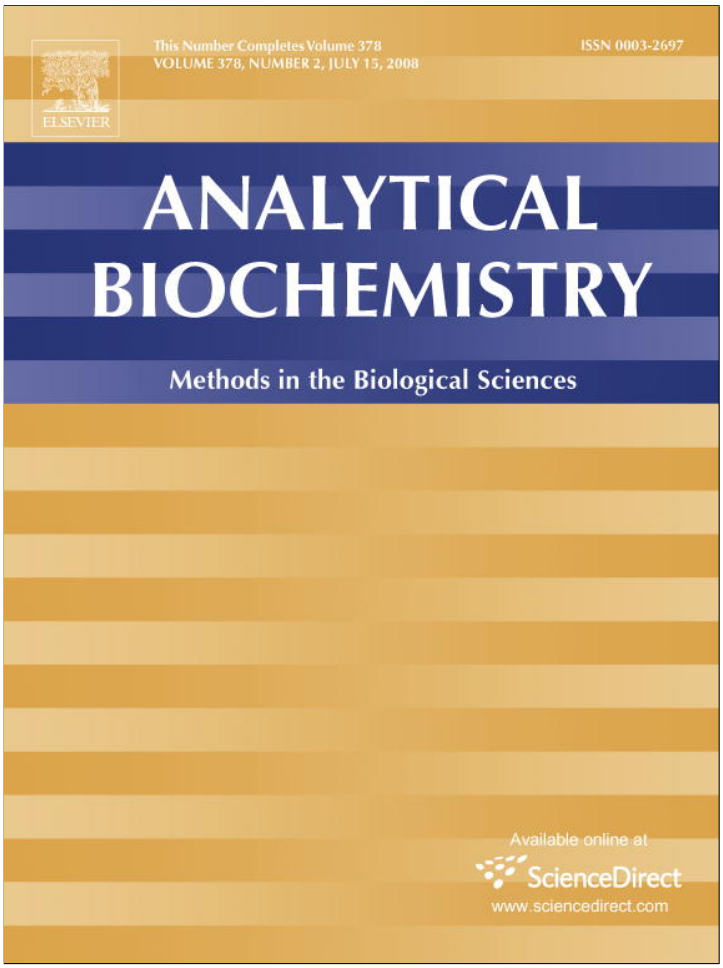

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Assessing the inhibitory potency of galectin ligands identified from combinatorial (glyco)peptide libraries using surface plasmon resonance spectroscopy

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ABSTRACT

Combinatorial (glyco)peptide libraries offer the possibility to define effective inhibitors of protein (lectin)–glycan interactions. If a (glyco)peptide surpasses the inhibitory potency of the free sugar, then the new peptide–lectin contacts underlying the affinity enhancement may guide further rational drug design. Focusing on the adhesion/growth regulatory human galectins 1 and 3, a screening of three combinatorial solid-phase (glyco)peptide libraries, containing Gal(β1-O)Thr, Gal(β1-S)Cys/Gal(β1-N)Asn, and Lac(β1-O)Thr, with the fluorescently labeled lectins had led to a series of lead compounds. To define the inhibitory potency of a selection of resynthesized (glyco)peptides systematically, a surface plasmon resonance-based inhibition assay with immobilized asialofetuin was set up. (Glyco)Peptides with up to 66-fold potency relative to free lactose as inhibitor were characterized. The presence of lactose in the most effective glycopeptides indicated the presence of affinity-enhancing peptide–lectin contacts. In addition to drug design, they may be helpful for fine-structural analysis of the binding sites.

The emerging functionality of glycans as sugar-encoded signals, translated into cellular responses by endogenous lectins, entails a medical perspective [1]. Explicitly, undesired interactions in vivo mediated by lectin–glycan recognition become a target for inhibitor development. Such attributes apply to the lectin family of galectins [1,2]. Due to their involvement in intercellular interactions in processes such as angiogenesis and tumor invasion/spread, as well as in intracellular recognition processes encompassing anti-apoptotic and promalignant activities via binding to distinct protein targets such as Bcl-2 and oncogenic H-ras, especially human galectins 1 and 3 (h-Gal-1[1] and h-Gal-3, respectively) are attractive models for innovative drug design. Fittingly, screening by library approaches to detect sugar-based inhibitors has started [3].

