

Thiodigalactoside–Bovine Serum Albumin Conjugates as High-Potency Inhibitors of Galectin-3: An Outstanding Example of Multivalent Presentation of Small Molecule Inhibitors

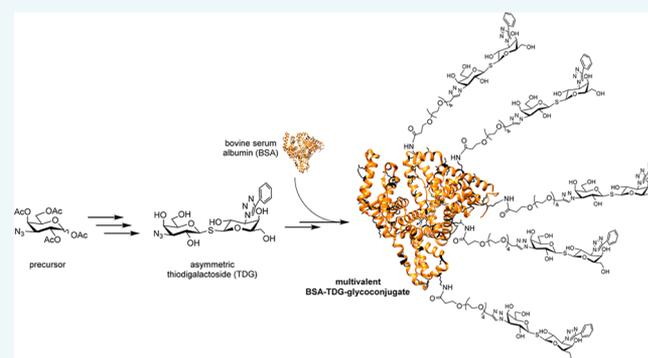
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Supporting Information

ABSTRACT: Galectin inhibitors are urgently needed to understand the mode of action and druggability of different galectins, but potent and selective agents still evade researchers. Small-sized inhibitors based on thiodigalactoside (TDG) have shown their potential while modifications at their C3 position indicated a strategy to improve selectivity and potency. Considering the role of galectins as glycoprotein traffic police, involved in multivalent bridging interactions, we aimed to create multivalent versions of the potent TDG inhibitors. We herein present for the first time the multivalent attachment of a TDG derivative using bovine serum albumin (BSA) as the scaffold. An efficient synthetic method is presented to obtain a novel type of neoglycosylated proteins loaded with different numbers of TDG moieties. A polyethylene glycol (PEG)-spacer is introduced between the TDG and the protein scaffold maintaining appropriate accessibility for an adequate galectin interaction. The novel conjugates were evaluated in galectin binding and inhibition studies *in vitro*. The conjugate with a moderate density of 19 conjugated TDGs was identified as one of the most potent multivalent Gal-3 inhibitors so far, with a clear demonstration of the benefit of a multivalent ligand presentation. The described method may facilitate the development of specific galectin inhibitors and their application in biomedical research.



A dense layer of carbohydrates is found on mammalian cells, and the variety of the attached glycans results in specific profiles for molecular recognition. This recognition involving the so-called “sugar code” is operational by reversible interaction of carbohydrate-binding proteins. Members of this protein class are described as lectins and fulfill a variety of effector functions in terms of cellular communication.¹ Galectins as one subtype of lectins that can specifically recognize β -galactosides are found in fungi, invertebrates, and vertebrates.² Fifteen different galectins have been identified in humans until now, and they play crucial roles in the organization of receptor-lectin complexes (lattices)^{3,4} and regulation of immune responses.⁵ Their concave-shaped groove for oligosaccharide binding, namely, the carbohydrate recognition domain (CRD), is highly preserved. Galectin-1 (Gal-1) and galectin-3 (Gal-3) are the most thoroughly studied galectins due to their involvement in angiogenesis, tumor progression, and metastasis.^{6–8} The participation in malignant processes makes them promising targets for anticancer therapy. In this regard, immense efforts have been spent on the synthesis of potent and specific ligands. The prevailing majority of drug discovery efforts have been focused on the synthetic

modification of lactose (methyl β -lactoside, $K_d = 220 \mu\text{M}$ for Gal-3, $K_d = 190 \mu\text{M}$ for Gal-1) and *N*-acetylglucosamine (methyl β -LacNAc, $K_d = 67 \mu\text{M}$ for Gal-3), which are the natural disaccharide ligands for both Gal-1 and Gal-3.^{9–11} Thiodigalactosides (TDG, $K_d = 49 \mu\text{M}$ for Gal-3, $K_d = 24 \mu\text{M}$ for Gal-1) were identified as more potent Gal-3 inhibitors with additional advantages such as their enhanced glycolytic stability while maintaining a similar binding mode compared to lactose and LacNAc.^{12,13} Especially, the introduction of triazole moieties at the C3'-position of the galactose molecules resulted in monovalent galectin inhibitors with outstanding high affinities showing K_d values in the low nanomolar range.^{14–17} Apart from designing small molecule inhibitors, a multivalency-based strategy was adopted to promote the interactions between ligands and protein, as this more closely mimics the natural way in which galectins interact with glycoproteins. So far, different scaffolds were reported to carry multiple galectin

