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# Discovery of galectin ligands in fully randomized combinatorial one-bead-one-compound (glyco)peptide libraries

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**Abstract**—The involvement of human lectins (galectins) in disease progression accounts for the interest to design potent inhibitors. Three fully randomized hexa(glyco)peptide libraries were prepared using the portion mixing method combined with ladder synthesis. On-bead screening with fluorescently labelled galectin-1 and -3 yielded a series of lead structures, whose inhibitory activity on carbohydrate-dependent galectin binding was tested in solution by solid-phase and cell assays. The various data obtained define the library approach as a facile route for the discovery of selective (glyco)peptide-based galectin inhibitors.
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Surface epitopes equip human cells with the versatility to interact with the environment. The frequent occurrence of glycoconjugates at this site signifies that they genuinely meet essential criteria to occupy this strategic position. Foremost, the glycoconjugate glycan chains have an unsurpassed capacity to encode biological information, thereby playing salient roles as molecular signals within the mentioned biochemical communication.[1,2] The already discovered effector/sensor functions raise numerous new opportunities for envisioning and establishing carbohydrate-based therapeutics.[3–6] Respective research in this area is given a clear direction, when the receptors for distinct glycan epitopes (lectins), which translate the sugar-encoded message into clinically relevant cellular responses, are known and available for testing.[7–9] Such a situation is now encountered in the case of the lectin family of galectins. Its members can drive malignant properties such as tumour spread, resistance to apoptosis or tissue invasion.[10–13] Consequently, this knowledge highlights the importance of defining the adhesion/growth-regulatory galectins as targets for drug design and, more specifically, to develop potent inhibitors.

To this end, combinatorial library techniques have widely superseded traditional screening approaches. If, for example, the one-bead-one-compound method for peptide randomization is combined with the ladder synthesis for compound characterization, it offers attractive, not yet tested, potential in this respect.[14–16] Why peptide libraries are a promising platform for testing becomes obvious when it is considered that galectins can specifically interact both with carbohydrate and peptide ligands, making them unusually versatile targets for the development of synthetic inhibitors.[8] Equally important, the synthetic preparation of peptides as glycomimetics still offers advantages over that of oligosaccharides. Moreover, since the carbohydrate recognition domain is not confined to binding a single residue (the term 'galectin' implies galactose as key site), the library's complexity can be instrumental in picking up potent inhibitors, which match the topology of more than the monosaccharide's docking site. Substantial increases in inhibitory activity on galectin binding have already been detected by adding a *p*-nitrophenyl group to a galactose residue[17,18] or by extending the sequence of the sugar structure.[19–21] The ability of the WYKYW pentamer to interfere with the carbohydrate binding of two avian galectins and galectin-1 supplies evidence for the possibility of peptides serving as glycomimetics.[22,23] Because structural analysis of the extended binding sites for carbohydrate ligands, more complex than

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lactose, is at an early stage,[24,25] experimental screening is the current means of choice to discover new lead structures, regardless of their chemical nature. Fully randomized one-bead-one-compound combinatorial libraries have so far not been tested for this class of human lectins. Here, the first application of this approach for discovering (glyco)peptides with reactivity to human galectins is reported.