Because galectins are involved in carbohydrate–protein interactions as well as in protein–protein interactions, (glyco)peptide ligands may provide versatile targets for the development of synthetic inhibitors. Previously, solid-phase peptide libraries and phage display libraries have been screened to identify high-affinity mimics [4]. Although it is known that peptides are able to bind specifically in the carbohydrate recognition domain (CRD) of a galectin [5], these peptides need to fulfill certain sequence requirements for specific binding in the CRD [6]. Glycopeptides may be interesting targets to modulate galectin binding because the carbohydrate part provides the specificity of the interaction, whereas the peptide backbone may actively participate in binding by, for example, hydrogen bonding or hydrophobic interactions. Glycopeptides can be generated in a library format via a combinatorial approach [7,8]. The most frequently implemented method for the generation of "one-bead-one-compound" (glyco)peptide libraries [9,10] is the split-and-mix method [11,12]. This method, combined with the ladder synthesis strategy, offers facile synthesis and characterization of thousands of possible ligands that can be used for interaction studies or the development of new therapeutic agents. Several glycopeptide libraries generated via this strategy have been screened with lectins, and effective mimics could be identified

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[1] *Abbreviations used:* h-Gal-1, human galectin 1; h-Gal-3, human galectin 3; CRD, carbohydrate recognition domain; SPR, surface plasmon resonance; PEGA, poly(ethylene glycol)dimethylacrylamide copolymer; BSA, bovine serum albumin; IMP, ionization–mass peptide; Fmoc, 9-fluorenylmethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; NEM, *N*-ethylmorpholine; Cha, cyclohexylalanine; *t*Bu, *tert*-butyl; OPfp, pentafluorophenyl; Dhbt–OH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; DMF, dimethyl formamide; MWCO, molecular weight cutoff; PBS, phosphate-buffered saline; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; α-CHC, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; TIS, triisopropylsilane; FWHM, full-width at half-maximum; p20, polyoxyethylenesorbitan; EDTA, ethylenediaminetetraacetic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; HBS–EP, HEPES-buffered saline with EDTA and p20; DTT, dithiothreitol; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide hydrochloride; RU, resonance units.

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[11,13,14]. Some of these mimics were shown to have a higher affinity toward a relevant receptor as compared with their original ligands, emphasizing the potential of glycopeptides in the development of pharmaceutically active compounds that are able to interfere with undesired interactions.

Recently, we described the screening of three glycopeptide libraries, containing Gal(β1-O)Thr (**A**), Gal(β1-S)Cys/Gal(β1-N)Asn (**B**), and Lac(β1-O)Thr (**C**), with h-Gal-1 and h-Gal-3 [15]. The inhibitory activity of some lead structures was tested in solid-phase and cell assays. In view of the potential of this substance class for medical applications, we set out to systematically examine the capacity of h-Gal-1- and h-Gal-3-binding (glyco)peptides to interfere with lectin–glycan interaction by surface plasmon resonance (SPR).

Materials and methods

Materials and general methods

PEGA1900 resin (0.2 mmol/g loading, 300–500 μm) and Wang resin (0.68 mmol/g loading, 200–400 mesh, prefunctionalized with a Rink amide linker) were obtained from NovaBiochem (Läufelfingen, Switzerland). Suitably protected N^{α}-Fmoc and N^{α}-Boc amino acids were purchased from Bachem (Bubendorf, Switzerland). The two human galectins were purified after recombinant production by affinity chromatography, and purity, molecular mass, and activity were rigorously controlled [16,17]. Bovine serum albumin (BSA) and asialofetuin were obtained from Sigma (Zwijndrecht, The Netherlands), and the Alexa Fluor 488 labeling kit was purchased from Molecular Probes (Leiden, The Netherlands). All solvents were of HPLC grade and were used without further purification.

Solid-phase library synthesis

Solid-phase glycopeptide library **A**, containing Gal(β1-O)Thr introduced via building block **2** [18] (Fig. 1), and library **B**, containing Gal(β1-S)Cys and Gal(β1-N)Asn using building blocks **3** and **4** [19] (Fig. 1), were previously synthesized according to the split-and-mix method combined with the ladder synthesis strategy [14]. Library **C**, containing Lac(β1-O)Thr introduced via building block **5** (Fig. 1), was synthesized according to the same procedure. Briefly, 250 mg (∼ 105,000 beads) of PEGA1900 resin, derivatized with the ionization–mass peptide (IMP) spacer Gly-Pro-Pro-Phe-Pro-Phe (general library construct **1** [Fig. 1], 0.4 mmol/g loading), was equally distributed over the wells of a 20-well multiple-column peptide synthesizer [20] (2.0 ml capacity). To each well, one of the following amino acids was coupled as a 9:1 mixture of 9-fluorenylmethoxycarbonyl (Fmoc) and *tert*-butyloxycarbonyl (Boc) amino acids after 5 min of preactivation (4 eq, 3.9 eq of TBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] and 6 eq of NEM [*N*-ethylmorpholine]): Ala, Arg, Asn, cyclohexylalanine (Cha), Gln, Gly, His(Boc), Ile, Met, Phe (2×), Pro, Ser(*t*Bu), Thr(*t*Bu), Trp, Tyr(*t*Bu) (2×), and Val. Glycosylated amino acid building block **5** (8 eq) and pentadecanoic acid pentafluorophenyl ester (OPfp) (11.2 eq) were coupled to the two remaining wells in the appropriate ratios to obtain 10% capping after activation with 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt–OH) (6.4 eq). After each coupling, the resin was pooled, mixed, and distributed again over all wells prior to Fmoc removal. Each coupling and deprotection step was followed by washings with dimethyl formamide (DMF, 10×). After six subsequent coupling steps, all Fmoc, Boc, and amino acid side chain protecting groups were removed as described previously, and the carbohydrates were de-O-acetylated [11,14].

