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Identification of peptide ligands for malignancy- and growth-regulating galectins using random phage-display and designed combinatorial peptide libraries

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Abstract—Members of the galectin family of endogenous lectins are involved in tumor growth regulation and in establishing characteristics of the malignant phenotype via protein–carbohydrate and protein–protein interactions. To identify peptide ligands with the potential to modulate these tumor-relevant interactions beneficially, complementary screening methods were employed, that is, both phage-display and a combinatorial pentapeptide library with the key YXY tripeptide core. Three representative prototype galectins were selected. The search for high-affinity ligands among phage-displayed random heptamers yielded enrichment after five selection cycles of the nonglycomimetic CQSPSARSC peptide in the case of the chicken homologue of galectin-1 but not the human protein, an indication for specificity. The most active glycomimetic from the combinatorial library of 5832 pentamers was WYKYW. Identification of peptide ligands for galectins with and without glycomimetic properties is thus possible. Our study documents the potential to combine the two library-based approaches for structural optimization of lead peptides.

1. Introduction

Galectins are becoming a major focus of research activity in medicinal chemistry.[1] This is due to their roles as cell-type-specific pro- or anti-apoptotic effectors, their roles in regulating tumor cell adhesion and migration and their role as prognostic markers in tumor pathology. Galectins bind to β-galactosides but the prototype galectin-1 and chimeric galectin-3 are also engaged in protein–protein (lipid) interactions with relevance for the malignant phenotype. The interaction of galectin-1 with oncogenic H-Ras facilitates the transforming protein's routing and ensuing cell activation.[2] This inherent versatility for binding partners requires a broad range of screening methods, when searching for pharmaceutically active modulators of galectin binding and signaling that either interfere with undesired interactions or enhance clinically useful recognition. Explicitly, identification of effective, readily synthetically accessible galectin-binding compounds is our goal, regardless of whether they harbor glycomimetic properties or not. In our previous pilot study, we provided proof-of-principle evidence for pentapeptide sequences containing an YXY core that affected carbohydrate-dependent galectin binding.[3] An active pentapeptide ligand was shown to bind in the vicinity of the central Trp residue in a galectin's carbohydrate recognition domain.[3] Interestingly, binding was regulated in an inhibitory or stimulatory manner, depending on the galectin type.[3] The latter case carries potential application to further enhance the negative growth regulation caused by galectin-1 seen for certain tumor cell types, for example, neuroblastoma, and activated or malignant T cells.[1i,4] In order to take the next step from this initial discovery toward identifying potent

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and selective peptide ligands for galectins, we pursued two approaches: phage-display screening and screening of a designed combinatorial library.

Screening of phage-display libraries has allowed the use of glycomimetic peptides for vaccination in order to elicit an anti-carbohydrate immune response, especially against capsular polysaccharides of infectious bacteria.[5] Access to such antibodies has started to answer the question on the individual binding modes of carbohydrate and peptide ligands to an antibody's combining site, and consequently the potential for rational design of the peptide lead structure arises.[6] Regarding lectins, phage-displayed peptide library screening was shown for E- and P-selectins.[7] However, the uncovered sequences may not be structural glycomimetics in all cases. In one case the screening was performed with an anti-Le^a antibody to overcome the inherent low affinity of individual lectin–carbohydrate contacts.[7] Ligand binding to E- and P-selectins takes place at a shallow surface area and is almost entirely electrostatic in nature, and involves Ca^{2+}–OH contacts typical of C-type lectins. Binding to galectins is considerably different with extensive van der Waals contacts, hydrogen bonds, and C–H/π-electron interactions.[8] Nonetheless, the work with selectins used random libraries of peptides presented in a conformationally restrained manner illustrating the thermodynamic advantage of such a presentation. After all, carbohydrates as ligands are not highly flexible molecules, and galectins select one of several conformers from solution, to avoid entropic costs.[9] We set out to search for galectin ligands using a disulfide-constrained random heptamer library as an N-terminal fusion to the phage coat protein pIII. To initially focus on high-affinity interactions, the low density of surface presentation with up to five copies of this epitope per phage was selected.

In parallel, we pursued an approach using a combinatorial peptide library with knowledge-based input. This input originated from the identification of the YPY motif as a crucial part of hexapeptides that acted as functional mimics of carbohydrate ligands for the mannose-binding plant lectin concanavalin A. Furthermore the sequence is also a potent epitope that elicits tumor-cell-binding antibodies and it also forms the central part of an anti-idiotypic antibody (CDR3 domain of the H chain of 6F9), which mimics meningococcal group C capsular polysaccharide.[10] The occurrence of the related PWLY or WMY motifs was also found to be critical in the binding to several carbohydrate binding proteins. Examples include octapeptide binding to the Le^y-specific monoclonal antibody B3, or a heptapeptide binding to the protective 2H1 monoclonal antibody against cryptococcal glucuronoxylomannan. Other relevant illustrations of the importance of these motifs are the conserved active-site-contacting WRY tripeptide in proteinaceous α-amylase inhibitors from *Streptomyces* (e.g., tendamistat), the WHW tripeptide as the smallest functional unit of a 15-mer peptide binding to ganglioside GD1a and the observed W cluster in a hexapeptide inhibitor of a sialyltransferase (ST3Gal I). These examples all make a strong case for the importance of aromatic residues with stacking as well as hydrogen bonding ability in the binding to various sugar receptors.[5a,11] The observed sequence variation of the peptides is linked to the variation of the hexopyranoses they mimic. This suggests the possibility to discover specific sequences for galectin ligands, that is β-galactosides. This notion along with our initial experience with a small library[3] that yielded sequences with galectin specificity and no affinity for concanavalin A bodes well for a successful expanded screening. In order to find improved sequences we increased the size of the combinatorial library more than 50-fold to a total of 5832 peptides, incorporating the YXY core as a fixed unit in all sequences. Despite the design feature, in principle, the peptides might also interact with the proteins at sites different from the carbohydrate recognition domain.