Received: January 18, 2018

Revised: February 22, 2018

Published: February 23, 2018

ligands.^{18–22} In our previous work, bovine serum albumin (BSA) was used as a plain protein carrier for neo-glycosylation.^{23,24} *N*-hydroxysuccinimidyl (NHS)-esters, the most commonly used amine-reactive reagents, offer a larger variety of amine-reactive ligands for labeling proteins.^{25–27} Benefiting from this prior information, we successfully approached a type of neo-glycoproteins through conjugation of NHS functionalized-TDG to lysine residues of BSA. The most obvious finding to emerge from the present study is that the TDG-conjugates exhibit outstanding high inhibitory potencies despite a low or moderate number of attached ligands. Combining a highly potent monovalent ligand with a beneficial multivalent presentation resulted in some of the most effective Gal-3 inhibitors. Besides, the multivalent TDG-conjugates represent the first example of decorating a nonglycosylated carrier with TDG derivatives.

RESULTS AND DISCUSSION

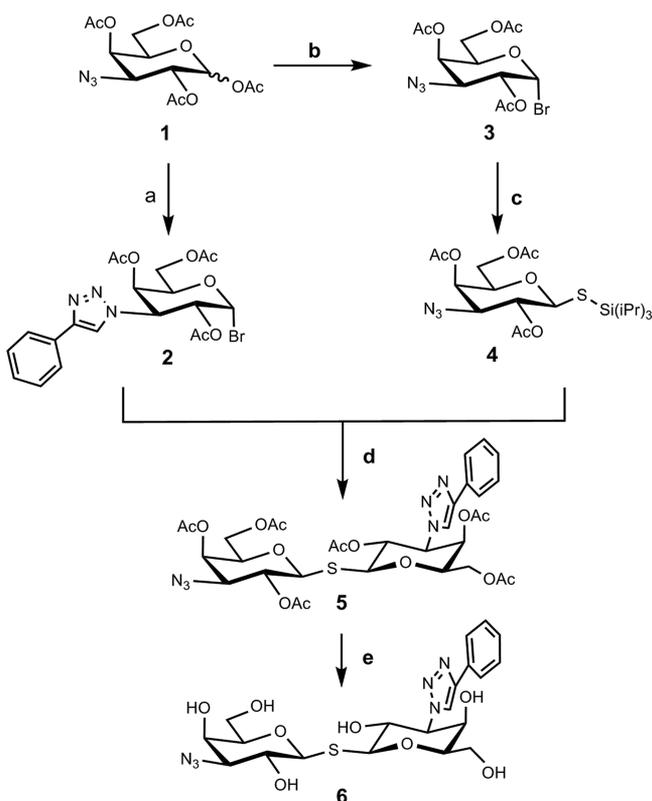
Synthesis of a Carboxy-Functionalized Thiodigalactoside Precursor. The synthesis of a carboxy-functionalized TDG precursor started as shown in Scheme 1. The key building block was the unsymmetrical thiodigalactoside (TDG) precursor 6 carrying phenyltriazole and azide at the 3- and 3'-positions. To construct TDG precursor 6, two building blocks, namely, tri-isopropylsilyl thio-glycoside (compound 4) and glycosyl halide (compound 2), were prepared using a published method of a one-pot desilylation and glycosyl thiol

alkylation with glycosyl halide.²⁸ Hence, compound 1, prepared from commercially available 1,2,5,6-diacetone- α -D-glucofuranoside through a known four-step reaction,²⁹ was converted to 4. Meanwhile, copper-catalyzed azide-alkyne cycloaddition of compound 1 with phenylacetylene provided the corresponding triazole analog crude, which reacted with HBr to obtain the glycosyl halide compound 2 (79% yield in two steps). Desilylating and activating with TBAF turned compound 4 into a thiol nucleophile and thus replaced the anomeric bromide of compound 2 through an S_N2 reaction. Purification by silica column chromatography gave the resulting compound 5 in 54% yield. After removing the acetyl protecting group, the resulting crude 6 was used for the next step without further purification (Scheme 1).