The solid support, that is, lysine-functionalized poly(ethylene glycol)dimethylacrylamide copolymer ($PEGA_{1900}$ resin), was loaded with a fully randomized series of hexamer extensions for on-bead screening. Using established spacer and photolabile linker chemistry, as well as encoded ladder synthesis, an unambiguous sequence assignment for each library constituent was feasible by MALDI-TOF mass spectrometry.[16,26] The three prepared libraries were built up of the 15 natural amino acids Ala, Arg, Asn, Gly, Gln, His, Ile, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val, and nonnatural cyclohexylalanine (Cha). To exploit the sugar's capacity to direct the compounds into the galectin's carbohydrate recognition domain, both unsubstituted and glycosylated amino acids were used. Library **A** contained the β-Gal derivative of Thr [Gal(β1-O)Thr], library **B** the β-Gal derivatives of Asn and Cys [Gal(β1-N)Asn and Gal(β1-S)Cys] and library **C** the β-lactose derivative of Thr [Lac(β1-O)Thr]. Accordingly, screening then covered galectin reactivity towards hexa(glyco)peptides, which may or may not contain an amino acid derivative carrying a sugar unit. While streptavidin-based screening would involve indirect on-bead monitoring, the assays described here were performed in a direct way with fluorescently labelled (Alexa Fluor 488) human galectin-1 and -3. These galectins were selected because of their roles in tumour progression, and also cardiac dysfunction in the case of galectin-3.[10–13,27] Each screening step involved approximately 10,500 beads per library (25 mg) and an overnight incubation with 45 μg/mL galectin. Following careful washing steps, the bead populations were thoroughly inspected under the microscope. This process was successful, and fluorescent beads were detected. The two galectins apparently had affinity for distinct (glyco)peptides. As an inherent measure of selectivity, less than 1% of the beads in libraries **A** and **B**, and about 1–2% of the beads in library **C**, were significantly positive when evaluated by fluorescence microscopy. It can be concluded that both galectins are very selective in terms of interactions to hexa(glyco)peptides, posing the question on the chemical nature of the positive hexamers.

Manual selection of the most intensely stained beads, followed by sequence determination of the coupled peptide products, led to the characterization of a series of galectin-binding (glyco)peptides. A representative list is presented in Table 1 (for the complete panel, see Supplementary Material in Table I). Each compound in this table is capable of mediating binding of the tested galectin when presented in clustered arrangement on a bead's surface. In principle, the interaction can take place either within the carbohydrate recognition domain or at other sites. Even for the lectin site as the target, peptide sequences may not necessarily be similar, making predictions problematic. Consequently, a close inspection of the determined sequences is warranted. Reflecting homology and sequence divergence between the two galectin family members, the structure profiles of the (glyco)peptides are different, but follow common themes. Cha, Ile and Phe/Tyr moieties frequently show up in the two populations of 27 and 33 peptide sequences from libraries **A** and **B**. As to the effect of the monosaccharide, galactose was apparently not a major factor to convey high affinity to glycopeptides. Only a few peptide sequences were favourable. In these cases, the peptide backbone can apparently contribute to the interaction. Such a phenomenon was also observed for the *p*-nitrophenyl group in *p*-nitrophenyl glycosides, especially as thioglycosides.[17,18] When comparing the amino acid parts of the glycosylated amino acids, Asn was represented twice in the list of galectin-1 hits, and Cys once for galectin-3. To address the question on the effect of the presence of lactose in a library composition, the identified active compounds from library **C** were inspected next.

In accord with the substantially increased reactivity of lactose relative to galactose for galectin-1 and -3,[17,18] lead compounds from library **C** predominantly contained the β-lactosylated derivative of Thr. Sixteen out of 18 compounds for galectin-1, and 14 out of 16 compounds for galectin-3 were glycopeptides. A key factor

**Table 1.** Sequences of (glyco)peptides identified by on-bead library screening with fluorescent galectin-1 (**1**–**17**) and galectin-3 (**18**–**25**) and inhibitory capacity of the compounds in solid-phase assay using a glycoprotein as matrix