In this study we combine the search for galectin ligands by a phage-displayed random heptamer library with a similar search using a designed combinatorial pentapeptide library with a fixed core. More than one galectin was screened in order to identify possible specificity. We focused on human galectin-1 (h-Gal-1) due to its documented roles in tumor growth regulation and invasion and on chicken galectin-16 (CG-16) with its distinct sequence but similar overall prototype structure.[1,4,12] Interestingly h-Gal-1 has a known capacity to bind a noncarbohydrate ligand.[2] Furthermore, with a human and a homologous avian galectin in the test panel we deliberately selected two evolutionarily separated members of this family to introduce a control for specificity, based on the rather early divergence of mammals and birds compared to different prototype lectins in a species. We added chicken galectin-14 (CG-14), a second avian prototype galectin, due to its fine-specificity differences, which reflects the slightly divergent topologies of the sugar-combining sites.[12] Including this galectin thus provides a sensitive measure for sensitivity of closely related galectins for possible hits from the library.

2. Results and discussion

2.1. Affinity selection by phage-display

The aim of this part was to detect peptide heptamers with affinity for a galectin. Approximately 2×10^{11} infectious phages originating from the original C7C library (cysteine flanked heptamers), containing about 200 copies of each sequence, were screened in repetitive cycles. Five consecutive rounds of affinity selection were carried out, using biotinylated galectins. Activity assays on the galectins after labeling had ensured that biotin labeling did not impair lectin activity. Incubation with the phage library was performed in solution followed by exposing the library to a streptavidin-coated surface. An excess of label-free galectin was used to dissociate phage–galectin complexes from the surface, thus selecting for peptides interfering with both protein–carbohydrate and protein–protein interactions. This setting, with consecutive cycles of affinity selection and a low surface density of the random peptides (only up to five proteins pIII are expressed by a single phage), is suitable for selecting

strong interaction processes. Subjecting 10 individual clones after the fifth selection round to sequencing yielded no enrichment for a distinct sequence in the case of h-Gal-1. In fact, 10 different sequences with no obvious consensus element were obtained, only one of them harboring a hydrophobic patch with CPTSLLWWC. Frequent presence of polar side chains as in the found CQRSPHSTC sequence is a sign for potential of directional hydrogen bonds but the lack of a consistent sequence preference emerging from sequencing casts doubt on the general relevance of these findings. In this sense, the situation for phage-displayed binding partners of CG-16 was notably different. Six clones had an identical sequence with CQSPSARSC, three other clones selected showed the heptamer sequence GAHRLHQ between the flanking cysteine residues. The remaining tenth clone had a hydrophobic patch with YSFPHTT.

Evidently, the phage-display screening identified a high-affinity peptide for CG-16 but not for h-Gal-1. Thus, the technical approach proved to be productive, although the positive data for CG-16 will not really account for an immediately obvious medical perspective. In this respect, the negative result supports the notion that galectin-1/oncogenic H-Ras may primarily interact via the suitably presented farnesyl chain, rather than through protein–protein interactions.[2c] Also, no selection of a potent glycomimetic sequence with a hydrophobic core was apparent. For h-Gal-1 modifications for improved screening are being implemented and include the use of dodecamer libraries, the use of major coat protein pVIII with up to 2700 copies per phage,[13] and switching from interaction in solution to panning on a lectin-presenting surface.

Emerging from the screening thus was one heptamer sequence with preferential representation in the phage population in the case of CG-16. In addition, a second sequence showed up three times in 10 clones, an indication for enrichment to be followed, too. Although the sequences were definitely part of the initial library, no equivalent selection was apparent for h-Gal-1, excluding the heptapeptides as pan-galectin ligands, but in contrast underscoring the inherent specificity. Hence, two potential candidates for CG-16 were identified, and further analysis in binding assays followed (Section 2.3).

2.2. Affinity selection of the combinatorial peptide library

We previously reported a proof-of-principle study identifying the YXY motif as part of lectin ligands mimicking galactose, by screening a small peptide population (100 pentamers). The spatial vicinity of the best ligand to the central Trp residue of the carbohydrate binding domain was established by an NMR spectroscopic technique.[3] Here we considerably extended the size of the library to a total of 5832 sequences. The split–mix technique enabled the preparation of a one compound–one bead library,[14] by solid-phase synthesis on PEGA resin. This hydrophilic copolymer has proven versatile in the screening of resin-bound compounds with proteins.[15] It is compatible with biomolecules of up to 35 kDa and, therefore, well-suited for on-bead screening with galectins.

The resin was loaded with glycine and the growing peptide chain was constructed in a stepwise manner using standard Fmoc chemistry with a set of 18 different amino acids.[16] It included unnatural (D) amino acids as well as a fluorinated phenylalanine. The unnatural building blocks were intentionally selected to complement the phage-display approach. Moreover, an enhanced in vivo stability of peptides containing D-amino acids is expected due to reduced proteolytic degradation. Any of the 18 amino acids were introduced at positions 1, 3, and 5 of the pentapeptides while keeping positions 2 and 4 constant as a tyrosine. Upon completion of the combinatorial library, an Fmoc determination was performed giving a value of ~0.4 mmol/g, after correction for the mass of the peptide, which was identical to the initial glycine loading. This result is consistent with the notion that the synthesis was successful. After deprotection of the terminal Fmoc group, acid-labile side chain-protecting groups were removed with a mixture of TFA, H_2O, TIS, and EDT.

Screening of the bead population with its multivalent display of peptides was performed using the biotinylated galectins CG-14 and h-Gal-1. After incubation of the library suspension overnight with the biotinylated galectins at a concentration of 1.5 μM, the solution was removed from the beads by filtration. The biotin label was exploited to direct the streptavidin-peroxidase conjugate to the galectin-containing beads. After removal of the conjugate by filtration, the peroxidase substrates *o*-phenylenediamine and H_2O_2 were added and the color-producing reaction was stopped with addition of a solution of 2 M H_2SO_4. A reaction time of 15 min made a clear distinction possible between intensely and weakly stained beads. For the h-Gal-1 screening, about 1% of the beads were markedly darker than the rest. Less selectivity was seen at increased protein concentrations (3–5 μM) with up to 4–5% of the beads being intensely colored. This result signifies that over 95% of the YXY-harboring peptides are not engaged in lectin binding, a clear measure of specificity.