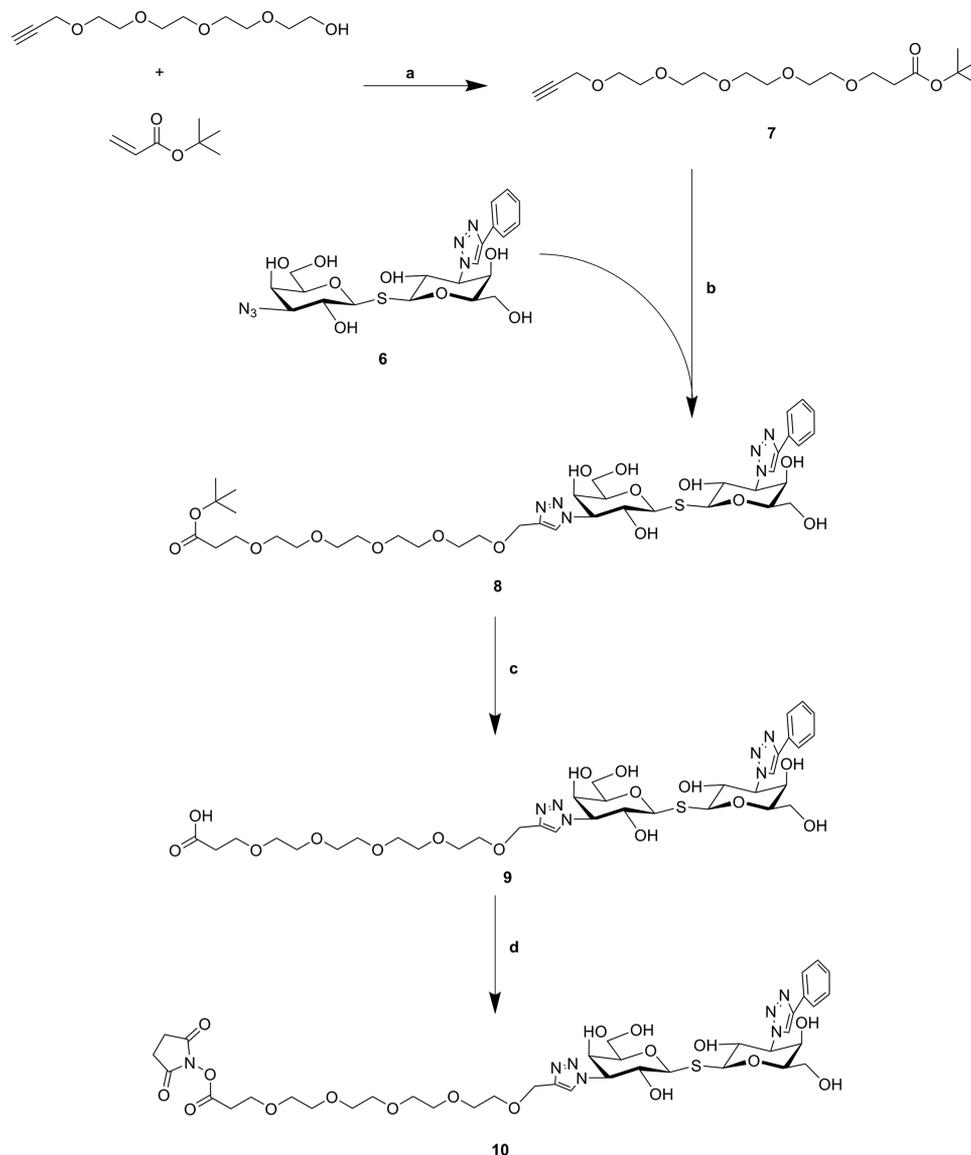
The synthesis of polyethylene glycol (PEG)-spacer (compound 7) was started from tetraethylene glycol. As previously reported, the reaction of tetraethylene glycol with an equal amount of propargyl bromide in the presence of NaH in THF at room temperature gave the monoalkyne terminated PEG4.³⁰ Then the Michael addition of the resulting compound to *tert*-butyl acrylate in the presence of catalytic sodium metal gave compound 7 in 70% yield.³¹ Having assembled the important intermediates (compounds 6 and 7), the next objective was their CuAAC conjugation assisted by copper iodide. After purification with size-exclusion, compound 8 was obtained. Removal of the *tert*-butyl group from compound 8 gave carboxyl compound 9 (90% yield), which was transferred to the corresponding NHS-ester 10 through coupling with TSTU (Scheme 2). Compound 10 is prone to hydrolysis (e.g., during purification); thus, it was directly used for further reaction.