| Compound | | % inhibition |
|---|---|---|
| # **1** | PFFISR | 9 |
| # **2** | PFIChaFQ | 14 |
| # **3** | IIAITCha | n.t. |
| # **4** | MFVChaChaR | 22 |
| # **5** | PTIFFF | n.i. |
| # **6** | GVFIChaA | n.i. |
| # **7** | YChaHChaYT | 9 |
| # **8** | PChaNChaVY | 67 |
| # **9** | IFRChaRY | 70 |
| # **10** | (Gal-)TIIQChaY | n.i. |
| # **11** | ChaVI(Gal-)NYQ | n.i. |
| # **12** | PIF(Lac-)TRR | n.i. |
| # **13** | FRPR(Lac-)TI | 3 |
| # **14** | (Lac-)TChaRRFI | n.i. |
| # **15** | AYRR(Lac-)TI | n.i. |
| # **16** | SASS(Lac-)TR | 22 |
| # **17** | (Lac-)TMRA(Lac-)TCha | 38 |
| | Gal/Lac | 17/54 |
| # **18** | ChaChaRPMR | 28 |
| # **19** | HHVYYH | n.i. |
| # **20** | PFFFFF | 76 |
| # **21** | N(Lac-)TFVRI | 44 |
| # **22** | P(Lac-)TVAPR | n.i. |
| # **23** | RVHY(Lac-)TR | n.i. |
| # **24** | MR(Lac-)TR(Lac-)TR | n.i. |
| # **25** | (Lac-)TANY(Lac-)TR | 15 |
| | Gal/Lac | n.i./44 |

Cha, cyclohexylalanine; galactosylated (Gal) or lactosylated (Lac) derivatives of threonine or asparagine are denoted by (Gal/Lac-)T or (Gal-)N; n.t., not tested; n.i., not inhibitory at 10 mM.

for galectin binding in these instances appeared to be the presence of a lactose unit, in five cases even two moieties. Comparison of the hits from the three libraries suggests that an amino acid from the peptide backbone cannot substitute for the glucose moiety in the case of the galactosylated glycopeptide hits from libraries **A** and **B**. The lactosylated glycopeptides likely are competitors of sugar binding. Looking at the peptide portion, Arg residues shared frequent occurrence with lactose (Table 1; see Supplementary Material in Table I). They may be involved in proper sugar presentation and/or in direct contact(s). Otherwise, no consensus element could be discerned. So far, the (glyco)peptides have proven their activity when presented on the beads. To address the issues as to whether lead compounds maintain activity when assayed in solution, and whether these discovered structures for inhibitor design can indeed interfere with sugar binding, 25 representative (glyco)peptide sequences (Table 1) were resynthesized case-by-case and characterized (for complete analytical data, see Supplementary Material, Table II).

The prepared peptides were tested in an optimized solid-phase assay.[28,29] In this assay, a surface-immobilized glycoprotein (asialofetuin) presents its three galectin-reactive N-glycans with type I/II *N*-acetyllactosamine termini as docking sites.[30] For the interpretation of the results of these experiments, it is important to note that the (glyco)peptides are no longer conjugated to the beads in the typically high density. The change of assay conditions not only reduces the density, but also has an impact on secondary interactions described previously.[31] The solid-phase assays were performed under constant conditions at an identical inhibitor concentration of 10 mM. Galactose and lactose were included as internal standards to compare relative inhibitory capacities. Invariably, these two cognate sugars reduced the extent of carbohydrate-dependent binding, their efficacy showing the already noted difference (Fig. 1). Mannose as osmolarity control was negative. When tested in solution as inhibitor, (glyco)peptide hits emerging from screening with the cross-linking galectin-1, a proto-type homodimer, showed different levels of activity (Table 1, Fig. 1A). There were even two cases of peptides, which were more effective than lactose. The galectin-3 hits also showed activity differences in solution (Table 1, Fig. 1B). Library screening on beads thus yielded several compounds with lectin-inhibitory capacity in solution.

To answer the question on selectivity among galectins, assays were completed with both galectins and the full panel of resynthesized (glyco)peptides. Obviously, cross-reactivity of the lead compounds was possible between galectin-1 and -3 (Fig. 1A and B). To broad-

