. Dell, H. Morris, D. Bartolo, P. Durdey, A. Corfield, *Glycobiology* **1994**, *4*, 267.
[67] S. A. Brooks, A. J. C. Leathem, *Invasion Metastasis* **1999**, *18*, 115.
[68] S. A. Brooks, A. J. C. Leathem, *Br. J. Cancer* **1995**, *71*, 1033.
[69] The generation of antibody HBTn1 has not been published. Some minimal information on the specificity is supplied in the product specification sheet from Dako Cytomation.
[70] Y. Cao, U. Karsten, H. Zerban, P. Bannasch, *Virchows Arch.* **2000**, *436*, 119.
[71] B. Davidson, A. Berner, J. M. Nesland, B. Risberg, G. B. Kristensen, C. G. Trope, M. Bryne, *Hum. Pathol.* **2000**, *31*, 1081.
[72] G. Barresi, G. Giuffre, E. Vitarelli, M. Grosso, G. Tuccari, *Pathology* **2001**, *33*, 298.
[73] S. Amaya, M. Sasaki, Y. Watanabe, W. M. S. Tsui, K. Tsuneyama, K. Harada, Y. Nakanuma, *Histol. Histopathol.* **2001**, *16*, 550.
[74] HBTn1 recognizes both monomeric and clustered Tn; see Y. Zhang, H. Iwasaki, H. Wang, T. Kudo, T. B. Kalka, T. Hennet, T. Kubota, L. Cheng, N. Inaba, M. Gotoh, A. Togayachi, J. Guo, H. Hisatomi, K. Nakajima, S. Nishihara, M. Nakamura, J. D. Marth, H. Narimatsu, *J. Biol. Chem.* **2003**, *278*, 573.
[75] In the context of a tissue sample or cell, other factors such as accessibility, spacing, and orientation can affect the affinity toward a particular epitope. Therefore, the Tn receptors might show enhanced binding for "off target" epitopes in some cellular environments.
[76] D. K. Podolsky, *J. Biol. Chem.* **1985**, *260*, 8262.
[77] S. D. Kuduk, J. B. Schwarz, X.-T. Chen, P. W. Glunz, D. Sames, G. Ragupathi, P. O. Livingston, S. J. Danishefsky, *J. Am. Chem. Soc.* **1998**, *120*, 12474.
[78] A. H. Andreotti, D. Kahne, *J. Am. Chem. Soc.* **1993**, *115*, 3352.
[79] R. Paruszewski, R. Matusiak, G. Rostafinska-Suchar, S. Gumulka, P. Krzaścik, *Pol. J. Chem.* **1987**, *61*, 127.
[80] P. Depouilly, A. Chenede, J. M. Mallet, P. Sinaÿ, *Bull. Soc. Chim. Fr.* **1993**, *130*, 256.
[81] V. Pavliak, P. Kovac, *Carbohydr. Res.* **1991**, *210*, 333.
[82] G. Malle, *Eur. Pat. Appl.* **1995**, EP 653202; [*Chem. Abstr. 123*, 17463].
[83] G. Grundler, R. R. Schmidt, *Liebigs Ann. Chem.* **1984**, 1826.
[84] A. Marra, J. Esnault, A. Veyrières, P. Sinaÿ, *J. Am. Chem. Soc.* **1992**, *114*, 6354.
[85] A. Fernandez-Mayoralas, A. Marra, M. Trumtel, A. Veyrières, P. Sinaÿ, *Carbohydr. Res.* **1989**, *188*, 81.
[86] V. Pozsgay, *J. Org. Chem.* **1998**, *63*, 5983.
[87] R. Szweda, U. Spohr, R. U. Lemieux, D. Schindler, D. F. Bishop, R. J. Desnick, *Can. J. Chem.* **1989**, *67*, 1388.
[88] W. Chai, E. F. Hounsell, G. C. Cashmore, J. R. Rosankiewicz, C. J. Bauer, J. Feeney, T. Feizi, A. M. Lawson, *Eur. J. Biochem.* **1992**, *203*, 257.

Received: April 19, 2005
Published online on October 27, 2005