Solid-phase library screening

Prior to library screening, the two galectins were labeled with the Alexa Fluor 488 fluorescent dye according to the manufacturer's protocol with two modifications. The excess of dye was removed by centrifugal filtration using a 10,000 molecular weight cutoff (MWCO) filter (Nalgene), and the repeated washing steps were performed with a 10-mM phosphate-buffered saline (PBS) buffer (pH 7.4) containing 2.7 mM KCl and 137 mM NaCl (10×). The library screening was carried out at room temperature. The beads (25 mg, ∼ 10,500 in number) were swollen in PBS buffer, and protein binding to their surface was blocked by the addition of 1% BSA to the PBS solution (1 ml, 30 min) to minimize (glyco)peptide-independent binding. The beads were then incubated with the fluorescently labeled galectin (1.5 μM) in PBS buffer containing 1% BSA (300 μl) overnight. The solution was removed by careful suction, and the resin was washed with PBS buffer (2×) and water (1×). Small portions of beads were transferred to

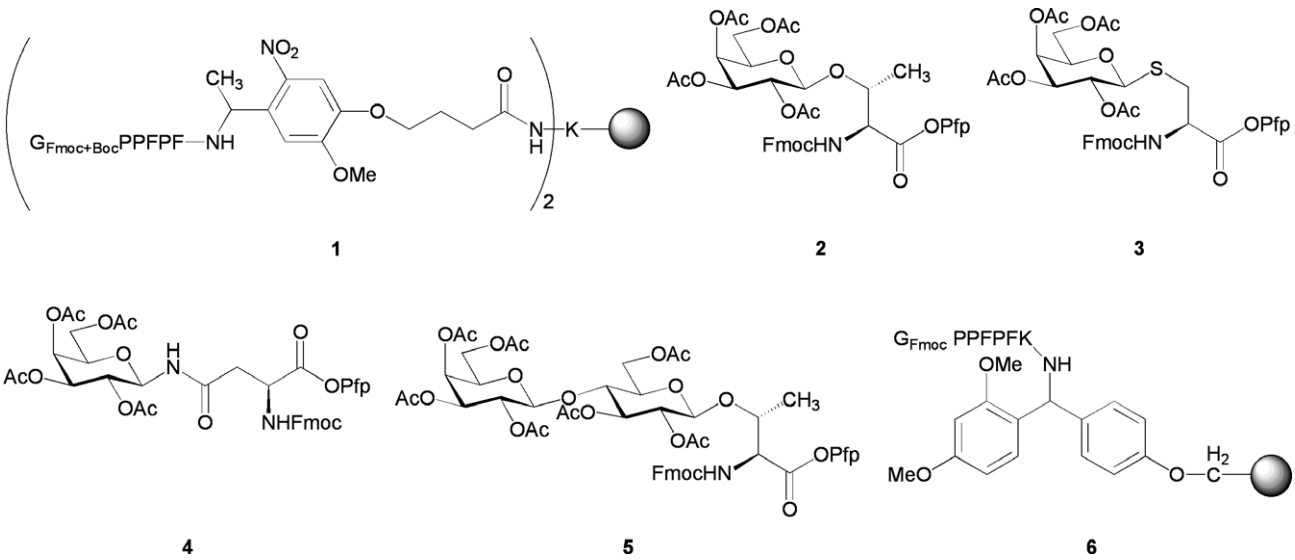

Fig. 1. General library construct **1**; glycosylated amino acid building blocks **2** (library **A**), **3** and **4** (library **B**), and **5** (library **C**); and general solid-phase construct **6**.

a glass plate, swollen in water, and inspected under a fluorescence microscope. The most fluorescent beads were manually collected and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS). The beads were placed on a stainless-steel target and irradiated with UV light (254 nm, 30 min). The (glyco)peptides were extracted from the beads using 0.2 μl of 50% aq acetonitrile, and 0.2 μl of α-cyano-4-hydroxycinnamic acid (α-CHC) in 50% aq acetonitrile (10 mg/ml) was added as a matrix.

