Altering the Modular Architecture of Galectins Affects its Binding with Synthetic α-Dystroglycan *O*-Mannosylated Core M1 Glycoconjugates *In situ*

Lareno L. Villones Jr.a, Anna-Kristin Ludwigb, Seiya Kikuchia, Rika Ochia, Shin-Ichiro Nishimuraa, Hans-Joachim Gabius†, Herbert Kaltnerb,\*, Hiroshi Hinoua,\*

This manuscript is dedicated to Professor Hans-Joachim Gabius.

[a] L.L. Villones Jr., S. Kikuchi, R. Ochi, Dr. S.-I. Nishimura, Dr. H. Hinou  
Graduate School of Life Science and Faculty of Advanced Life Science, Frontier Research Center for Advanced Material & Life Science  
Hokkaido University  
N21, W11, Sapporo 001-0021, Japan  
E-mail: hinou@sci.hokudai.ac.jp

[b] Dr. A.-K. Ludwig, Dr. H.-J. Gabius, Dr. H. Kaltner  
Physiological Chemistry, Department of Veterinary Sciences, Faculty of Veterinary Medicine  
Ludwig-Maximilians-University Munich  
82152 Planegg-Martinsried, Germany

E-mail: kaltner@tiph.vetmed.uni-muenchen.de

†Deceased on August 2, 2021

Supporting information for this article is given via a link at the end of the document.

**Abstract:** The multifunctionality of galectins helps regulate a broad range of fundamental cellular processes via *cis*-binding and *trans*-bridging activities and has gained widespread attention with respect to the importance of the natural specificity/selectivity of this lectin family to its glycoconjugate receptors. Combining galectin (Gal)-1, -3, -4, and -9 variant test panels, achieved via rational protein engineering, and a synthetic α-dystroglycan (DG) *O*-Mannosylated core M1 glycopeptide library, a detailed comparative analysis was performed, utilizing microarray experiments to delineate the design-functionality relationships within this lectin family. Enhancement of prototype Gal-1 and chimera-type Gal-3 *cis*-binding toward the prepared ligands is possible by transforming these lectins into tandem-repeat type and prototypes, respectively. Furthermore, Gal-1 variants demonstrated improved *trans*-bridging capabilities between core M1 α-DG glycopeptides and laminins in microarray, suggesting the possible translational applications of these galectin variants in the treatment of some forms of α-dystroglycanopathy.

Introduction

The functional pairing of tissue lectins and cellular glycoconjugates is receiving increasing attention because these interactions are involved in a wide range of (patho)physiological processes [1–4]. The glycan-encoded biochemical signals (“sugar code”) are translated into their distinct cellular effects (“special meaning”) by lectins (“the readers”) [5]. As driven by evolution, the diversity and specificity of this molecular recognition entails that structural and topological aspects on both sides influence this functional pairing. Lectins are protein receptors consisting of a carbohydrate recognition domain (CRD) that accommodates glycan epitopes with strong avidity. This capability efficiently facilitates cell-cell or cell-matrix adhesion (*trans*-bridging) or signal transduction (*cis*-binding), which is critically dependent on the molecular presentation of lectin CRD. Lectins are classified according to the protein fold by their CRD, which is diversified at the sequence and modular design level, with phylogenetic variation in each group, displaying distinct characteristics [6,7].

Focusing on adhesion/growth-regulatory galectins (Gal), this lectin family preferentially binds to galactose-terminated glycoconjugates. Naturally, this family of carbohydrate-binding protein has three phylogenetically conserved structural designs (Fig. 1A): i) a non-covalently linked homodimer (prototype), ii) two non-identical but homologous CRDs associated associated by a peptide linker (tandem-repeat type), and iii) a CRD connected to an N-terminal tail with non-triple-helical collagen-like repeats (chimera-type). These natural forms of galectins can be further modified via alternative splicing and proteolytic cleavage, resulting in multiple isoforms within the lectin family [8,9]. With this panel of galectin architecture, CRD-counterreceptor interactions can form lattices of unique spatial characteristics, dependent on CRD presentation, eliciting post-binding events [10]. Galectin CRDs have been extensively studied; however, it remains unclear why galectin CRDs are limited to these modular designs. Thus, rational protein engineering is a promising method for delineating this natural preference and the impact of CRD structural alterations on lectin activity. To probe the effects of CRD organization and spacing on receptor cross-linking, newly engineered galectins have been successfully tested using cells, glycoclusters, glycodendrimers, glycopolymers, and glycopeptides [11–14]. This approach will provide further insights into the design-functionality correlation of the target specificity of wild-type galectins and the prospect of translational therapeutic applications of these human lectin variants.

For instance, several modifications of Gal-1 have been attempted to improve the oxidation sensitivity and bioactivity of wild-type protein. A cysteine-less mutant of Gal-1 (C3/17/43/61/89/131S, CSGal-1), with functional and structural properties similar to that of the parent protein, was prepared as a stable substitute for wild-type Gal-1 (Gal-1WT) [15]. Glycine-glycine (GG)-linked Gal-1, which displays Gal-1 as a single-chain bivalent galectin, demonstrated a 10-fold increase in apoptotic activity in both murine thymocytes and mature T cells of Gal-1WT [16]. The Gal-1 tandem-repeat type mutant with 14 amino acid Gal-9 short isoform random-coil linker (Gal-1-9-1) exhibited a 30-fold enhancement in triggering Jurkat E6-1 cell death over wild-type Gal-1 [17]. In oysters and bay scallops, a Gal-1 isoform with

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Fig 1.tif

**Figure 1.** (A)Structural design of galectin wild-type and variants. [8S] = Gal-8, 33-aa peptide linker; [8L] = Gal-8, 74-aa peptide linker; Gal-3NT = Gal-3 N-terminal Tail; V = shorten Gal-4 linker; P = Prototype; N = N-terminal CRD; C= C-temrinal CRD. (B) Classification of *O*-Mannosylated glycans in α-DG.

quadruple-CRD has been identified and is involved in the recognition, phagocytosis, and elimination of several pathogenic microbes in these invertebrates [18,19]. Incorporating this architectural design into human Gal-1 (homo-oligomer variants ((Gal-1)4-GG/8S)) results in a 25-fold higher cell-bridging activity than Gal-1WT, as revealed by hemagglutination and erythrocyte aggregation experiments [12]. Furthermore, a heterotetramer galectin variant with two Gal-1 and Gal-3 domains (G1/G3 Zipper) exhibited a higher apoptotic activity than wild-type Gal-1 and Gal-3 alone or in combination; thus, it can be a good therapeutic candidate for regulating innate and adaptive immunity [20,21].