Neo-Glycoprotein Synthesis and Analysis. Compared to click chemistry³² and squarate linker chemistry,^{23,24,33–35} NHS-mediated coupling^{25,27,36} is another straightforward and convenient coupling strategy for the modification of protein carriers. The lysine residues of bovine serum albumin (BSA) react with the NHS ester moiety of the TDG derivative, but a crucial factor that needs to be taken into consideration is the distance between the TDGs and BSA. Benefiting from our previous work on chito-oligomer spacers in neo-glycoproteins,³⁴ we concluded that a suitable spacer of a certain length would be recommended to maintain an appropriate ligand accessibility and proper galectin interaction of final products. As a logical consequence thereof, a PEG as placeholder with a similar spacing distance was incorporated into conjugation agent 9. Furthermore, the modification of carbohydrates or derivatives thereof with PEG as a biocompatible molecule is a commonly used technique.^{37,38} The NHS functionalized TDG 10 readily reacted with amino groups of BSA using a reaction buffer that contained 35 mM HEPES (pH 7.0). The total amount of compound 10 was divided into three and added batchwise after every 24 h. As a result, we obtained compound 11 that was verified by the TNBSA-assay²³ to carry 7.0 ± 1.0 TDG moieties per BSA molecule (Scheme 3). The coupling efficiency was 7.8%. Shifting the pH to slightly higher values (pH 8.0–9.0) by the addition of triethylamine (TEA) gave compound 12. The TNBSA-assay confirmed that the number of attached TDGs was now 18.7 ± 1.6 corresponding to a coupling efficiency of 20.1%. The elevation of the pH may deprotonate the amino groups of lysine residues to a degree sufficiently high for fast and efficient coupling. In accordance with our previous findings, reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the attachment of TDGs (Figure S15, Supporting Information).

Scheme 1. Synthesis of Thiodigalactoside 6^a



^aReagents and conditions: (a) (i) Phenylacetylene, CuSO₄, sodium ascorbate, DMF/H₂O, 80 °C, microwave, (ii) HBr, CH₂Cl₂, r.t., 79% yield in two steps; (b) TiBr₄, CH₂Cl₂/EtOAc, 25 °C, 67%; (c) TIPSSH, K₂CO₃, CH₃CN, 25 °C, 30%; (d) TBAF, CH₃CN, 25 °C, 62%; (e) NaOMe, CH₃OH, 25 °C.

Scheme 2. Preparation of Carboxy- (9) and NHS-Functionalized (10) TDG Derivatives^a

^aReagents and conditions: (a) Na, THF, 0–25 °C, 39%; (b) CuI, CH₃OH, 25 °C, 64%; (c) TFA/DCM, 25 °C, 98%; (d) TSTU, DiPEA, DMF, 25 °C.

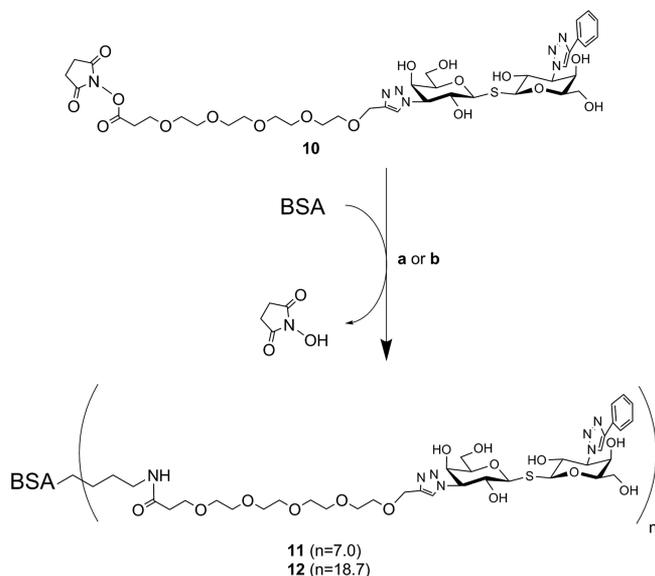
Evaluation of Multivalent BSA-Conjugates as Galectin Ligands. Multivalent TDG-conjugates **11** and **12** were utilized as immobilized ligands in solid-phase binding assays for recombinant human His₆-tagged galectin-1 (Gal-1) and galectin-3 (Gal-3) (Figure 1). Our binding studies revealed that Gal-1 bound ligand **11** in a 3-fold more efficient manner than Gal-3 did. Gal-1 showed higher affinity for conjugate **11** with a 6-fold reduced apparent dissociation constant K_d in comparison with Gal-3 with $p < 0.001$ (Student's *t* test). However, Gal-3 showed an elevated capacity when binding to conjugate **11**, indicated by the higher B_{max} value (Table 1). This is putatively caused by cluster glycoside effects,³⁹ which may lead to galectin oligomerization and thus to an increased binding signal (Figure 1). This effect might be more pronounced for Gal-3, which is generally considered to form higher oligomers when binding to multivalent ligands. Gal-1 aggregation is likely limited to the formation of dimers.