Resynthesis of soluble lead (glyco)peptides

The lead (glyco)peptides were synthesized using a previously described protocol [14]. Briefly, soluble lead (glyco)peptides were synthesized on IMP-derivatized Wang resin (general solid-phase construct **6** [Fig. 1]) on a 12.2-μmol scale using TBTU/NEM activation for the coupling of Fmoc-Aa-OH (2 eq) and Dhbt–OH activation for the building blocks **2** to **5** (3 eq) [18,19]. After the last coupling step, the glycopeptides were de-O-acetylated, and in a single step, using a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95.0:2.5:2.5, 4 × 30 min), the amino acid side chains were deprotected and the (glyco)peptides were released from the resin. The free (glyco)peptides were extracted from the resin with 10% aq acetonitrile (4×), concentrated, and purified by reverse-phase HPLC. Preparative HPLC was performed on a Knauer HPLC system using a reverse-phase Polaris C18-A column (250 × 4.6 mm, Varian, Middelburg, The Netherlands) with UV detection at 214 nm. Eluent A (0.1% TFA in 5% aq acetonitrile) and eluent B (0.08% TFA in 90% aq acetonitrile) were mixed using a linear gradient starting from 90% A to 60% A with a slope of 1.5%/min and a flow rate of 1 ml/min. After purification, the (glyco)peptides were lyophilized and analyzed by MALDI–TOF MS. MALDI–TOF mass spectra were recorded using a Voyager–DE Pro instrument (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) in the reflector mode at a resolution of 5000 full-width at half-maximum (FWHM). Exact masses of soluble lead (glyco)peptides were measured by using α-CHC as a matrix, and a mixture of peptides (Peptide Calibration Mix4 [Proteomix], 500–3500 Da, LaserBio Labs, Sophia-Antipolis, France) was added as the internal standard.

SPR experiments

General

SPR measurements were carried out on a BIAcore 2000 instrument using a CM5 sensor chip. A 10-mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) buffer (pH 7.5) containing 150 mM NaCl, 0.005% polyoxyethylenesorbitan (p20), and 3 mM ethylenediaminetetraacetic acid) (EDTA) (HBS–EP), was used for all experiments (surface preparation, affinity determination, and competition assays). For the experiments with soluble h-Gal-1, an appropriate stock solution was prepared in HBS–EP containing 100 μM dithiothreitol (DTT) to preclude loss of lectin activity by oxidation. Subsequent dilutions were made with HBS–EP without DTT. The data collected were deconvoluted using BIAevaluation 4.1 software. All experiments were carried out at a flow rate of 5 μl/min. All assays were carried out in duplicate, and the average of the two results is reported.

Surface preparation

The carboxymethylated dextran layer in flow cell 2 was activated by injecting 35 μl of a 1:1 mixture of 0.05 M *N*-hydroxysuccinimide (NHS) and 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), and asialofetuin was immobilized (2 μl, 12.5 μg/ml in 10 mM NaOAc buffer, pH 4.5) to a level of 106 resonance units (RU). The remaining NHS esters were blocked by the injection of a 1.0-M ethanolamine hydrochloride solution (35 μl, pH 8.5). The untreated carboxymethylated dextran layer in flow cell 1 was used as the reference surface for h-Gal-1. A reference surface for h-Gal-3 was created in flow cell 3 by applying the activation procedure described above, followed by immobilization of periodate-treated BSA (5 μl, 25 μg/ml in 10 mM NaOAc buffer, pH 4.5) to a level of 260 RU. The remaining NHS esters were blocked as described above.

Binding experiments

The binding potential of the surface was titrated with a series of galectin concentrations (20–0.16 μM, obtained by twofold dilution) in HBS–EP buffer, allowing 10 min for dissociation. The surfaces were not regenerated because the galectins dissociated completely from the ligand. The data were double reference subtracted, and K_D and F_{im} values were calculated by nonlinear fitting of the plot of R_{eq} versus the galectin concentration using the average model with steady-state affinity as given in BIAevaluation 4.1.

Competition experiments

For the competition experiments, a (glyco)peptide or free lactose was added to a solution of 5 μM galectin, and the mixture was injected over the sensor chip surface for 2 min, allowing 5 min for dissociation. The surfaces were not regenerated because the galectins dissociated completely. Inhibitor concentrations tested were as follows: 800, 600, 400, 200, and 100 μM for **7**, **22**, and **23**; 4000, 3000, 1500, 800, and 500 μM for **14** and Lac (h-Gal-1); 400, 300, 200, 100, and 50 μM for **17**; 80, 60, 40, 20, and 10 μM for **18**, **20**, **21, 24, 25, 26, 28, 29**, and **30**; 40, 30, 25, 20, and 10 μM for **27**; 1000, 800, 500, 300, and 100 μM for Lac (h-Gal-3). The binding response values at equilibrium were taken at the average equilibrium binding between 50 and 100 s postinjection. The IC_{50} values were derived from the K_i values, which were obtained by fitting the data to the fractional inhibition equation: $f = [I]/([I] + K_i(1 + F_{im}/K_D))$ [21,22].