Dystroglycan (DG) is a transmembrane protein that links the extracellular matrix and intracellular cytoskeleton via α-DG and β-DG, respectively [22]. α-DG is highly *O*-Mannosylated (*O*-Man); this plays an important role in its ability to bind basement membrane proteins containing laminin G (LamG) domains (Fig. 1B) [23]. The matriglycan of α-DG has been identified as the receptor for laminins, which maintains skeletal muscle integrity and normal development of the central nervous system [24]. Disruption of α-DG glycosylation leads to various forms of muscular dystrophy; these have been known for several decades, but there are still no effective treatments for this group of diseases [25]. In our previous study, we demonstrated that the LacNAc-terminated *O*-Man core M1 glycopeptide of the α-DG fragment 372TRGAIIQ**T**P**T**LGPIQP**T**RV390 interacts with galectin via *cis*-binding (peptide- and carbohydrate-protein interactions) *in situ* [26]. The systematic variation of headgroup, density, and position presentation on the peptide scaffold and the type of galectin native structure affected lectin affinity. The *trans*-bridging capability of wild-type Gal-1 with core M1 glycoconjugates and laminins was also revealed *in situ*. Here, we combined α-DG glycopeptide and galectin variant libraries to analyze the impact of switching galectin design and valency on the binding process using glycan microarray technology. Furthermore, we tested the influence of the alteration of Gal-1 structure (presence of linker, increasing the CRD per protein molecule, and transforming to tandem-repeat type heterodimer) on the observed α-DG-laminin *trans*-bridging activities compared to that of the wild-type protein.

Results and Discussion

**Galectin variant toolbox**

Our galectin test panel encompasses three types of galectin architectural design found in humans. We selected prototype Gal-1, chimera-type Gal-3, and two tandem-repeat types, Gal-4 and

-9, as the test groups (Fig. 1A). To examine the architecture-dependent functionality of galectins, engineering by modular transportation allowed us to switch between galectin classes and access new combinations [4,27–30]. For Gal-1, an engineered covalently linked homodimer, that is, **(Gal-1)2**, and homotetramer, that is, **(Gal-1)4**, of Gal-1 connected by dipeptide Gly-Gly (**GG**) and 33-aa linker of tandem-repeat-type Gal-8 (**8S**) and a chimera

variant inspired by transplanting the mono Gal-1 CRD to Gal-3 NT, that is, **Gal-3NT/1**, were prepared.

The wild-type Gal-3 was turned into a homodimer by directly linking the C- and N- terminal amino acids of two Gal-3 CRDs (**Gal-3\_Gal-3**) or by inserting a tandem-repeat-type presentation Gal-8 linker, i.e., 33-aa (**8S**) or 74-aa (**8L**), yielding **Gal-3[8S/L]Gal-3** variants. Heterodimers consisting of the Gal-3 and Gal-1 CRDs were also constructed. Here, the structural variables were the spatial order of the CRD, that is, **Gal-1/-3** or **Gal-3/-1** (from the N-to C-terminus), in the absence or presence of a linker (**8S**).

For tandem-repeat type Gal-4, to address the issue of the relevance of the linker length for its binding capacity, two variants, that is, with reduced size (**Gal-4V**) and complete linker removal mimicking a dimer of the proto-type group (**Gal-4P**), were designed and produced. In addition, the Gal-4 C-terminal CRD was incorporated into a Gal-3-like design, that is, **Gal-3NT/4**. For Gal-9, CRDs with no linker (**Gal-9N/C**) were constructed.

The Lac/LacNAc binding properties of these galectin variants were found to be similar to their wild-type counterparts, thus proving the complete activity of all test lectins [29–32]. With this highly diverse galectin toolbox, an extensive comparative analysis of the three architectural designs of human galectins, including evaluation of the impact of linker length and mode of CRD presentation, is achievable. These proteins were isolated under activity-preserving conditions and employed in growth regulation, bridging, and aggregation assays using cells and glycodendrimersomes [28,33]. Here, we tested the interaction profiles of this lectin family in an array-based screening with core M1 α-DG glycopeptides with distinct structural variations.

**α-DG *O-*Man core M1 glycopeptide library**

Surface-immobilized glycopeptides in microarrays serve as effective tools for investigating galectin-carbohydrate interactions. The advantage of this method is that it can determine the precise binding epitope of galectins and provide insights into the effect of other glycan moieties, position, and density along the peptide scaffold. Here, we utilized our previously reported microarray platform based on the 19 amino acids along the mucin-like

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Fig 2.tif

**Figure 2.** Versatile core M1 *O*-Mannosylated α-DG glycopeptide library utilized in microarray experiment.

domain of α-DG (372TRGAIIQ**T**P**T**LGPIQP**T**RV390) displaying *O*-Man core M1 and extended glycan structures [26,34]. This chemoenzymatically synthesized glycopeptide library can investigate various evaluations at the same time (Fig. 2, Supplemental Table S1): i) the individual contribution of glyco-amino acids and peptide scaffolds to lectin binding (**1**–**4**) and MUC1 peptide scaffold as negative control (**30**); ii) the impact of individual glycopeptides and position effect of mono core M1 (GlcNAcβ1-2Man, **5**–**7**) LacNAc-terminated core M1 (Galβ1-4GlcNAcβ1-2Man, **12**–**14**) and sialyl-LacNAc-terminated core M1 (NeuAcα2-3Galβ1-4GlcNAcβ1-2Man, **19**–**21**); and iii) the multivalency effect of bis- and tris-core M1 and extended structures (**8**–**11**, **15**–**18**, **22**–**25**); iv) the effect of different neighboring glycan structure (**26**–**29**). The 5-oxohexanoic acid N-terminus-functionalized compounds were printed on a hydroxylamine-coated microarray chip in quadruplets. They were then immobilized via glycoblotting method [35]. To ensure the validity of this protocol, positive control plant lectins *Concanavalia ensiformis agglutinin* (ConA) and *Glycine max* (soybean) agglutinin (SBA) were utilized as previously reported [26]. The stage was set to determine the binding profile of the prepared glycoconjugates with galectin variants.

**Binding profile of prototype (Gal-1) variants**

The Gal-1 variants could bind to glycans of this test panel, so strong signals were recorded (Fig. 3 and Supplemental Figs. S1–S2). Similar to the wild-type, Gal-1 variants maintained preferential contact formation with core M1 LacNAc-terminated epitopes of α-DG, irrespective of the modular design. The GG-linked homodimer (**(Gal-1)2[GG]**) and tetramer (**(Gal-1)4[GG]**) exhibited the same binding profile as that of the wild-type protein. They strongly interact with glyco-amino acids (**2**–**3**), unglycosylated peptide (**4**),and glycopeptides presenting mono-core M1 (**5**). In addition, glycopeptides terminated with LacNAc (**12**–**18, 25**) or sialyl-LacNAc (**19**–**25, 27**–**29**) were solid binders, but not MUC1 peptide **30** even at low lectin concentration (3.2 μg/mL) (Fig. 3 and Supplemental Figs. S1–S2). No binding with compounds **6**–**11** was observed, specifically at 32 μg/mL lectin, differentiating the activity of the parent protein under ambient oxidizing conditions [16]. The rigid short glycine-glycine linker fixates the homodimer Gal-1, prohibiting it from dissociation and preventing internal cysteine residue oxidation [36].