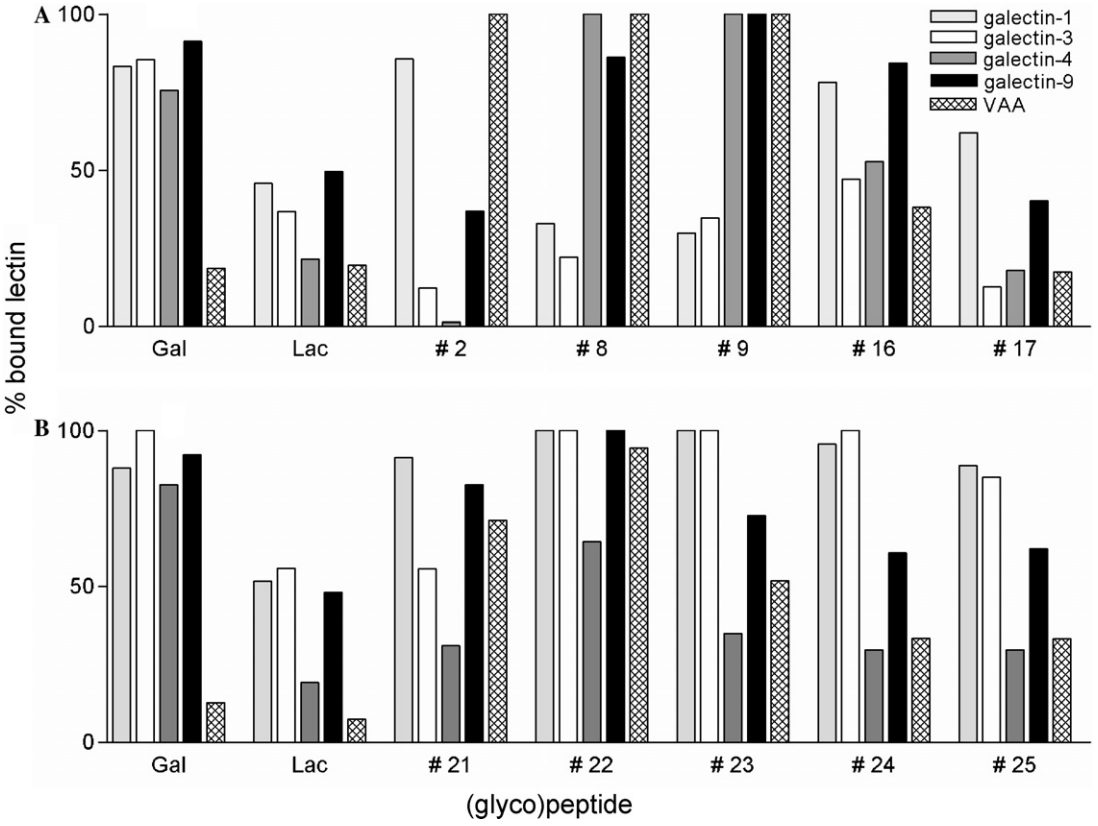


**Figure 1.** Representative illustration of inhibitory potency of resynthesized glyco(peptides) detected by on-bead library screening with fluorescent galectin-1 (A) or galectin-3 (B) in a solid-phase assay. The extent of carbohydrate-dependent binding of four human galectins and the plant toxin from *Viscum album* L. (VAA) to a matrix of asialofetuin (0.25 μg/well for the plant lectin and 0.5 μg/well for the human lectins) was assessed spectrophotometrically in the absence and presence of 10 mM inhibitor; the standard deviation in each case does not exceed 12%. As internal controls, galactose (Gal) and lactose (Lac) were used at the same concentration.

en the data basis beyond these two family members, two tandem-repeat-type proteins, namely, galectin-4 and -9, were also added to the test panel established by the proto/chimera-type proteins.[32,33] Differences in activity were seen and were pronounced for certain peptides from the galectin-1 hits, that is, compounds # **8** and # **9** (Fig. 1A). In contrast, the similar grading of activity relative to free lactose intimated that the disaccharide appears to be the main and commonly used contact of glycopeptides detected by galectin-3 during screening (Fig. 1B). So far, four members of the galectin family were tested, and it could be concluded that several lead compounds effectively reduced the extent of carbohydrate binding. When implying specificity of interaction, it can then be predicted that a lectin without similarity to galectins at the level of sequence and folding should react differentially to the presence of these compounds. To test this assumption, a plant agglutinin with β-trefoil folding, that is, toxin/agglutinin from *Viscum album* L. (VAA), was included in the assays.[34,35] The peptides failed to interfere with carbohydrate-dependent binding (Fig. 1A), whereas the glycopeptides showed a variable degree of inhibitory potency (Fig. 1B). On average, there is a clear trend for reducing the disaccharide's ligand activity by the peptide content. To proceed from the solid-phase measurements with a rigid model matrix and a single glycoprotein to the medically relevant situation of a native cell surface with natural complexity of glycan chains, some of the resynthesized lead compounds were subsequently tested as inhibitors of galectin binding to tumour cell surfaces.[28,36]