Results and discussion

Initial screening of the (glyco)peptide libraries (**A** and **B**, containing Gal(β1-O)Thr and Gal(β1-S)Cys/Gal(β1-N)Asn, respectively [14], and library **C**, containing Lac(β1-O)Thr) by solid-phase assays had yielded a series of compounds interfering with galectin binding (for a survey of sequences, see supplementary material) [15]. Briefly, the galectins shared a specificity for peptides having Cha, Phe, and Ile but did not show selectivity for galactose given that approximately 90% of the active compounds identified after the screening of libraries **A** and **B** with h-Gal-1 and h-Gal-3 were peptides. In contrast, nearly 90% of the identified ligands after the screening of library **C** with both galectins were glycopeptides, showing a strong preference for Arg and Lac(β1-O)Thr.

To assess the potency of (glyco)peptides to interfere with glycan-dependent binding, a series of representative h-Gal-1-reactive (glyco)peptides from each library (library **A: 7–13**; library **B: 14–16**; library **C: 17–23** [Table 1]) was resynthesized. In detail, (glyco)peptides were prepared on Wang resin containing the general solid-phase construct **6** (Fig. 1). Exact masses (Table 1), determined by high-resolution MALDI–TOF MS with internal calibration, confirmed the purity and identity of all synthetic compounds.

To determine the affinity of the representative galectin-reactive (glyco)peptides for the two galectins, interaction studies were initially performed using soluble (glyco)peptides and surface-immobilized galectins. Although for h-Gal-1 binding data were successfully obtained (data not shown), no binding data could be

Table 1
Analytical data of resynthesized h-Gal-1 lead structures

Sequence[a]		M + H calculated	M + H determined	Yield (%)	IC_{50} (μM)
7	PFFISR	1535.853	1535.986	57	385 ± 5
8	PFIChaFQ	1573.894	1573.897	38	NI
9	IIAITCha	1452.898	1452.896	54	NI
10	MFVChaChaR	1627.955	1627.958	64	NI
11	(Gal-)TIIQChaY	1721.952	1721.964	36	NI
12	PTIFFF	1540.836	1541.010	70	NI
13	GVFIChaA	1428.841	1428.871	69	NI
14	ChaVI(Gal-)NYQ	1720.931	1720.980	90	580 ± 20
15	YChaHChaYT	1658.910	1658.925	51	NI
16	PChaNChaVY	1567.904	1567.941	62	NI
17	PIF(Lac-)TRR	1883.007	1883.013	67	113.5 ± 13.0
18	FRPR(Lac-)TI	1883.007	1883.178	81	25 ± 1
19	IFRChaRY	1676.979	1676.974	77	NI
20	(Lac-)TChaRRFI	1939.069	1939.663	83	59 ± 9
21	AYRR(Lac-)TI	1872.986	1873.012	99	46 ± 4
22	SASS(Lac-)TR	1701.834	1701.850	93	234 ± 10
23	(Lac-)TMRA(Lac-)TCha	2150.047	2150.371	70	235 ± 15
	Lactose				675 ± 5

Note. NI, not inhibitory.
[a] All lead structures were synthesized on general solid-phase construct **6** and contain the IMP spacer (GPPFPFK) on the C-terminal side.

generated for h-Gal-3 because this galectin lost its activity on immobilization on the sensor chip. Therefore, an SPR-based competition assay was set up with asialofetuin as ligand to establish that the (glyco)peptides identified from the library screenings are able to inhibit carbohydrate-dependent binding. Note that asialofetuin is a pan-galectin ligand, with its reactivity being dependent on binding site occupancy exhibiting a gradient of decreasing binding constants [23]. The glycoprotein was immobilized on a flow cell of a CM5 chip (106 RU), and an untreated flow cell was used as the reference surface for h-Gal-1. The IC_{50} values of the (glyco)peptides and lactose were derived from the K_i values, which were determined by analyzing the SPR data using the following equation [21]:

$$f = [I]/([I] + K_i(1 + F_{im}/K_D)), \quad (1)$$

where f is the fractional inhibition, I is the inhibitor concentration, K_i is the solution affinity of the inhibitor for the galectin, K_D is the dissociation constant of the galectin for the surface, and F_{im} is the concentration of available binding sites. Prior to the inhibition experiments, the K_D of h-Gal-1 binding to asialofetuin was determined by titration of the surface with various galectin concentrations (20–0.16 μM [Fig. 2A]). The K_D value was obtained from a nonlinear fit of the data in a plot of R_{eq} (response at equilibrium) versus the galectin concentration (Fig. 2B). The value of R_{max} (14.7 RU) was also obtained from this plot and was used to calculate F_{im} by using the equation