Extending the linker length of Gal-1 variants (homodimer (**(Gal-1)2[GG]**) and tetramer (**(Gal-1)4[GG]**)) to 33 amino acids of Gal-8 (**8S**), yielding **(Gal-1)2[8S]**) and **(Gal-1)4[8S]**), respectively, demonstrated analogous binding capacity (Fig. 3 and

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Fig 3.tif

**Figure 3.** (A.)Fluorescence image of core M1 α-DG glycopeptide printed microarray chip taken after treatment with **10.0 μg/mL** Gal-1 variants. (B.) Signal intensities: stacked chart of **200 μM** core M1 α-DG glycoconjugates with **3.20 μg/mL** Gal-1 variants. Relative interaction of 20 and 200 μM core M1 α-DG glycoconjugates with 0.10–32.0 μg/mL of Gal-1 variants are shown in Supplemental Figs. S1–S2, respectively.

Supplemental Figs. S1–S2). The presence of the linker resulted in enhanced binding of Gal-1 to the test compounds, and the length of the linker seemed to matter; the longer the size, the higher the binding activity (**(Gal-1)2[GG]** vs. **(Gal-1)2[8S]** and **(Gal-1)4[GG] vs. Gal-1)4[8S]**). The increase in linker length allowed flexibility in the orientation and lateral movement of the CRDs [37]. In addition, the **[8S]** linker perturbs the F-face of CRD, making it more accessible for binding, thereby enabling more effective crosslinking with glycan ligands, specifically to core M1-terminated glycopeptides **6**–**11** (**(Gal-1)4[GG]** vs. **(Gal-1)4[8S]**,Supplemental Figs. S1–S2) [38]. Furthermore, the increased number of CRDs per molecule did not change the binding properties, but an enhanced signal intensity relative to the parent homodimer was observed (**Gal-1WT** vs. **(Gal-1)4[GG]** vs. **(Gal-1)4[8S]**).

Bringing the Gal-1 CRD into the Gal-3-like design (**Gal-3NT/1**) markedly reduced the signal intensities. Only at high concentrations this variant retained interaction with peptide (**4**), and glycopeptides terminated with core M1 (**5**–**7**) or LacNAc (**12**–**18,26,**and **18**) (Fig. 3 and Supplemental Figs. S1–S2). In this modular design, Gal-1 CRD can use the N-terminal part of Gal-3 WT to oligomerize inaconcentration-dependent manner, forming disorganized crosslinked complexes with its ligand, similar to Gal-3WT [39]. However, weak intensity signals were recorded, suggesting that Gal-1 CRD prefer its wild-type or be in a tandem-repeat type variant form to bind to the surface-immobilized core M1 α-DG ligands.

**Binding profile of chimera type (Gal-3) variants**

Wild-type Gal-3 interacted weakly with LacNAc-terminated glycopeptides **12**–**18** (Fig. 4 and Supplemental Figs. S3–S4). The

removal of the collagenous N-terminal tail (**trGal-3**) resulted in a weak enhancement of binding for LacNAc-terminated (**12**–**18**, **26**) and sialyl-LacNAc (**3**, **19**–**25**, **27**) core M1 α-DG glycoconjugates (Fig. 4 and Supplemental Figs. S3–S4). The loss of the N-terminal tail in Gal-3 disables its tendency to self-aggregate forming dimers or higher-order oligomers [40]. In addition, unlike the prototype Gal-1 and Gal-2, which can form dimers via hydrophobic interactions between the N- and C-terminal residues of two monomeric units, **trGal-3** cannot form dimers via the same interactions [41]. Thus, the slight increase in affinity suggests that the CRD-only modular architecture is desirable for Gal-3 so that it can interact with the immobilized ligands. The results further reflect the preference of Gal-3 CRD for reducing-end polyLacNAc structures, not on the mono-terminal LacNAc structures, as displayed by the α-DG glycoconjugates [42,43].

The homodimer (**Gal-3\_Gal-3**) and heterodimer (**Gal-1\_Gal-3** and **Gal-3\_Gal-1**) prototype variants are highly associated with LacNAc-terminated (**2** and **12**–**19**), and its α2,3-sialylated derivatives (**3** and **19**–**25** and **27**–**29**) even at 3.2 μg/mL lectin concentration (Fig. 4 and Supplemental Figs. S3–S4). A very weak interaction was recorded for core M1 glyco-amino acid **1** and the unglycosylated peptide **4**. Conjugation of **1** to the glycosylation site of **4** (**5**–**11**) did not result in the enhanced binding of these variants. The spatial orientation of Gal-1/-3 in heterodimer prototype variants (**Gal-1\_Gal-3** vs. **Gal-3\_Gal-1**) affects the binding interaction with α2,3-sialylated core M1 α-DG glycopeptides (**19**–**25** and **27**–**29**).

Transforming homodimeric Gal-3 (**Gal-3\_Gal-3**) to tandem-repeat-types **Gal-3[8S]Gal-3** and **Gal-3[8L]Gal-3 negatively influenced its interaction with the test glycoconjugates** (Fig. 4 and Supplemental Figs. S3–S4). The tandem-repeat type variants both exhibited a decrease in affinity for LacNAc- and sialyl-

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Fig 4.tif

**Figure 4.** (A.)Fluorescence image of core M1 α-DG glycopeptide printed microarray chip taken after treatment with **10.0 μg/mL** Gal-3 variants. (B.) Signal intensities stacked chart of **200 μM** core M1 α-DG glycoconjugates with **3.20 μg/mL** Gal-3 variants (b). The relative interaction of 20 and 200 μM core M1 α-DG glycoconjugates with 0.10–32.0 μg/mL of Gal-3 variants are shown in Supplemental Figs. S3–S4, respectively.

LacNAc-terminated core M1 glycoconjugates (**Gal-3\_Gal-3** vs. **Gal-3[8S]Gal-3** vs. **Gal-3[8L]Gal-3**). The **8L**-linked Gal-3 (**Gal-3[8L]Gal-3**), but not **8S**-linked Gal-3 (**Gal-3[8S]Gal-3**), interacted with glyco-amino acids **1-3**, unglycosylated α-DG peptide **4**, and core M1 glycopeptide **5**. In the case of **8S**-linked Gal-1/Gal-3 heterodimers, the presence of an **8S**-linker also negatively influenced the binding activity of this variant against sialyl- LacNAc-presenting α-DG glycopeptides **19**–**25** and **27**–**29** (**Gal-1\_Gal-3** vs. **Gal-1[8S]Gal-3** and **Gal-3\_Gal-1** vs. **Gal-3[8S]Gal-1**) (Fig. 4 and Supplemental Figs. S3–S4). The consequence of the Gal-1/Gal-3 CRD spatial orientation is more prominent in the tandem-repeat type than in the prototype variants, specifically at low ligand concentration (20 μM).