In detail, binding parameters (percentage of positive cells, staining intensity) were determined after fluorescent staining. The cells were incubated with the lectin in the absence or presence of inhibitors, special care being taken to test aliquots of the same cell batch to avoid variations. Using lactose as a standard, control carbohydrate-inhibitable binding of galectin to cells, and concentration dependence of the inhibitory activity, were first ascertained (Fig. 2, left panel). Differential activities among compounds were measured. The galectin-3 binding could even be blocked by lead compounds with a higher efficiency than lactose (Fig. 2, top and middle). The cell assays also revealed the efficacy of compounds # **13** and # **17** to interfere with the cell surface binding of galectin-1 (Fig. 2, bottom). Differences in ligand display may preclude a direct correlation between the solid-phase and the cell assays. Based on these results, on-bead screening-derived lead compounds, which reduce the association of galectin to tumour cell surfaces with the potency of lactose or even better, have thus been discovered. This result may suggest evidence for contacts between lectin and inhibitor beyond the sugar part of the glycopeptides.

In summary, this study has demonstrated that the tested approach works for the detection of (glyco)peptide ligands for human galectins. To pursue structural optimization with glycopeptides it is important that the activity of the sugar is maintained when adding a glycosylated amino acid. The case of 5-hydroxylysine, a common site