$$R_{max} = (k'_p M_{galectin}) F_{im}, \quad (2)$$

where k'_p is the proportionality constant of 60,000 response units per unit concentration (g/L) of protein [22]. Values of 7.1 μM and 8.17 nM were obtained for K_D and F_{im}, respectively. It should be noted that the solid-phase assay with 1 μg asialofetuin in the coating solution or neoglycoproteins with triantennary *N*-glycans (0.5 μg) resulted in K_D values of approximately 0.5 to 1 μM [24,25] and that respective SPR data are known to vary with loading densities [26,27].

In the competition assays, immobilized asialofetuin and soluble (glyco)peptides competed for the binding to 5 μM h-Gal-1. A typical binding response in the presence of increasing inhibitor concentrations (structure **20**) is shown in Fig. 3A. Fractional inhibition values were calculated using the equilibrium response in the presence and absence of inhibitor. IC_{50} values (Table 1) were determined from nonlinear fits of the data in plots (Fig. 3B) of fractional inhibition versus the inhibitor concentration according to Eq. (1).

As can be seen from Table 1, only peptide **7** from library **A** and glycopeptide **14** from library **B** inhibited the binding of h-Gal-1 to asialofetuin slightly stronger than free lactose. In contrast, all Lac(β1-O)Thr-containing glycopeptides from library **C** (structures **17, 18,** and **20–23**) inhibited the binding 3- to 27-fold stronger

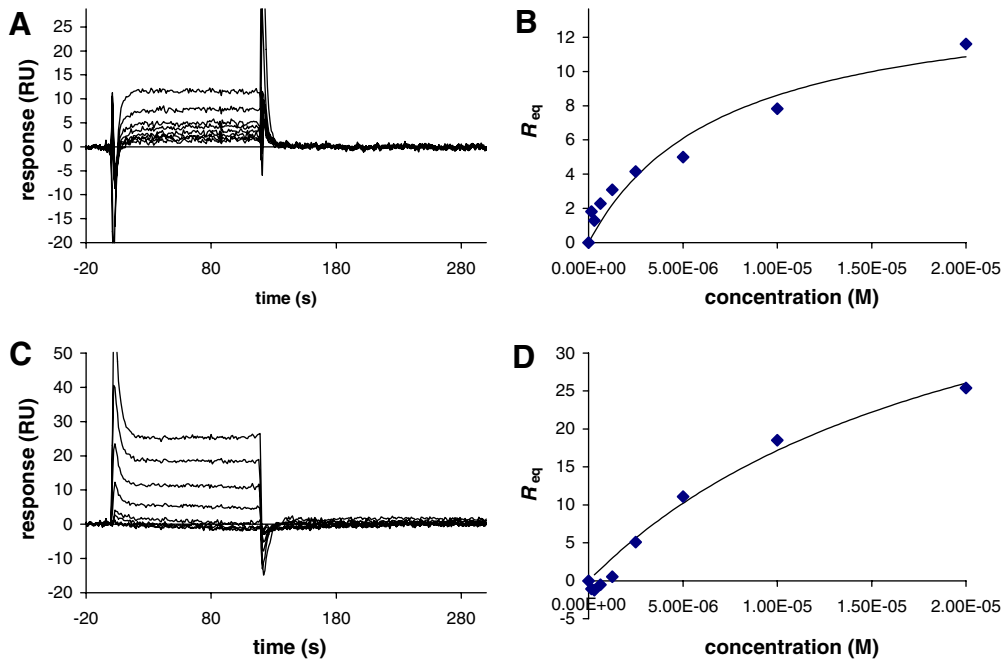

Fig. 2. (A) Binding of h-Gal-1 to immobilized asialofetuin at eight different concentrations between (top to bottom) 20 and 0.16 μM obtained by twofold dilution. (B) Nonlinear plot of R_{eq} versus the h-Gal-1 concentration. The experimental data were fitted using the steady-state model to obtain values for K_D (7.1 μM), R_{max} (14.7 RU), and F_{im} (8.17 nM). (C) Composite sensorgrams for h-Gal-3 as described in panel A. (D) Nonlinear plot for h-Gal-3 as described in panel B. K_D = 21.5 μM; R_{max} = 54 RU; F_{im} = 30 nM.