Overall, these results suggest that removing the N-terminal tail of Gal-3 resulted in increased affinity for α-DG core M1 glycoconjugates. Converting the chimera-type parent protein into directly conjugated prototype-like homo- (Gal-3) and heterodimeric (Gal-1/Gal-3) variants led to positive binding. Incorporating linkers into Gal-3 prototype variants generally leads to decreased affinity. Furthermore, the order of modular assembly of Gal-1/ Gal-3 CRD from N- to C-terminus had an effect. These observed activities reflected the binding properties and obtained *K*d values for these Gal-3 variants in neuroblastoma cells [28].

**Binding profile of tandem-repeat type (Gal-4 and -9) variants**

Wild-type Gal-4 interacts with peptide **4**, mono-core M1 substituted (**5**–**7**), and LacNAc-terminated core M1 glycopeptides (**12**–**18**) (Fig. 5 and Supplemental Figs. S5–S6). Reducing the size of the peptide linker (**Gal-4V**) and conversion to a prototype (**Gal-4P**) reduced the level of reactivity to identical ligands

(**4**, **12**–**18**) than that of **Gal-4WT** (Fig. 5 and Supplemental Figs. S5–S6). The linker is not just a connector of the CRDs but it also influences lectin‘s affinity because it provides spatial arrangement flexibility of the CRDs, as observed with Gal-4 interaction with glycoclusters [31,44]. Transformation of the parent protein to a Gal-3-like protein (**Gal-3NT/4C**) abolished its binding activity to the core M1 glycopeptides (Fig. 5 and Supplemental Figs. S5–S6). These results demonstrated that a reduced linker length or its complete removal in Gal-4, or prensenting the Gal-4 C-terminal CRD in a chimera type architecture decreased the susceptibility of Gal-4 towards the surface-immobilized ligands (**Gal-4WT**>**Gal-4V**>**Gal-4P**>**Gal3NT/4C**).

Wild-type Gal-9 revealed moderate affinity to unglycosylated peptide **4,** core M1-presenting glycopeptides (**5**–**11**), and LacNAc-terminated compounds (**12**–**18**) (Fig. 5 and Supplemental Figs. S7–S8). These observations were modulated by sugar epitope positioning and density. The α2,3-sialylation of LacNAc units (**19**–**25, 27**–**29**) impaired the binding of Gal-9 to glycoconjugates. The N-terminal CRD (**Gal-9N**) was bound to a few glycoconjugates; here, only compounds **15** and **18** (Supplemental Figs. S7–S8). On the other hand, **Gal-9C** revealed an analogous binding profile to Gal-9WT, with increased interaction at lower lectin concentrations and enhanced affinity to sialyl-LacNAc-terminated glycopeptides (**19**–**25, 27**–**29**) (Supplemental Figs. S7–S8). These results suggest the difference in the binding activity of the C- and N-terminal CRD of Gal-9, where **Gal-9C** likely contributes to the binding of its wild-type form andthe presence of linker and N-terminal CRD affected its overall affinity.

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Fig 5.tif

**Figure 5.** (A.)Fluorescence image of core M1 α-DG glycopeptide printed microarray chip taken after treatment with **10.0 μg/mL** Gal-4 and -9 variants. (B.) Signal intensities stacked chart of **200 μM** core M1 α-DG glycoconjugates with **3.20 μg/mL** Gal-4 and -9 variants (b). The relative interaction of 20 and 200 μM core M1 α-DG glycoconjugates with 0.10 to 32.0 μg/mL of Gal-4 and -9 variants are shown in Supplemental Figs. S5–S6 and S7–S8, respectively.

**Gal-1 variants crosslink α-DG core M1 glycoconjugates and laminins in microarray**

Gal-1 is a promising candidate for reducing muscular dystrophy; however, the exact mechanisms are still unknown [25,45,46]. Previously, we demonstrated the crosslinking capabilities of wild-type Gal-1 between various laminins and α-DG peptide and core M1 glycopeptides in microarray [26]. We attribute this to the protein-peptide interaction of Gal-1 F-face with α-DG peptide **4** and the protein-carbohydrate interaction of Gal-1 S-face with polyLacNAc-terminated *N*-glycans presented by laminin. This finding suggests a possible mechanism by which Gal-1 ameliorates several forms of congenital muscular dystrophy, where α-DG is highly hypoglycosylated. As described above, Gal-1 variants (except **Gal-3NT/1**) and Gal-1/Gal-3 heterodimer-tandem-repeat type variants (**Gal-1[8S]Gal-3**, **Gal-3[8S]Gal-1**) revealed enhanced affinity for the prepared core M1 glycoconjugates of α-DG (Figs. 3–4). For possible translational therapeutic applications in treating α-dystroglycanopathy by *trans-*bridging laminin and core M1 glycans of α-DG, we selected Gal-1 presenting variants (**(Gal-1)2[8S], Gal-1[8S]Gal-3**, **Gal-3[8S]Gal-1**, **(Gal-1)4[8S]**, and **Gal-3[8S]Gal-3** (negative control)).

The parent Gal-1 exhibited high *trans*-bridging activity between laminin-111, -121, -211, and -221 (but little with -511) and the α-DG peptide (**4**) and core M1 glycopeptides (**5**–**11**) in a glycan position- and density-dependent manner (Fig. 6). Presenting Gal-1 into a tandem-repeat type with an **8S**-linker (**(Gal-1)2[8S]**) maintained its crosslinking activity with laminin and α-DG peptide **4**, but attenuated *trans*-bridging with core M1

glycopeptides (**5**–**11**) was observed.Interestingly, a noticeable increase in *trans*-bridging towards LacNAc-terminated (**12–18, 26**) and sialyl-LacNAc-terminated (**19–25**, **27–29**) was demonstrated by **(Gal-1)2[8S]** which is attributed to the high affinity of this Gal-1 variant to the ligands compared to **Gal-1WT** (Fig. 6). This difference in crosslinking activity is associated with the Gal-8 linker, 33 aa (**8S**), between the two Gal-1 monomers providing Gal-8-like properties to Gal-1. Gal-8 interacts with the receptor peptide fragment of NDP52, activating antibacterial autophagy [47]. The X-ray structure of this interaction revealed that the C- and N-terminal CRDs could be associated with the ligand in a back-to-side orientation, forming dimeric structures and thus possessing four carbohydrate recognition domains available for binding [44,48]. As a result, **(Gal-1)2[8S]** is expected to demonstrate improved crosslinking activity towards the LacNAc- and sialyl-LacNAc-terminated core M1 glycopeptides of α-DG and laminin than **Gal-1WT**. Moreover, **(Gal-1)2[8S]** can *trans*-bridge the core M1 glycoconjugates of α-DG with Lam-511, which was not exhibited by the wild-type protein. Overall, these results suggest that the *trans­-*bridging activities of **(Gal-1)2[8S]** occur via F-face (Gal-1–α-DGpeptide) – to – S – face (Gal-1–LacNAc-terminated glycans of laminin) and S-face (Gal-1–α-DG LacNAc-terminated glycopeptides)-to-S-face (Gal-1–LacNAc-terminated glycans of laminin) interactions (Fig. 7).