Conjugate **12** carries an increasing number of TDG derivatives and was therefore bound with a higher affinity and

capacity by Gal-3. The lower apparent K_d value and raised B_{max} value led to the highest binding efficiency observed for this binding assay (Table 1). The enhancement of Gal-3 binding efficiency for compound **12** was significant with $p < 0.001$ (Student's *t* test). In contrast, the higher TDG loading of compound **12** did not affect Gal-1 binding much and the capacity (B_{max}) was only slightly increased while the affinity was even slightly reduced. The corresponding binding efficiencies for Gal-1 toward conjugates **11** and **12** are similar within experimental error (Table 1).

To summarize these assay results, prototype Gal-1 and chimera-type Gal-3 bind compounds **11** and **12** with high affinity through recognizing the conjugated TDGs. Our findings corroborate previous studies, which reported on the interaction of both tested galectins with the corresponding monovalent and symmetrical TDG-based compounds by a fluorescence polarization assay.¹⁷ However, we did not detect any specificity differences as seen for previously synthesized neo-glycoproteins carrying poly-*N*-acetylglucosamine (poly-LacNAc) derivatives.²⁴

Scheme 3. Loading of BSA Protein Carriers with NHS-Functionalized Compound 10 Resulting in Compounds 11 and 12^a



^aReagents and conditions: (a) compound 11: BSA (0.06 mM, 150 μ L) in HEPES buffer (35 mM, pH 7.0), compound 10 in DMF (54 mM, 3 \times 5 μ L), 72 h, 4 $^{\circ}$ C; (b) compound 12: BSA (0.06 mM, 50 μ L) in HEPES buffer (pH 8.0–9.0, adjusted with TEA); compound 10 in DMF (54 mM, 3 \times 1.67 μ L), 72 h, 4 $^{\circ}$ C.

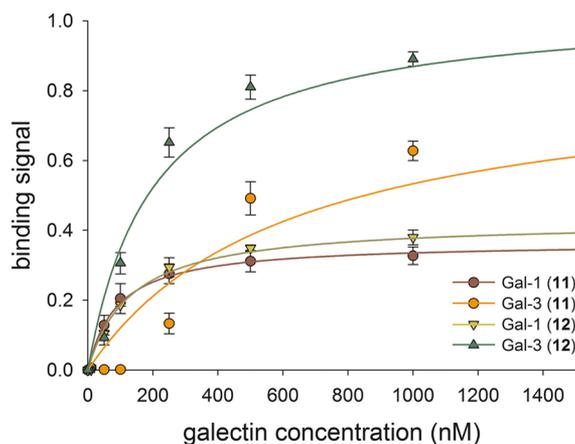


Figure 1. Behavior of recombinant human Gal-1 and Gal-3 for binding to immobilized neo-glycoconjugates 11 and 12. The subtracted blank value (no Gal-1 and/or Gal-3) was 0.047 ± 0.003 .