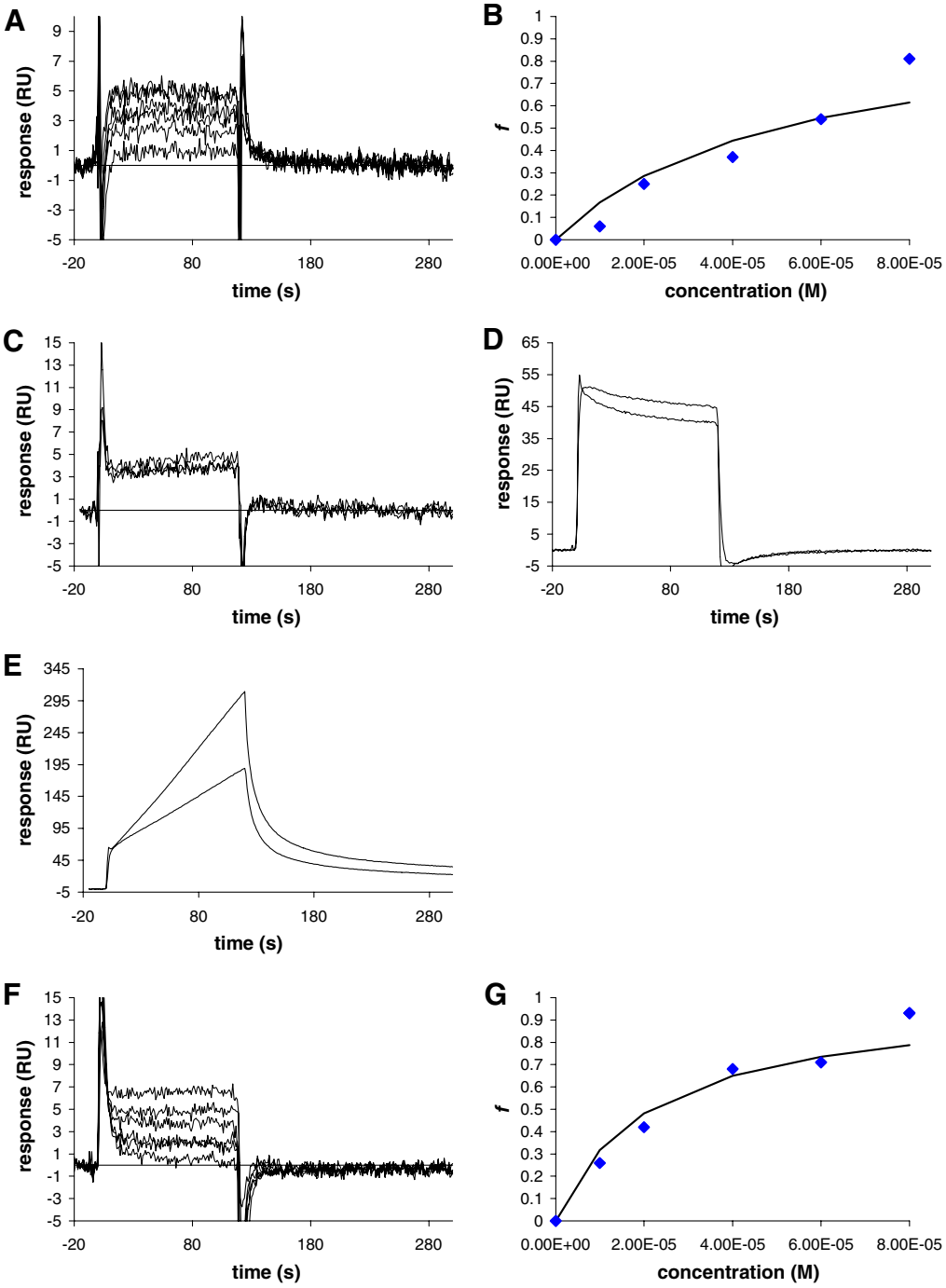

Fig. 3. (A) Inhibition of h-Gal-1 binding at 5 μM lectin concentration to asialofetuin in the presence of (top to bottom) 0, 10, 20, 40, 60, and 80 μM of glycopeptide **20**. (B) Fractional inhibition curve for the inhibition of h-Gal-1 binding to asialofetuin with glycopeptide **20**. (C) h-Gal-1 binding to asialofetuin in the presence of 200 μM (top) and 0 μM (bottom) peptide **13**. (D) Response of h-Gal-1 binding to asialofetuin (top) and reference surface (bottom) in the presence of 200 μM peptide **13**. (E) Response of h-Gal-1 binding to reference surface (top) and asialofetuin (bottom) in the presence of 400 μM peptide **13**. (F) Inhibition of h-Gal-3 binding at 5 μM lectin concentration to asialofetuin in the presence of (top to bottom) 0, 10, 20, 40, 60, and 80 μM of glycopeptide **25**. (G) Fractional inhibition curve for inhibition of h-Gal-3 binding to asialofetuin with glycopeptide **25**.