On the other hand, the hetero-tandem-repeated type 8S-linked Gal-1/-3 variants (**Gal-1[8S]Gal-3** and **Gal-3[8S]Gal-1**)exhibited a considerable reduction in the *trans*-bridging activity on the surface-immobilized ligands and in solution laminins (Fig. 6). The **trGal-3** showed a very weak interaction with the prepared

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Fig 6_2.tif

**Figure 6.** Fluorescence image of core M1 α-DG glycopeptide printed microarray chip (**a**) and stacked chart of signal intensities (**b**) taken after treatment with various 32.0 μg/mL **laminin–Gal-1** **variant** solution for 30 min. The relative interaction of 200 μM core M1 α-DG glycoconjugates with 0.10 to 32.0 μg/mL of various **laminin–Gal-1** **variant** solution are shown in Supplemental Supplementary Fig. S9–S14.

ligands compared to **Gal-1WT** (Fig. 4 and Supplemental Figs. S3–S4). Incorporating Gal-3 CRD with Gal-1 CRD via an 8S-linker (**Gal-1[8S]Gal-3** or **Gal-3[8S]Gal-1**) resulted in increased bindingactivity because of the high affinity of Gal-1 CRD with the test α-DG core M1 glycoconjugates (Fig. 4 and Supplemental Figs. S3–S4). Gal-3 is also known to bind laminin through its numerous poly-LacNAc structures [49]. It can bridge neutrophils to laminin *in vitro,* which is dependent on both the CRD and N-terminal tail region [50]. However, **Gal-1[8S]Gal-3** and **Gal-3[8S]Gal-1** failed to effectively crosslink the core M1 glycoconjugates of α-DG with laminin. These results suggest that at least two Gal-1 CRD are required to effectively *trans*-bridge α-DG glycopeptides with

laminins. As expected, the **Gal-3[8S]Gal-3** variant did not exhibit *trans*-bridging activity to any of the prepared α-DG ligands and laminins (Fig. 6).

The **(Gal-1)4[8S]** exhibited higher crosslinking ability towards LacNAc-presenting α-DG core M1 glycoconjugates than **Gal-1WT**and **(Gal-1)2[8S]**, whilea substantial decrease in signal intensity was observed in the α-DG peptide **4** and core M1-terminated glycopeptides (**5–11**) (Fig. 6). Given the increased number of Gal-1 CRD sites per protein, enhancement in the *trans*-bridging of **(Gal-1)4[8S]** is anticipated. In this case, the high activity towards LacNAc-terminated core M1 glycoconjugate indicates that S-Face (Gal-1 – α-DG LacNAc / sialyl - LacNAc-

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Fig 7.tif

**Figure 7.** (A.)X-raycrystal structure ofGal-1 (PDB access code: 1GZW) showing the S- and F-face of the CRD. (B.) *cis-* and *trans-*bridging activity activities of Gal-1 variants with core M1 α-DG and laminin *in situ*.

terminated glycopeptides) to S-Face (Gal-1–LacNAc-terminated

glyco-peptides of laminin) *trans*-bridging interaction with laminins is involved as shown in Fig. 7.

Conclusion

Changes in galectin architecture, such as i) turning a non-covalently associated homodimer into a covalently linked homodi- or tetramer or a heterodimer, ii) bringing a CRD into a different structural context, or iii) reducing the linker length of a tandem repeat-type galectin can modulate the binding towards core M1 α-DG glycoconjugates. These classes of glycans disclosed that Gal-1’s CRD maintains its binding pattern (with LacNAc and its oligomers) irrespective of the tested alterations of the protein architecture when interacting with surface-immobilized glycans in an array. In the case of Gal-3, removing the N-terminal tail and conversion to a homo/hetero pototype can enhance its affinity to the prepared ligands, and the presence of a peptide linker between the CRDs (transforming it to a tandem-repeat type) influenced the activity. For Gal-4, maintaining the full-length linker is crucial, and its transformation to prototype and chimera type is undesirable for its binding. On the other hand, the C-terminal Gal-9 CRD mainly contributed to the overall binding of the wild-type protein to the core M1 α-DG core glycoconjugates.

Furthermore, Gal-1 variants **(Gal-1)2[8S]** and **(Gal-1)4[8S]** can effectively *trans*-bridge core M1 α-DG glycoconjugates and laminins (111, -121, -211, -221, and -511) compared to Gal-1 wild-type. This enhancement of crosslinking activity is attributed to the additional peptide linker between the two Gal-1 CRD and the increased number of Gal-1 CRD sites per protein. Similar to Gal-1WT, these Gal-1 variants are potential candidates for treating α-dystroglycanopathy. However, further tests are required to validate this hypothesis.

Overall, this experimental setup demonstrated that the alteration of galectin structures can provide additional insights into the preferential modular architecture and binding behavior of this lectin family towards specific ligands. In addition, rational protein engineering is a useful tool for redesigning lectins with possibly higher therapeutic potential than their wild-type counterparts. Here, Gal-1 has a proof-of-principle character.

Experimental Section

**Production of labeled galectin variants** [4,28,51]

Galectins were prepared by recombinant expression using the *E.coli* strain BL21 (DE) pLysS and the pGEMEX-1 vector (Promega, Walldorf, Germany). Galectin-expressing bacteria were grown at 37°C until an OD600 of 0.6-0.8 is acheived. The expression of i) Gal-1, (Gal-1)2[GG], (Gal-1)4[GG], (Gal-1)2[8S], and (Gal-1)4[8S] was induced with 100 µM isopropyl ß-d-1-thiogalactopyranoside (IPTG) at 37°C; ii) Gal-3 (400 mM IPTG), trGal-3, Gal-3⎯Gal-3, Gal-3⎯Gal-1, Gal-1⎯Gal-3, Gal-3⎯8S⎯Gal-3, Gal-3⎯8L⎯Gal-3, Gal-3⎯8S⎯Gal-1, Gal-1⎯8S⎯Gal-3, Gal-9N, and Gal-9C were induced with 100 µM IPTG at 22°C; and iii) Gal-4, Gal-4V, Gal-4P, and Gal-3NT/4C were induced at 30°C with 75 µM IPTG. Induced bacteria were grown for 16 h. Proteins were purified after cell lysis by sonication (three times, each stroke for 1 min) through affinity chromatography on a homemade lactose-sepharose resin. Subsequently, the bound proteins were eluted from the resin using 20 mM PBS (pH 7.2) containing 50 mM lactose and 20 mM iodoacetamide. PBS was exchanged using a PD10 column in 10 mM sodium carbonate buffer, pH 8.5. The proteins (2-3 mg/mL) were directly conjugated in the dark and in the presence of activity-preserving 20 mM lactose to NHS-ester Alexa 555 fluorescent dye at 25°C for 4 h. Unbound dye was removed by gel filtration using a Sephadex G-25 column. Protein purity was assessed using gel electrophoresis and western blotting. Activity was verified using solid-phase assays, flow cytometry, and hemagglutination assays. Labeled proteins were lyophilized in aliquots and stored at -20°C until reconstitution. Stock probed galectins (1 mg/mL) were prepared in 1x PBS (pH 7.40) containing 1% (w/v) BSA, 0.09% (w/v) NaN3, and 50% (w/v) glycerol, and stored at -20oC until use.