A pre-selection of about 20 of the most intensely stained beads was made by using a microscope. From this pool, again the most intensely stained beads were picked out and the sequences of its attached peptide were determined by standard automated Edman degradation.[17] This procedure was followed for three separate screening runs per galectin. In order to conveniently decipher a trend for the nature of the amino acids in each position that was varied, 10 of the darkest beads were mixed together and subjected to a single standard Edman degradation procedure. This bulk-decoding method does not identify distinct individual sequences but assigns a series of amino acids to the positions 1, 3, and 5 of the peptides (Fig. 1). In addition to the two analyses by bulk decoding for both galectins, seven beads were individually decoded as well (Fig. 1). As a negative control to exclude lectin binding to the matrix, unfunctionalized PEGA-NH_2 resin was subjected to the same screening procedure. As expected, this analysis yielded only colorless beads, indicating the absence of nonspecific binding of the lectins to the resin itself.

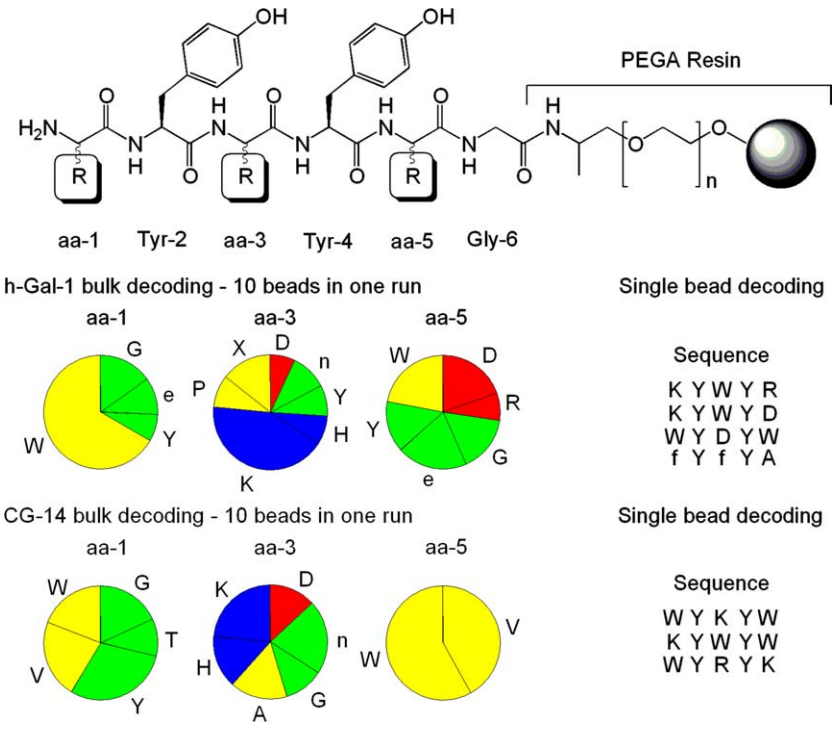
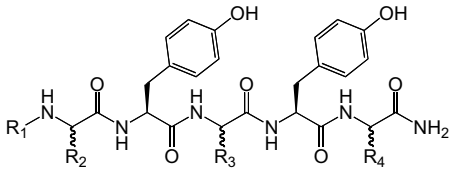


Figure 1. The structure of the peptide library synthesized on PEGA resin showing the regions of variability (top panel). Seen below is the summary of the results obtained from Edman degradation sequencing. The pie charts illustrate results for sequencing 10 beads in one run. A notable preference for Trp is seen in positions 1 and 5, whereas position 3 between the two central tyrosine residues is primarily occupied by either charged or at least polar residues. Amino acids are given in the one letter code and the lower case indicates the D-form. Color code: yellow—hydrophobic residues; green—polar residues; blue—basic residues; red—acidic residues.

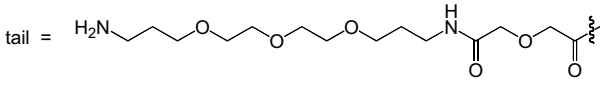
Standard Edman degradation cannot differentiate between D and L amino acids. Therefore, in order to obtain unambiguous results D-Ser and L-Thr, D-Glu and L-Asp, D-Asn and L-Gln, D-Leu and L-Val were used. The unnatural amino acid 4-fluorophenylalanine was observed to co-elute with leucine upon standard Edman degradation analysis. Consequently, an ambiguity in sequencing was inevitable, a concern which was actually only relevant once, that is, at position 3 when screened with h-Gal-1 in the decoded beads. As an internal control for the integrity of the bead-attached peptides and the quality of the synthesis, tyrosine had to be found at positions 2 and 4. Indeed, all sequenced beads contained tyrosine at these positions.

Compilation of the obtained sequence information allowed us to discern any preferential occurrence of a certain amino acid in the pentamer. A distinct preference for Trp was observed for the variable positions, profoundly extending data of our previous report where we had not included Trp into the panel of amino acids.[3] The representation of the charged amino acid residues Lys, Arg or Asp signified that this independent screening is in accord with our previously identified sequences from the 100-member library with predominance of charged and aromatic residues. Figure 1 illustrates the distinct preference for Trp from the panel of 18 different moieties at position 1 (in 12 of the 27 decoded beads), Lys at position 3 (8/27) and Trp at position 5 (11/27). As predicted from these data, the resulting WYKYW sequence was in fact also found by peptide sequencing of an individual bead. Although Arg and Pro were present in the amino acid mixture, Lys was clearly selected versus Arg and Pro in contrast to reported peptides binding concanavalin A or 6F9 antibody, while the preference for an aromatic moiety at the N-terminal flank is shared.[10] Compatible with the overall structural homology of the galectins and their common specificity for D-galactose, a similar pattern of peptide selection emerged between the two galectins tested (Fig. 1). This situation is different from the picture established by the data of phage-display screening. When next comparing selectivity of similar functional groups with different stereochemistry, Asp (found 6×) was preferred over D-Glu (3×), D-Asn (3×) over Gln (0×), and Val (6×) over D-Leu (1×). All together, these results considerably broadened the basis for validating the concept of an extended YXY motif serving as potentially glycomimetic peptide ligand. In fact, both stacking interactions with the galectins' central and essential Trp, an invariant characteristic of the binding pocket, and electrostatic interactions with polar side chains positioned around this moiety are hereby possible.