than free lactose. Of interest, (glyco)peptides that did not show any inhibitory activity affected the h-Gal-1 binding to asialofetuin in a different way. Peptide **13,** as a typical example, did not significantly influence the extent of h-Gal-1 binding at concentrations up to 200 μM (Figs. 3C and 3D). At higher concentrations, the signal changed, resulting in a response on the untreated flow cell that exceeded the response on the asialofetuin surface (Fig. 3E). As noted for hamster Gal-3 and increased homophilic interactions [28] and the KYWYR pentapeptide [4], aggregation might explain this phenomenon. Alternatively, these (glyco)peptides may interact nonspecifically or at a site other than the CRD given that initial binding studies using soluble (glyco)peptides and surface-immobilized h-Gal-1 indicated nonsaturable binding of the (galactosylated glyco)peptides to the galectin, whereas the binding of all lactosylated glycopeptides to immobilized h-Gal-1 was saturable (data not shown).

With respect to h-Gal-3, in addition to three peptides (**28, 30,** and **31** [Table 2]), five representative glycopeptides containing Lac(β1-O)Thr (**24–27** and **29**) were selected for resynthesis. Prior to the inhibition assays, the values for K_D (21.5 μM) and F_{im} (30 nM) of the binding of h-Gal-3 to asialofetuin (R_{max} = 54 RU) were determined as described for h-Gal-1 (Figs. 2C and 2D). A reduced affinity relative to h-Gal-1 properties is in accord with previous measurements using neoglycoproteins with triantennary *N*-glycans and the glycoprotein laminin [25,28]. Because an untreated reference surface did not give reproducible results, a flow cell containing immobilized BSA (260 RU) was used as the reference surface. As can be seen from Table 2, the subsequent inhibition assays showed that peptides **28** and **30** inhibited the binding of h-Gal-3 to asialofetuin approximately 7- and 10-fold stronger than free lactose; the IC_{50} value of compound **31** could not be determined because of low solubility. The compounds containing Lac(β1-O)Thr inhibited the binding of h-Gal-3 10.0- to 66-fold stronger than free lactose (e.g., glycopeptide **25** [Figs. 3F and 3G and Table 2]). Of note, the glycopeptide **26** was an efficient blocking compound of h-Gal-3 to human colon adenocarcinoma cells in vitro [15].

The presented results from the inhibition assays revealed differences in fine-specificity and affinity for h-Gal-1 and h-Gal-3, whereby h-Gal-3 generally displayed a higher affinity for its lead structures than did h-Gal-1. Although a few carbohydrate-free peptides were also effective, mostly peptides presenting lactose as ligand surpassed the free disaccharide's inhibitory potency. The finding that the lactosylated glycopeptides have a higher inhibitory potency than lactose itself indicates that the peptide backbone presents the carbohydrate favorably and most likely is engaged in interactions in additional contacts with the binding site of the lectins.

Conclusions

In this study, effective galectin inhibitors could be obtained from the screening of combinatorial solid-phase glycopeptide libraries despite the difference in (glyco)peptide presentation during the screening (multivalent) and in the SPR-based inhibition assays (monovalent).

In comparison with the haptenic disaccharide, especially lactose-exposing glycopeptides showed enhanced potency. These results reveal beneficial effects of presenting the carbohydrate ligand in distinct hexapeptide contexts. The further structural analysis of these complexes may disclose peptide–galectin contacts beyond the central lactose moiety. Whether they involve sites of the lectin active in binding of histo-blood group A/B-tetrasaccharides or the ganglioside GM1-derived pentasaccharide [29,30] is an open question.

Table 2
Analytical data of resynthesized h-Gal-3 lead structures

Sequence[a]		M + H calculated	M + H determined	Yield (%)	IC_{50} (μM)
24	N(Lac-)TFVRI	1842.964	1843.131	59	50[c]
25	P(Lac-)TVAPR	1733.911[b]	1733.885	59	24.8 ± 3
26	RVHY(Lac-)TR	1946.974	1947.157	63	11 ± 1
27	MR(Lac-)TR(Lac-)TR	2238.096	2238.101	81	3.8 ± 0.8
28	ChaChaRPMR	1634.972	1635.006	76	25 ± 5
29	(Lac-)TANY(Lac-)TR	2142.997	2143.015	74	37 ± 3
30	HHVYYH	1624.818	1624.793	84	34[c]
31	PFFFFF	1620.841	1621.001	52	ND
	Lactose				253 ± 2

Note. ND, not determined because of low solubility.
[a] All lead structures were synthesized on general solid-phase construct **6** and contain the IMP spacer (GPPFPFK) on the C-terminal side.
[b] Na^+ form.
[c] Not measured in duplicate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2008.04.023.

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