**Development of α-dystroglycan core M1 (glyco)peptide library** [14,34]

The *O*-Man core m1 α-DG glyco-amino acids (**1**), peptide (**4**), glycopeptides (**5-11**), and MUC1 peptide (**30**) were synthesized manually by microwave-assisted solid-phase synthesis by using Fmoc-amino acids (Novabiochem), Fmoc(Ac3GlcNAcβ1🡪2Ac3Manα)Thr (Medicinal Chemistry Pharmaceuticals, Sapporo, Japan), and H-Rink Amide ChemMatrix® (0.48 mmol/g, 24 μmol) resin. The resin was swollen with CH2Cl2 in a polypropylene tube equipped with a filter (LibraTube; Hipep Laboratories, Kyoto, Japan) for 1 h at room temperature. The protected Fmoc-amino acid (4.0 equiv) was pre-activated by treating with HBTU (4.0 equiv), HOBt (4.0 equiv), and DIEA (6.0 equiv) in DMF (455 μL) for 9 min under microwave irradiation (Green Motif 1 microwave synthesis reactor, IDX Corp, Japan) and then attached to the resin. In every step, the N-fluorene-9-ylmethoxycarbonyl (Fmoc) groups at the N-terminus were removed with 20% piperidine in DMF (1 mL) for 3 min under microwave irradiation. All coupling reactions were performed for 10 min and the solvents were removed using PP syringes fitted with a porous disk. For glycosylated amino acid, Fmoc-Thr(Ac3GlcNAcβ1→2Manα1)-OH (1.2 equiv) was treated with PyBOP (1.2 equiv), HOAt (1.2 equiv) and DIEA (3.0 equiv) in DMF (275 μL) subjected to MW irradiation for 9 min at 50°C. PyBOP-HOAt (1.2 equiv) was then added and allowed to react for another 9 min. As the final synthesis step, 5-oxohexanoic acid (3 equiv) was introduced at the N-terminus of each glycopeptide resin according to the above coupling procedure for Fmoc-amino acids. Removal of side-chain protecting groups and cleavage of glycopeptides from the resin occurred in parallel by treatment with 95% aqueous TFA (1 mL) for 1 h at ambient temperature. The crude peptides and glycopeptides were precipitated in a cold water bath using tert-butyl methyl ether (5 mL). The solution was then centrifuged at 3000 rpm for 1 min and the supernatant was carefully removed. The precipitate was dissolved in Milli-Q water (5 mL) and lyophilized. Deacetylation of the glycan moiety was then performed by dissolving the lyophilized material in methanol; the pH was adjusted to 12.5, with dropwise addition of 1M NaOH, and the solution was stirred at room temperature for 1 h. After deprotection, the solution was neutralized with 20% AcOH in methanol and the flow of nitrogen gas displaced the solvent. The crude peptides and glycopeptides were purified by RP-HPLC using a preparative C18-reversed-phase column (Intersil ODS-3 10×250 mm) on an HPLC (HITACHI, Japan) equipped with an L7150 pump, at a flow rate of 5 mL/min, monitored by a UV detector at 220 nm at room temperature. Eluent A was distilled water containing 0.1% TFA and eluent B was acetonitrile containing 0.1% TFA. Each product was analyzed by Ultraflex MALDI-TOF MS (Bruker Daltonics, Germany) using DHB as a matrix.

Galactosylation of compounds 1, 5, and 11 in a 24 h incubation step yielded compounds 2 and 12–18. The 50 mM HEPES buffer (pH 7.0) contained 10 mM MnCl2, 0.1% BSA, galactosyltransferase from bovine milk (Sigma Aldrich), and UDP-Gal (Yamasa Corporation, Chiba, Japan). Subsequently, compounds **2,** **12**–**18** were sialylated using α2,3-sialyltransferase from *Pasteurella multocida* (Sigma Aldrich) and CMP-NANA (Yamasa Corporation, Chiba, Japan) in 50 mM Tris buffer (pH 6.5) and 500 mM NaCl, and incubated for 36 h to yield compounds **3, 19**–**25**. Compounds **26**–**29** were synthesized using solid-phase synthesis and enzymatic sugar elongation, as described previously. Each product was purified by RP-HPLC as described above using an appropriate solvent system and identified by MALDI-TOFMS.

**Lectin Microarray Binding Experiment** [14,26,52]

The AO/PC copolymer microarray slides (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were deprotected using 2N HCl overnight at room temperature, rinsed with MilliQ H2O, and dried by centrifugation. The test compounds were robotically printed in quadruplets at two concentrations (20 µM and 200 µM) in 25 mM AcOH-Pyr (pH 5.0) and 0.0025% (w/v) Triton X-100 using an Arduino-based CNC machine handcrafted robot. A cyanin3-keto-BSA (Cy3-keto-BSA) at 25 µg/mL was printed on the slide as a grid. Subsequently, the slide was incubated at 80°C for 1 h to complete oxime bond formation. The mixture was washed once with Milli-Q H2O and dried by centrifugation at 2,000 rpm for 2 min.

A silicon rubber sheet with six chambers is attached to the printed slide. Next, slide was pretreated with reaction buffer {Phosphate-Buffered Saline solution (PBS, 1X) [10 mM Na2HPO4, 1.8 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl] pH7.4 containing 0.05% (v/v) Tween-20} for 15 min and dried by centrifugation. Plant lectin or galectin solutions in PBS were prepared and maintained in a cold ice bath before use. A cover glass was then placed in each chamber, and 12 µL of 0.10 µg/mL lectin solution in reaction buffer was added through the gap of the slide and cover. After 1 h of incubation with the lectin solution at room temperature in a humidified chamber, the slide was rinsed with washing buffer and fluorescence intensity was measured using a GlycoStation System (GlycoStation Reader 1200, GlycoTechnica Ltd., Yokohama, Japan). To determine additional interactions at higher Gal concentrations, the reaction buffer solution was carefully removed and replaced with the next lectin test concentration, incubated for 5 min at room temperature, washed with the reaction buffer, and fluorescence intensity was obtained. This step was repeated until all chosen test concentrations were completed (0.32, 1.00, 3.20, 10.0, and 32.0 µg/mL).