To validate our peptide selection obtained by the two different screening systems, we proceeded to synthesize particular representatives of the peptide library. In detail, resynthesis for screening included mostly pentamers identified from individual bead decoding. The only exception was compound **5** with an Asp placed at the fifth position to reflect data from bulk decoding and to test the influence of an acidic residue in this position.



R_1	R_2	R_3	R_4	Compound
H	D-Ser	Lys	D-Ser	**1**
tail	D-Ser	Lys	D-Ser	**1a**
H	Lys	Trp	Arg	**2**
tail	Lys	Trp	Arg	**2a**
H	D-Phe	D-Phe	Ala	**3**
tail	D-Phe	D-Phe	Ala	**3a**
H	Trp	Asp	Trp	**4**
tail	Trp	Asp	Trp	**4a**
H	Trp	Lys	Asp	**5**
tail	Trp	Lys	Asp	**5a**
H	Trp	Lys	Trp	**6**
tail	Trp	Lys	Trp	**6a**



Sequence	Compound
Ac-CQSPSARSC-NH_2	**7**
Ac-CQSPSARSC-NH_2 (S–S)	**8**

Figure 2. Structures of the resynthesized peptide sequences identified with the on-bead screening (**2**–**6a**) and with the phage-display library (**7**, **8**). Compounds **1** and **1a** are control peptides.

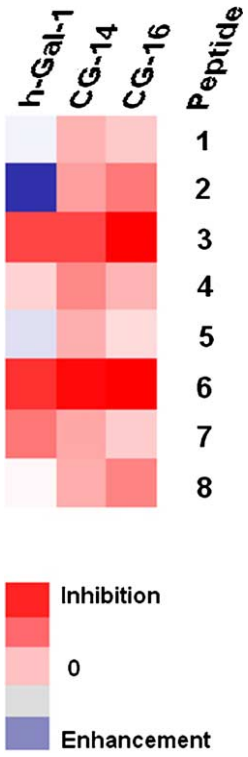


Figure 3. Summary of results of the solid-phase inhibition assay. The gradient to red indicates increasing inhibition of lectin binding to the glycan chains of asialofetuin, the gradient to dark blue indicates enhanced lectin binding. Results are averaged over at least two independent assays. The peptides were screened at 4 mg/mL with h-Gal-1 (0.5 μM), CG-14 (1.5 μM), and CG-16 (1.5 μM) at room temperature.

As a control we included compounds **1** and **1a** into the panel. Because serine was clearly not represented in the population of galectin-binding peptides obtained from on-bead screening (Fig. 1) but prominently resided in the phage-display defined peptide, we placed D-Ser twice herein (Fig. 2). In order to overcome solubility problems of the hydrophobic YXY-containing sequences a hydrophilic tail intended to improve solubility was covalently attached.[3] Indeed, it increased the solubility considerably. The list in Figure 2 also includes the CG-16-specific heptamer identified by phage-display and its cyclic version containing the disulfide bridge, which we prepared to mimic the peptide's presentation in the coat protein of the M13 phage. This panel was used as test substances in solid-phase assays to determine their capacity to interfere with galectin binding to glycans whose presentation on a plastic surface is a model for cell surface properties.

2.3. Activities of peptide penta- and heptamers as ligands

In the solid-phase assay the peptides were tested for their ability to interfere with carbohydrate-dependent binding of the galectins. Both functional and structural mimicry will thus become detectable. The results were visualized by using the Treeview program v. 1.60 developed by Eisen[18] (Fig. 3). The presence of peptides caused different effects, the control peptide (**1**) having at best weak activity. A general stickiness of peptides with a core of aromatic amino acids affecting sugar binding could be excluded. Also, the data show a non-identical response of the three galectins and thus indicate fine-specificity differences.

Clearly, compound **6** stands out amongst the other sequences by showing strong inhibition with CG-14, CG-16, and h-Gal-1. To address the concern that the polar tail might influence binding properties, sequences screened with the tail showed rather similar results when compared to their underivatized counterparts (data not shown). The only exception is the sequence fYfYA (**3**) that yielded strong inhibition with h-Gal-1, whereas its derivatized counterpart **3a** led to strong enhancement, indicative of a delicate balance between the two processes. We also monitored the effect of the individual peptides on carbohydrate-dependent binding of h-Gal-1 to an asialofetuin functionalized chip in a surface plasmon resonance setting. The binding of the galectins could be easily observed and the effect of the peptides on this process was monitored. Again the WYKYW sequence, present in **6a** showed the strongest activity, however, instead of inhibition a signal enhancement was observed (Fig. 4A). The enhancement is concentration dependent and saturates with an IC_{50} of around 1 mM (not shown). Peptide **2** also showed a signal enhancement while the control peptide **1** as well as the other identified sequences showed only minor effects. The same experiments were repeated with CG-14 and showed a similar