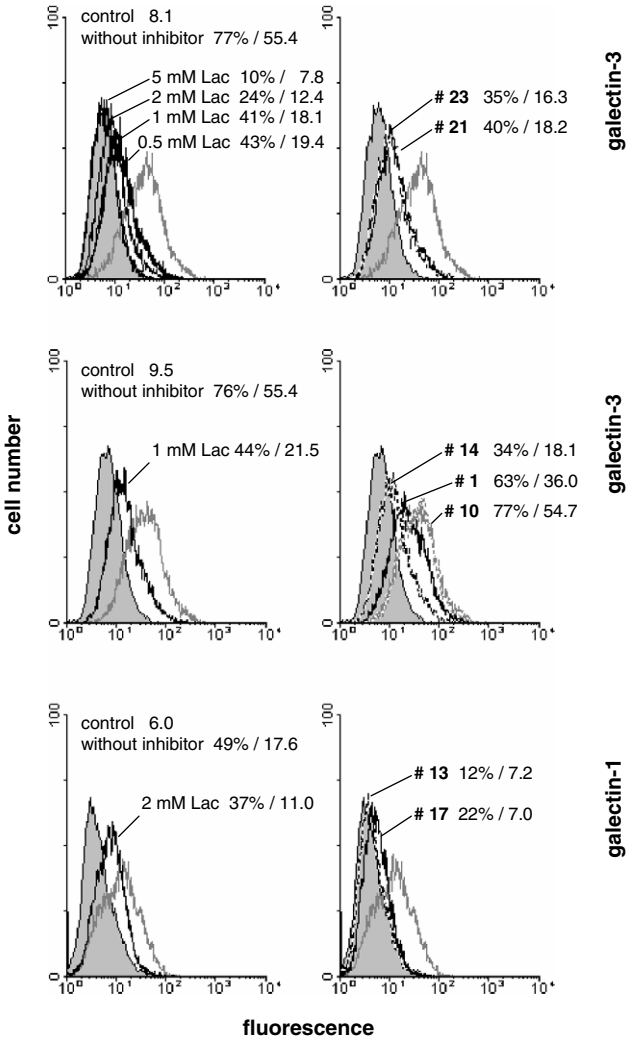


**Figure 2.** Semilogarithmic representation of the fluorescent surface staining of cells of the human colon adenocarcinoma line SW480 (top and middle panels) and the human pancreatic carcinoma line Capan-1, reconstituted for p16 expression (bottom panel), using 10 μg/mL labelled galectin-3 (top and middle panels) and 40 μg/mL labelled galectin-1 (bottom panel). The control values representing 0% in the absence of lectin and 100% in the absence of inhibitor are given as shaded area and grey line, respectively. Inhibition was tested at concentrations of 0.5, 1, 2 and 5 mM lactose, and 1 mM of the resynthesized glycopeptides # **21** and # **23** (top panel); 1 mM lactose and 1 mM of the resynthesized (glyco)peptides # **1**, # **10** and # **14** (middle panel); and 2 mM lactose and 2 mM of the resynthesized glycopeptides # **13** and # **17** (bottom panel). Quantitative data on the percentage of positive cells (%) and mean channel fluorescence are given in each panel.

of glycosylation in collagen, reveals the occurrence of negative effects in this respect.[37] In addition, two α-mannose-specific plant lectins (concanavalin A, *Lens culinaris* agglutinin) lose their sugar specificity when confronted with glycopeptides containing Man(β1-N)Asn.[38] The feasibility of the tested approach for this clinically relevant class of human lectins is thus proven. In comparison to other screening techniques, the search for hits was more successful than with a C7C phage-display library.[23] The parallel screening with galectin-1 and -3 and the addition of two other members of this lectin family furthermore revealed that products of the screen-

ing can apparently distinguish between galectins, at least to a certain extent. Computational work by modelling, using crystallographic information and homology-based calculations,[39,40] can help to attribute distinct sequence substitutions to differential (glyco)peptide reactivity. However, structural dynamics in solution, detectable as carbohydrate-ligand-induced changes in gyration radius or as loop rearrangements for galectin-1 and -3, respectively,[41,42] call for adequate consideration of this structural mobility in computational procedures. Next, not only the carbohydrate recognition domain matters for biological relevance, and thus as target for drug design. Although intuitively restricted to carbohydrates as ligands, galectins are engaged in protein–protein interactions besides their activity as carbohydrate receptors.[8,43] A blocking (glyco)peptide may well be derived from such a screening when occupying the site for interaction between oncogenic H-Ras and galectin-1 or the bcl-2-like NWGR quartet in the carbohydrate recognition domain with assumed relevance for anti-apoptotic potency of human galectin-3.[44,45]

In order to proceed from this proof-of-principle study, adding a new aspect of clinical relevance to glycopeptide research,[46] the following routes to drug design can be pursued:

(a) Specificity can be enhanced by elaborating the sugar part, for example, by exploiting differences to natural ligands such as (α2-6)-sialylated *N*-acetyllactosamine dimers (DiLacNAc) or to 3′-functionalized *N*-acetyllactosamine.[25,47,48] Also, the peptide part of the lead compounds may, in contrast to wheat germ agglutinin[38] and sialoadhesin,[26,49] play a more active role than simply acting as a scaffold for carbohydrate presentation. The example of selectin ligands, derivatized with a branched hydrophobic anchor to gain intrafamily selectivity, encourages respective efforts.[50,51]
(b) Affinity can be enhanced by restricting the intramolecular flexibility of peptides, also a conundrum for devising peptides as mini-lectins.[52] Cyclization of lead compounds via disulfide bridging or deliberate screening of libraries of cyclic (glyco)peptides[53] will be instrumental to explore the impact of this parameter change.
(c) Affinity and intrafamily selectivity can be enhanced by exploiting the different cross-linking capacities of galectins and their avidity to multivalent ligand display.[28,54,55]

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.10.067.

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