The slides were imaged in the presence of a reaction buffer. The fluorescence intensities were analyzed using ArrayVision software (V8.0; GE Healthcare, Tokyo, Japan). Background correction was applied to obtain the net intensity. The average relative fluorescence unit (RFU) was plotted as a bar graph, and the error bars represent the standard deviation using Microsoft Excel.

**Laminin–Gal-1 Binding Assay** [26]

The printed test compounds were added to 12 µL of premixed Cy3-laminin (Biolamina and BroadPharm) and unlabeled-Gal-1(variants) solutions at a final concentration of 0.10 µg/mL and incubated for 1 h in a humidified chamber. The slide was rinsed with washing buffer, and fluorescence intensity was measured using the GlycoStation System, as described above. To determine additional interactions at higher laminin-Gal-1 concentrations, we carefully removed the reaction buffer and replaced it with the next test concentration, incubated for 5 min at room temperature, washed with the reaction buffer, and measured the fluorescence intensity. This step was repeated until all chosen test concentrations were completed (0.32, 1.00, 3.20, 10.0, and 32.0 µg/mL). For weak interactions, incubation with 32.0 µg mL-1 of laminin-Gal-1 solution was extended for 30 min. Slide images were captured and analyzed as described above.

Acknowledgments

Valuable discussions with Dr. Takahiko Matsushita and Dr. Fayna Garcia-Martin regarding peptide synthesis are acknowledged. We thank Cornelia Lange for her support with galectin expression and purification. Generous funding from JSPS Scientific Research (22H02191, 17K05920, to H.H.; 25220206, to S.-I.N.), JST A-STEP (JPMJTM20JB, H.H.), JKA Foundation (2019M-165, H.H), and Hoansha Foundation (H.H.) for their financial support is greatly appreciated.

Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** Galectin, Protein Engineering, α-Dystroglycan, Laminin

[1] C. Reily, T. J. Stewart, M. B. Renfrow, J. Novak, *Nat. Rev. Nephrol.* **2019**, *15*, 346–366.

[2] H. Kaltner, S. Toegel, G. G. Caballero, J. C. Manning, R. W. Ledeen, H.-J. Gabius, *Histochem. Cell Biol.* **2017**, *147*, 239–256.

[3] K. Kasai, *Trends Glycosci. Glycotechnol.* **2018**, *30*, SE221–SE223.

[4] G. García Caballero, H. Kaltner, T. J. Kutzner, A.-K. Ludwig, J. C. Manning, S. Schmidt, F. Sinowatz, H.-J. Gabius, *Histol. Histopathol.* **2020**, *35*, 509—539.

[5] H.-J. Gabius, J. Roth, *Histochem. Cell Biol.* **2017**, *147*, 111–117.

[6] H.-J. Gabius, S. André, J. Jiménez-Barbero, A. Romero, D. Solís, *Trends Biochem. Sci.* **2011**, *36*, 298–313.

[7] J. C. Manning, A. Romero, F. A. Habermann, G. García Caballero, H. Kaltner, H.-J. Gabius, *Histochem. Cell Biol.* **2017**, *147*, 199–222.

[8] R. Heusschen, I. A. Schulkens, J. van Beijnum, A. W. Griffioen, V. L. Thijssen, *Biochim. Biophys. Acta* **2014**, *1842*, 284–292.

[9] N. Nishi, A. Itoh, H. Shoji, H. Miyanaka, T. Nakamura, *Glycobiology* **2006**, *16*, 15C-20C.

[10] G. A. Rabinovich, M. A. Toscano, S. S. Jackson, G. R. Vasta, *Curr. Opin. Struct. Biol.* **2007**, *17*, 513–520.

[11] H. Kaltner, J. C. Manning, G. García Caballero, C. Di Salvo, A. Gabba, L. L. Romero-Hernández, C. Knospe, D. Wu, H. C. Daly, D. F. O’Shea, H.-J. Gabius, P. V Murphy, *RSC Adv.* **2018**, *8*, 28716–28735.

[12] J. Kopitz, Q. Xiao, A.-K. Ludwig, A. Romero, M. Michalak, S. E. Sherman, X. Zhou, C. Dazen, S. Vértesy, H. Kaltner, M. L. Klein, H.-J. Gabius, V. Percec, *Angew. Chemie Int. Ed.* **2017**, *56*, 14677–14681.

[13] C. Zhou, H. L. Reesink, D. A. Putnam, *Biomacromolecules* **2019**, *20*, 3704–3712.

[14] G. Artigas, H. Hinou, F. Garcia-Martin, H.-J. Gabius, S.-I. Nishimura, *Chem. Asian J.* **2017**, *12*, 159–167.

[15] N. Nishi, A. Abe, J. Iwaki, H. Yoshida, A. Itoh, H. Shoji, S. Kamitori, J. Hirabayashi, T. Nakamura, *Glycobiology* **2008**, *18*, 1065–1073.

[16] P. Bättig, P. Saudan, T. Gunde, M. F. Bachmann, *Mol. Immunol.* **2004**, *41*, 9–18.

[17] L. A. Earl, S. Bi, L. G. Baum, *Glycobiology* **2011**, *21*, 6–12.

[18] S. Tasumi, G. R. Vasta, *J. Immunol.* **2007**, *179*, 3086–3098.

[19] X. Song, H. Zhang, J. Zhao, L. Wang, L. Qiu, C. Mu, X. Liu, L. Qiu, L. Song, *Fish Shellfish Immunol.* **2010**, *28*, 326–332.

[20] M. M. Fettis, S. A. Farhadi, G. A. Hudalla, *Biomater. Sci.* **2019**, *7*, 1852–1862.

[21] S. A. Farhadi, M. M. Fettis, R. Liu, G. A. Hudalla, *Front. Chem.*  **2020**, *7*.

[22] J. M. Ervasti, K. Ohlendieck, S. D. Kahl, M. G. Gaver, K. P. Campbell, *Nature* **1990**, *345*, 315–319.

[23] T. ENDO, *Proc. Japan Acad. Ser. B* **2019**, *95*, 39–51.

[24] T. Yoshida-Moriguchi, K. P. Campbell, *Glycobiology* **2015**, *25*, 702–713.

[25] P. M. Van Ry, T. M. Fontelonga, P. Barraza-Flores, A. Sarathy, A. M. Nunes, D. J. Burkin, *Compr. Physiol.* **2017**, *7*, 1519–1536.

[26] L. L. Villones, A.-K. Ludwig, H. Kumeta, S. Kikuchi, R. Ochi, T. Aizawa, S.-I. Nishimura, H.-J. Gabius, H. Hinou, *Sci. Rep.* **2022**, *12*, 17800.

[27] A.-K. Ludwig, H. Kaltner, J. Kopitz, H.-J. Gabius, *Biochim. Biophys. Acta - Gen. Subj.* **2019**, *1863*, 935–940.