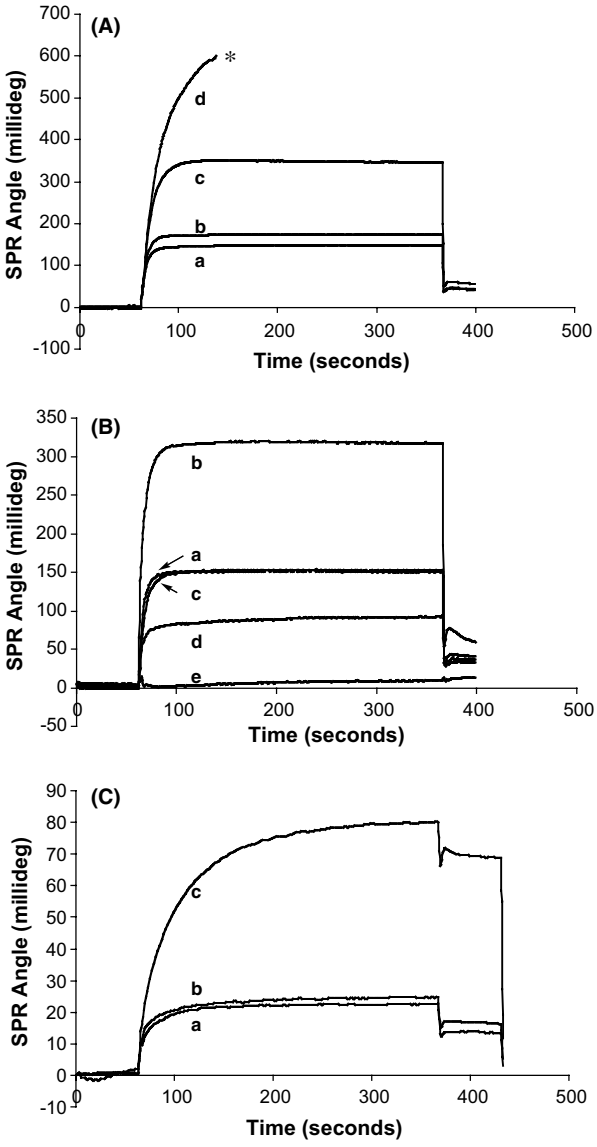


Figure 4. Observed SPR signal as a function of time for the following experiments (HBS, pH 7.4, 25 °C): (A) (a) 1 μM h-Gal-1, (b) 1 μM h-Gal-1 + 5 mM **1**, (c) 1 μM h-Gal-1 + 5 mM **2**, (d) 1 μM h-Gal-1 + 5 mM **6a**, * out of the SPR detector range; (B) (a) 1 μM h-Gal-1, (b) 1 μM h-Gal-1 + 1 mM **6a**, (c) 1 μM h-Gal-1 + 1 mM **6a** + 0.2 mM lactose, (d) 1 μM h-Gal-1 + 1 mM **6a** + 0.7 mM lactose, (e) 1 μM h-Gal-1 + 1 mM **6a** + 4 mM lactose; (C) (a) 2.5 M CG-16, (b) 2.5 μM CG-16 + 1 mM **7**, (c) 2.5 μM CG-16 + 1 mM **8**.

profile, yet with a smaller degree of the signal enhancement with peptide **6a** and **2**.

The strongest enhancement of the SPR signal effect was seen for **6a** in combination with h-Gal-1. This effect could be reduced by the addition of the galectin ligand lactose (Fig. 4B). A 1 μM solution of h-Gal-1 with 1 mM **6a** (tail-WYKYW), whose sequence was an inhibitor in the solid-phase assay, showed a doubling of the equilibrium SPR signal as compared to a run without the peptide present. However, when increasing amounts of lactose were added as well, the signal size at equilibrium progressively decreased and full inhibition was observed at a lactose concentration of 4 mM (Fig. 4B). A cross-linking/aggregating ability might account for this behavior. It is presently not clear whether the peptide itself can aggregate in solution, hereby forming more than monovalent ligands, or whether it remains monomeric when binding to the lectin. Since the affinity of the peptide–galectin interaction is still relatively weak, studying the effects of ordered multivalent presentation of the peptides by their attachment to a dendrimeric scaffold may address this question. Further information could come from structural analysis to pinpoint functional or structural mimicry. As previously found, the topology of dendrimers is also a determining factor for modulation with preference for distinct galectins.[21] Also, a functional mimetic might offer the way to the design of a bifunctional ligand homing in on different sites of a galectin, a further means to enhance selectivity. Because more than one galectin is commonly expressed by human tumor cells,[1a,22] it is essential to have tools in hand to target individual lectin types.

Compared with the most potent pentamers from the combinatorial library the phage display-derived heptamers were only weakly active in the solid-phase inhibition assay (Fig. 2). However, it should be kept in mind that the selection process was deliberately designed not to only favor glycomimetic peptides by elution with lectin instead of lactose. Peptide **8** at a concentration of 4 mM had negligible effects with CG-14, slightly enhancing effects for h-Gal-1, and slightly inhibitory effects for CG-16. We tested the hypothesis given above that h-Gal-1 should be a negative control by going up to 10 mM. Binding of h-Gal-1 was not measurably altered, a result expected from the screening data. In contrast, the level of signal for CG-16 was decreased by 15%, an indirect indication for peptide binding and an effect of this process. The linear peptide had to be added at 16 mM to reach 15% inhibition. For comparison, lactose presence at 3 mM already inhibited binding by 85%. Reflecting the decrease in frequency of occurrence in sequenced clones, assays with the second peptide from phage-display screening, tested in linear and cyclic peptide versions, invariably yielded results which were not significantly different from the control values obtained in parallel. Of note, we tested concentrations up to 16 mM, where presence of the more predominant heptamer had slightly but consistently inhibited binding. Thus, compared to lactose the heptamers were at best very weak inhibitors of carbohydrate-dependent binding, rendering it rather unlikely that the screening process has come up with phage-displayed glycomimetic shapes. Conformational restraint by disulfide cyclization excluded the possibility that the flexibility of the free peptide was the cause of the weak binding. Although rarely tested comparatively, conformational constraint was advantageous for affinity and specificity of oligopeptides binding to monoclonal antibodies against menigococcal lipooligosaccharide.[19] At any rate, a certain degree of effect in agreement with the selectivity toward CG-16 was apparent. Also, the assays with both heptamers from clone sequencing indicated a difference in target specificity. Because lactose as a ligand triggers a conformational change detected for h-Gal-1 by NMR spectroscopy and by small angle neutron scattering,[20] it

is possible that association of a peptide at a different site might account for topological changes in the combining site for the sugar. Fittingly, testing of CG-16 with compounds **7** and **8** by surface plasmon resonance provided further evidence for an effect on the galectin. The cyclic peptide strongly increased the equilibrium SPR signal intensity and it did so with slow kinetics (Fig. 4C). The presence of the linear peptide did not affect the signal size. The signal enhancement observed with the cyclic peptide is an indication for an aggregation of CG-16 in the cyclic peptide's presence.

3. Conclusion

We have examined two independent approaches to identify peptide ligands for the medically relevant family of galectins. Initial screening for high-affinity ligands by phage-display yielded one hit for CG-16 but not for h-Gal-1, underscoring target specificity. Due to the low activity of the sequence in the solid-phase inhibition study and its high aggregation power in the SPR assay where no washing steps are involved, it is likely that the peptide is not a glycomimetic, but rather binds at another site of the galectin. The parallel screening of the combinatorial peptide library with fixed YXY core significantly corroborated and profoundly extended our initial observation of pentapeptides as a new class of glycomimetic ligands. Tryptophan (Trp), which was now present in the library, was also prominently present in the identified hit sequences, especially at the flanking positions. Lys (but not Arg) featured prominently in the central position. The WYKYW sequence was identified as a potent glycomimetic in solid-phase inhibition assays. Under the conditions of the surface plasmon resonance experiment, lectin aggregation was favored. Our study revealed that it is possible to identify peptides as galectin ligands. Moreover, concerted action of the two screening methods by exploiting the information from further extended phage-display screenings for design input into a combinatorial library is likely a promising strategy for identifying even more potent and selective galectin ligands.

4. Experimental

4.1. Reagents and analytical/preparative procedures

Chemicals and resins were obtained from commercial sources and were used without further purification unless stated otherwise. *N*-Methylpyrrolidone (NMP), CH_2Cl_2, trifluoroacetic acid (TFA), CH_3CN, and MeOH were purchased from Biosolve (Valkenswaard, The Netherlands). Piperidine and *i*Pr_2NEt, were obtained from Acros Organics. Triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), divinyl sulfone, and HPLC grade TFA were obtained from Merck (Darmstadt, Germany). The base *i*Pr_2NEt was distilled from ninhydrin and KOH. Amino acids were purchased from Multisyntech. The coupling reagents 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[23] and benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP)[24] were obtained from Richelieu Biotechnologies Inc. (Montreal, Canada). Streptavidin-peroxidase conjugate (S-5512) was ordered from Sigma (Munich, Germany). All SPR experiments were performed on a double channel surface plasmon Autolab ESPRIT instrument (Ecochemie, Utrecht, The Netherlands) at 25 °C. The cuvet-based instrument was mounted with a CMD6 biosensor chip (XanTec bioanalytics GmbH, Münster, Germany) containing a carboxymethylated dextran layer with a molecular weight of 6000. Electrospray ionization (ESI) mass spectrometry was carried out using a Shimadzu LCMS QP-8000 single quadrupole bench top mass spectrometer (m/z range <2000), coupled with a QP-8000 data-processing system. Analytical HPLC was performed on a Shimadzu Class-VP automated HPLC using an analytical reversed-phase column (Alltech Adsorbosphere C18, 300 Å, 5 μm, 250 × 4.6 mm or Alltech Adsorbosphere C8, 90 Å, 5 μM, 250 × 4.6 mm) and a UV detector operating at 220 nm and 254 nm. Preparative HPLC was carried out on a Gilson automated HPLC using a preparative reversed-phase column (Alltech Adsorbosphere C18, 10 μM, 250 × 22 mm) and a UV detector operating at 220 and 254 nm. For elution an appropriate gradient from 0.1% TFA in H_2O to 0.085% TFA in CH_3CN/H_2O (95/5, v/v) using a flow rate of 1 mL/min (analytical) or 11.5 mL/min (preparative) was run. General solid-phase synthesis procedure: Fmoc deprotection: 20% piperidine in NMP (3 × 8 min); washing: NMP, then CH_2Cl_2 (3 × 2 min each). A Kaiser test was performed after each round of coupling and deprotection steps. All coupling steps were performed twice with 4 equiv amino acid, 4 equiv BOP, and 8 equiv *i*Pr_2NEt, followed by thorough washing.

4.2. Purification and labeling of galectins

cDNAs for the two chicken galectins were cloned from total kidney mRNA either with suitable primer sets by RT-PCR for CG-14 or by consecutive primer-directed RT-PCR followed by 3′-RACE-PCR to complete the terminal sequence section of CG-16, and recombinant expression was performed in TB or 2YT medium at 37 °C with the system combinations of pQE60 (Qiagen, Hilden, Germany)/*E. coli* strain M15[pREP4] or pUC540/*E. coli* strain HB101. The avian galectins and human galectin-1 were purified to homogeneity by affinity chromatography on lactosylated Sepharose 4B, obtained by divinyl sulfone activation, as crucial step, routine quality controls were run by one- and two-dimensional gel electrophoresis, gel filtration, and nano-electrospray ionization mass spectrometry.[1i,21b,25] Biotinylation under activity-preserving conditions, assessment of degree of labeling by a proteomics approach and activity assays by hemagglutination and solid-phase and cell binding were carried out as described.[21a,26]

4.3. Affinity selection of the phage-display library

The PhD C7C library based on a M13 phage-display system in which random 7-mer peptides flanked by

two cysteine residues are fused to the N-terminus of the minor coat protein pIII of the bacteriophage was purchased from New England Biolabs (Frankfurt a. M., Germany). Disulfide bridging between the flanking cysteine pair affords conformational constraint for the random peptides under nonreducing conditions. Affinity selection followed the manufacturer's instructions with previously published modifications.[27] In detail, the marker-binding matrix was established by adsorbing streptavidin (100 μg/mL) to the surface of microtiter plate wells (Immunolon 4; Nunc, Wiesbaden, Germany) overnight at 4 °C. The coating solution was removed by aspiration, unbound streptavidin washed off with 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl (TBS) and remaining protein-binding sites on the plastic surface were saturated by an incubation step for 1 h at 4 °C with carbohydrate-free bovine serum albumin (5 mg/mL) in 0.1 M $NaHCO_3$, pH 8.5, containing 0.2% NaN_3 and 0.1 μg/mL streptavidin to block access of any contaminating biotin in the albumin preparation. The wells were finally ready for the selection step after careful removal of the blocking solution and a series of six washing steps with TBS containing 0.1% Tween-20 (TBST). Binding of biotinylated galectin to streptavidin on the matrix was performed for 1 h at 4 °C on a rocking platform after co-incubation of biotinylated galectin (10 μL; 10 ng/μL) and phage-library-containing solution (10 μL; approximately 2×10^{11} infectious particles) in a total volume of 100 μL TBST overnight at 4 °C. Unbound material was first removed by aspiration and then by further thorough washing steps (4× with TBST, 2× with TBS). Dissociation of lectin–phage complexes was induced by using the galectin (100 μg/mL TBS) for 10 min. Aliquots of the eluant-containing solution were used for titration and amplification.