[28] A.-K. Ludwig, M. Michalak, Q. Xiao, U. Gilles, F. J. Medrano, H. Ma, F. G. FitzGerald, W. D. Hasley, A. Melendez-Davila, M. Liu, K. Rahimi, N. Y. Kostina, C. Rodriguez-Emmenegger, M. Möller, I. Lindner, H. Kaltner, M. Cudic, D. Reusch, J. Kopitz, A. Romero, S. Oscarson, M. L. Klein, H.-J. Gabius, V. Percec, *Proc. Natl. Acad. Sci.* **2019**, *116*, 2837 LP – 2842.

[29] T. J. Kutzner, A. Gabba, F. G. FitzGerald, N. V Shilova, G. García Caballero, A.-K. Ludwig, J. C. Manning, C. Knospe, H. Kaltner, F. Sinowatz, P. V Murphy, M. Cudic, N. V Bovin, H.-J. Gabius, *Glycobiology* **2019**, *29*, 593–607.

[30] G. García Caballero, D. Beckwith, N. V Shilova, A. Gabba, T. J. Kutzner, A.-K. Ludwig, J. C. Manning, H. Kaltner, F. Sinowatz, M. Cudic, N. V Bovin, P. V Murphy, H.-J. Gabius, *Histochem. Cell Biol.* **2020**, *154*, 135–153.

[31] S. André, G.-N. Wang, H.-J. Gabius, P. V Murphy, *Carbohydr. Res.* **2014**, *389*, 25–38.

[32] D. Solís, M. J. Maté, M. Lohr, J. P. Ribeiro, L. López-Merino, S. André, E. Buzamet, F. J. Cañada, H. Kaltner, M. Lensch, F. M. Ruiz, G. Haroske, U. Wollina, M. Kloor, J. Kopitz, J. L. Sáiz, M. Menéndez, J. Jiménez-Barbero, A. Romero, H.-J. Gabius, *Int. J. Biochem. Cell Biol.* **2010**, *42*, 1019–1029.

[33] Q. Xiao, A.-K. Ludwig, C. Romanò, I. Buzzacchera, S. E. Sherman, M. Vetro, S. Vértesy, H. Kaltner, E. H. Reed, M. Möller, C. J. Wilson, D. A. Hammer, S. Oscarson, M. L. Klein, H.-J. Gabius, V. Percec, *Proc. Natl. Acad. Sci.* **2018**, *115*, E2509 LP-E2518.

[34] H. Hinou, S. Kikuchi, R. Ochi, K. Igarashi, W. Takada, S.-I. Nishimura, *Bioorg. Med. Chem.* **2019**, *27*, 2822–2831.

[35] T. Matsushita, W. Takada, K. Igarashi, K. Naruchi, R. Miyoshi, F. Garcia-Martin, M. Amano, H. Hinou, S.-I. Nishimura, *Biochim. Biophys. Acta - Gen. Subj.* **2014**, *1840*, 1105–1116.

[36] Y. Nonaka, T. Ogawa, H. Shoji, N. Nishi, S. Kamitori, T. Nakamura, *Glycobiology* **2022**, *32*, 251–259.

[37] S. Bi, L. A. Earl, L. Jacobs, L. G. Baum, *J. Biol. Chem.* **2008**, *283*, 12248–12258.

[38] S. Vértesy, M. Michalak, M. C. Miller, M. Schnölzer, S. André, J. Kopitz, K. H. Mayo, H.-J. Gabius, *Protein Eng. Des. Sel.* **2015**, *28*, 199–210.

[39] N. Ahmad, H.-J. Gabius, S. André, H. Kaltner, S. Sabesan, R. Roy, B. Liu, F. Macaluso, C. F. Brewer, *J. Biol. Chem.* **2004**, *279*, 10841–10847.

[40] Y.-H. Lin, D.-C. Qiu, W.-H. Chang, Y.-Q. Yeh, U.-S. Jeng, F.-T. Liu, J.-R. Huang, *J. Biol. Chem.* **2017**, *292*, 17845–17856.

[41] J. Seetharaman, A. Kanigsberg, R. Slaaby, H. Leffler, S. H. Barondes, J. M. Rini, *J. Biol. Chem.* **1998**, *273*, 13047–13052.

[42] P. M. Collins, K. Bum-Erdene, X. Yu, H. Blanchard, *J. Mol. Biol.* **2014**, *426*, 1439–1451.

[43] M. J. Moure, A. Gimeno, S. Delgado, T. Diercks, G.-J. Boons, J. Jiménez-Barbero, A. Ardá, *Angew. Chemie Int. Ed.* **2021**, *60*, 18777–18782.

[44] S. Kamitori, *Trends Glycosci. Glycotechnol.* **2018**, *30*, SE41–SE50.

[45] P. M. Van Ry, R. D. Wuebbles, M. Key, D. J. Burkin, *Mol. Ther.* **2015**, *23*, 1285–1297.

[46] R. D. Wuebbles, V. Cruz, P. Van Ry, P. Barraza-Flores, P. D. Brewer, P. Jones, D. J. Burkin, *Mol. Ther. Methods Clin. Dev.* **2019**, *13*, 145–153.

[47] T. L. M. Thurston, M. P. Wandel, N. von Muhlinen, Á. Foeglein, F. Randow, *Nature* **2012**, *482*, 414–418.

[48] B.-W. Kim, S. Beom Hong, J. Hoe Kim, D. Hoon Kwon, H. Kyu Song, *Nat. Commun.* **2013**, *4*, 1613.

[49] F. A. van den Brûle, C. Buicu, M. E. Sobel, F. T. Liu, V. Castronovo, *Neoplasma* **1995**, *42*, 215–219.

[50] I. Kuwabara, F. T. Liu, *J. Immunol.* **1996**, *156*, 3939–3944.

[51] J. Kopitz, S. Ballikaya, S. André, H.-J. Gabius, *Neurochem. Res.* **2012**, *37*, 1267–1276.

[52] A. Kuno, N. Uchiyama, S. Koseki-Kuno, Y. Ebe, S. Takashima, M. Yamada, J. Hirabayashi, *Nat. Methods* **2005**, *2*, 851–856.

**Entry for the Table of Contents**

C:\Users\laren\Desktop\Project 1\2nd Paper\ChemBioChem\Final Figures\Table of Contents.tif

The alteration of galectin modular architecture effectively revealed the design-functionality relationship of lectin binding towards α-dystroglycan O-Mannosylated core M1 glycopeptides *in situ*.

Altering galectin modular architecture via rational protein engineering revealed the design-functionality relationship within this lectin family towards its affinity to α-dystroglycan *O*-Mannosylated core M1 glycopeptides *in situ*. Transforming galectin-1 and -3 into tandem-repeat- and proto-types, respectively, significantly enhanced *cis*-binding with the prepared ligands. Moreover, Gal-1 variants demonstrated higher *trans*-bridging capabilities between α-dystoglycan glycoconjugates and laminins compared to Gal-1 wild-type.

Institute and/or researcher Twitter usernames: ((optional))