4.4. Phage amplification and DNA sequencing

Eluant solution with galectin-binding phages was added to a suspension containing *E. coli* (strain ER2738) cells grown in early-log phase ($OD_{600} \sim 0.2$) in LB medium and incubated for 4.5 h at 37 °C with vigorous shaking. Following two consecutive centrifugation steps 1.7 mL of the bacteria-free supernatant was mixed with 0.28 mL 20% polyethylene glycol-8000/2.5 M NaCl (PEG/NaCl) and phage precipitation proceeded overnight at 4 °C. Again, two centrifugation steps were used for phage preparation and a further mild centrifugation step (1 min) was included to remove any contaminating bacteria. A further precipitation step with PEG/NaCl and a 1 min centrifugation step to discard insoluble material yielded the amplified eluate of the first round. It was titrated and subjected to the second and then further rounds of affinity selection. After five rounds of affinity selection individual plaques were picked for amplification, phage pellets were obtained as described above and single-stranded DNA was prepared by extraction with iodide buffer (100 μL; 10 mM Tris–HCl, pH 8.0, containing 4 M NaI and 1 mM EDTA–Na_2) and precipitation with ethanol (250 μL) for 10 min at room temperature, followed by centrifugation, washing with 70% ethanol, and drying in vacuum. The DNA was then suspended in nuclease-free water and the 5′-CCCTCATAGTTAGCGTAACG-3′ primer was used for sequencing in the UC Davis Division of Biological Sciences Automated DNA Sequencing Facility exploiting ABI 3730 capillary electrophoresis genetic analyzer and ABI BigDye Terminator v3.1 Cycle Sequencing chemistry.

4.5. Synthesis of the combinatorial library

PEGA-NH_2 resin was obtained from PolymerLabs and swollen in MeOH. The resin was measured by volume (5.85 mL) and washed with NMP and CH_2Cl_2 (2×2 min). To the reaction vessel containing the resin Fmoc-Gly-OH (4 equiv, 0.72 mmol, 213 mg) in NMP (3 mL), BOP (4 equiv, 0.72 mmol, 318 mg), and iPr$_2$NEt (8 equiv, 1.44 mmol, 251 μL) were added and the suspension was agitated by bubbling N_2 through the vessel for 60 min. After washing, the loading was determined to be 0.41 mmol/g. After deprotection and washing, the resin was split into 18 equal portions and one of the following Fmoc-protected amino acids (D-Ser, Thr, D-Glu, Asp, D-Asn, Gln, Ala, Arg, Lys, His, Val, D-Leu, Trp, Pro, Tyr, D-Phe, D-(4F)-Phe, Gly) was coupled to each portion. After washing, the resin portions were mixed, deprotected, washed, and Fmoc-Tyr-OH was coupled. After deprotection and washing the resin was split again and the next position in the sequence was filled by a variable amino acid as carried out before. Washing, mixing of resin portions, deprotection, and washing followed. Then Fmoc-Tyr-OH was coupled to the growing peptide chains on the resin. After deprotection and washing, the resin was split and the fifth position was occupied by a variable amino acid as before. Loading was determined to be 0.42 mmol/g. After terminal Fmoc deprotection, the side chains were deprotected with TFA/H_2O/TIS/EDT (85/8.5/2/4.5, 3×45 min) and washed with NMP, CH_2Cl_2 and H_2O (3×2 min) each.

4.6. Screening of the library

Biotinylated CG-14 (3 mL, 25 μg/mL phosphate buffer, pH 7) and h-Gal-1 (1.5 mL, 50 μg/mL phosphate buffer, pH 7) were added to a small amount of resin (25 mg), and the suspension was gently shaken overnight. The galectin-containing solution was filtered and the resin incubated with streptavidin-peroxidase conjugate (1 mL, 1 μg/mL) for 2 min. After filtration, a solution of *o*-phenylenediamine (1 μg/mL) and H_2O_2 (1 μL/mL) was added, and the suspension gently agitated for 15 min. After filtration, the reaction was stopped with 2 M H_2SO_4 (1 mL, 1 min), filtrated, and the beads were spread on a glass petri dish. Using a microscope, the most intensely stained beads were selected for decoding by Edman degradation.

4.7. General synthesis of pentapeptides 1–6a

These compounds were synthesized using general SPPS procedures on Argogel™ resin outfitted with a rink linker.[28] The amino acids used were *N*-Fmoc protected and the side chains were protected with an acid-labile group. For peptides bearing the solubility-improving tail: 3 equiv Boc-*tail*-OH[29] was used with 3 equiv BOP

and 6 equiv. iPr$_2$NEt for a reaction time of 2 h. After acidic cleavage from the resin, the peptides were precipitated in MTBE/hexane 1/1 at −20 °C, the solution was centrifuged, the precipitate washed with cold MTBE/hexane 1/1 (4×), and preparative HPLC was performed when necessary. Compounds were analyzed as pure peptides on HPLC and isolated as TFA salts. **sYKYs (1)**: t_R = 14.6 min; MS m/z: 646.7 $[M+H]^+$. **tail-sYKYs 1a**: t_R = 15.9 min; MS m/z: 964.8 $[M+H]^+$, 483.0 $[M+2H]^{2+}$. **KYWYR (2)**: t_R = 17.2 min; MS m/z: 814.8 $[M+H]^+$, 407.7 $[M+2H]^{2+}$. **tail-KYWYR (2a)**: t_R = 18.3 min; MS m/z: 1133.6 $[M+H]^+$, 567.1 $[M+2H]^{2+}$. **fYfYA (3)**: t_R = 19.8 min; MS m/z: 709.8 $[M+H]^+$. **tail-fYfYA (3a)**: t_R = 19.8 min; MS m/z: 1027.8 $[M+H]^+$. **WYDYW (4)**: t_R = 19.2 min; MS m/z: 831.5 $[M+H]^+$. **tail-WYDYW (4a)**: t_R = 19.5 min; MS m/z: 1149.7 $[M+H]^+$. **WYKYD (5)**: t_R = 16.8 min; MS m/z: 773.6 $[M+H]^+$. **tail-WYKYD (5a)**: t_R = 18.0 min, MS m/z: 1191.6 $[M+H]^+$, 546.9 $[M+2H]^{2+}$. **WYKYW (6)**: t_R = 19.4 min; MS m/z: 844.6 $[M+H]^+$. **tail-WYKYW (6a)**: t_R = 19.8 min; MS m/z: 1162.7 $[M+H]^+$, 582.3 $[M+2H]^{2+}$.

4.8. Synthesis of Ac-CQSPSARSC-NH$_2$ (7)

The phage-display-defined peptide was synthesized by the FastMoc protocol[30] on a 0.25 mmol scale using Argogel^{TM} Rink-NH-Fmoc resin on an ABI 433A peptide synthesizer coupled to a 759A absorbance detector. Fmoc detection was monitored by UV absorbance (λ = 301 nm) of the dibenzofulvolene–piperidine adduct. Each synthetic cycle consisted of N^{α}-Fmoc removal by a 10 min treatment with 20% piperidine in NMP, a 6 min NMP wash, a 45 min coupling step with pre-activated Fmoc amino acids (1.0 mmol) in the presence of 2 equiv iPr$_2$NEt, and a 6 min NMP wash. N^{α}-Fmoc amino acids were activated in situ with HBTU/HOBt (1.0 mmol, 0.36 M in NMP) in the presence of iPr$_2$NEt (2 mmol). After the final Fmoc removal the free amine was acetylated with an excess of acetic acid anhydride/iPr$_2$NEt/HOBt in NMP. The resin was split into two equal portions and to one portion was added a deprotection/cleavage mixture of TFA/H$_2$O/TIS/EDT 85/8.5/2/4.5 (7 mL) at 0 °C, whereafter the suspension was allowed to warm to room temperature for a total reaction time of 3 h. The peptide was precipitated with MTBE/hexane 1/1 at −20 °C and the solution was centrifuged. The precipitate was washed with cold MTBE/hexane 1/1 (4 × 15 mL) and lyophilized for a crude yield of 111.3 mg. The crude product was purified by preparative HPLC and lyophilized to yield the TFA salt (65.5 mg). HPLC: (C8, buffers A: H$_2$O/CH$_3$CN/TFA 95/5/0.1, B: 5/95/0.1) t_R = 12.7 min; 100%. MS: m/z = 979.6 $[M+H]^+$.

4.9. Synthesis of cyclic Ac-CQSPSARSC-NH$_2$ (8)

Ac-CQSPSARSC-NH$_2$ **7** (27.5 mg) was dissolved in H$_2$O (47.5 mL) and AcOH (2.5 mL). The pH was adjusted to 6 with (NH$_4$)$_2$CO$_3$, DMSO was added (2.5 mL) and the solution was stirred without stopper at room temperature for 1 day. Upon cyclization (identified by mass spectrometry), H$_2$O/CH$_3$CN/TFA 95/5/0.05 (50 mL) was added and the solvents were removed in vacuo. H$_2$O (7 mL) was added to the residue, the solution was lyophilized, and the cyclic peptide **8** was purified by preparative HPLC in 50% yield (12.5 mg). HPLC: (C8, buffers A: H$_2$O/TFA 100/0.1, B: H$_2$O/CH$_3$CN/TFA 5/95/0.85) t_R = 14.4 min 100%. MS: m/z = 977.7 $[M+H]^+$.

4.10. Solid-phase lectin–peptide inhibition assay

Following adsorption of the glycoprotein asialofetuin, obtained by acidic desialylation of commercial fetuin (Sigma, Munich, Germany), onto the plastic surface of microtiter plate wells the competition assay was performed under optimal conditions described previously.[21a,31] Competition experiments with at least duplicates were performed using a concentration of 4 mg/mL of the peptide. Signal generation to quantitate carbohydrate-dependent binding of the labeled lectins to the glycan-presenting matrix was achieved by streptavidin-peroxidase conjugate (0.5 μg/mL) and o-phenylenediamine (1 mg/mL)/H$_2$O$_2$ (1 μL/mL). The extent of peptide-inhibitable binding was determined by controls, where incubation of wells proceeded in the absence of the peptide mixture. Additionally, the extent of inhibition by galactose and lactose used at the same molarity was assessed.

4.11. Surface plasmon resonance monitoring

The lyophilized peptides and lectins were dissolved in HEPES-buffered saline (HBS; 10 mM HEPES, 4 mM EDTA-Na$_2$, 150 mM NaCl, pH 7.4, 0.005% Tween-20) and the lectin-containing solutions were frozen, and thawed only once to prevent activity loss. Asialofetuin (from a 400 μg/mL solution in 10 mM acetate buffer, pH 4.0) was immobilized onto a SPR sensor chip using the standard procedure as described by the manufacturer. The control channel was treated identically but without asialofetuin being present. The first channel of the cuvette was used as the measuring channel and the second as a control channel, where the signal obtained was subtracted to account for unspecific binding effects of the lectin and/or peptide ligand to the nonfunctionalized chip surface. Level of unspecific binding of the peptides to the surface-presented glycoprotein, which occurred in some cases at high peptide concentrations, was assessed in separate experiments and was also subtracted. The chip surface was regenerated with a short pulse (1 min) of 10 mM NaOH + 0.2% SDS after each sample run. Experiments were performed at room temperature. A competition experiment consisted of measuring a mixture of lectin and peptide at different peptide concentrations.

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