Table 1. Binding Behavior of Gal-1 and Gal-3 Using Multivalent TDG Conjugates as Immobilized Ligands

| galactin | ligand | apparent K_d (μ M) ^a | B_{max} (-) ^a | galactin binding efficiency [μ M ⁻¹] ^b |
|----------|--------|--|----------------------------|--|
| Gal-1 | 11 | 0.090 ± 0.012 | 0.37 ± 0.01 | 4.1 ± 0.8 |
| Gal-1 | 12 | 0.131 ± 0.017 | 0.43 ± 0.01 | 3.3 ± 0.6 |
| Gal-3 | 11 | 0.616 ± 0.246 | 0.86 ± 0.12 | 1.4 ± 0.5 |
| Gal-3 | 12 | 0.199 ± 0.045 | 1.04 ± 0.06 | 5.2 ± 0.3 |

^aDetermined in ELISA. ^bRatio of B_{max} and apparent K_d .

In the present case, the TDG ligands may primarily interact with the conserved region in the galectin's CRD as reported before and thus show only little variation.^{40,41} The conjugation of TDGs with larger aromatic substituents (e.g., 4-phenox-

ylphenyl)¹⁷ may help to design specialized glycoconjugates for Gal-3 selective targeting. In this context, multivalency could greatly maintain an outstanding strong Gal-3 interaction.

Neo-Glycoproteins Acting as Gal-3 Inhibitors. The univalent TDG derivative 9 and multivalent glycoconjugates 11 and 12 were evaluated in terms of their capability to block the binding of Gal-3 to immobilized asialofetuin (ASF). The ASF glycoprotein is used as a standard galectin ligand as reported before.^{23,24,34,42,43} Gal-3 was incubated together with increasing amounts of inhibitors 9, 11, or 12 aiming at a complete prevention of ASF–galectin interaction. Nonmodified BSA was utilized as negative control and confirmed to be not interfering with Gal-3 binding to ASF. Hence, we conclude that the observed inhibition phenomena were only due to the TDG cargo of compounds 11 and 12. As depicted by Figure 2, a

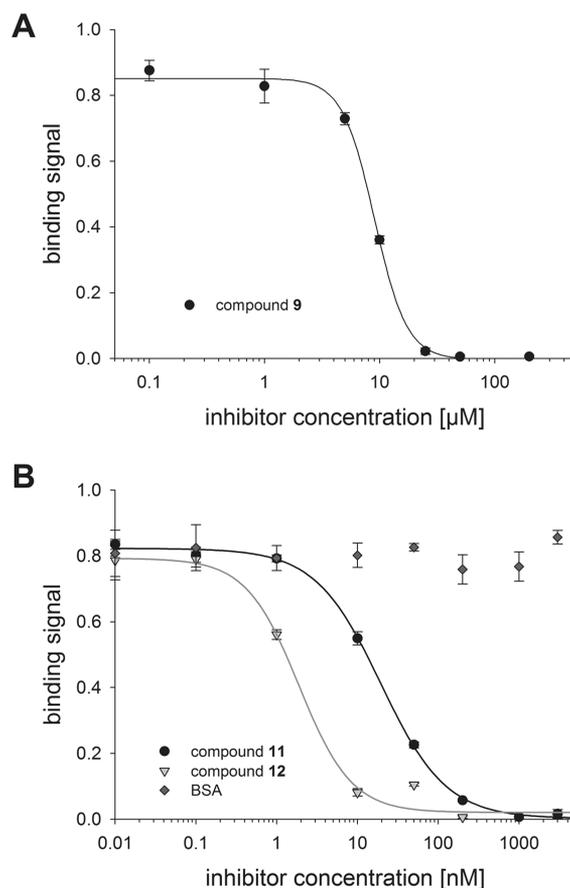


Figure 2. Competitive inhibition of Gal-3 (5.56 μ M) binding to ASF using monovalent carboxy-functionalized compound 9 (A) and multivalent TDG-conjugates 11 and 12 at indicated concentration (B). Refer to Scheme 2 and Scheme 3 for compound structures. The subtracted blank value (no Gal-3) was 0.091 ± 0.005 .

complete inhibition of Gal-3 binding to ASF was reached when using inhibitor 9 (>25 μ M) and conjugates 11 and 12 (>200 nM). The resulting sigmoidal inhibition curves were the basis for the calculation of the IC_{50} value, defined as the inhibitor concentration at which half-maximal inhibition was reached (Table 2). The inhibition strength of compound 9 was in the low micromolar range and fits the range of N',N'' -diacetylactosamine (LacdiNAc)-LacNAc tetrasaccharide, which was identified as a specific ligand of Gal-3.²³ However, we assume that the NHS-/PEG-modification of one C3 atom

Table 2. Inhibition Constants and Inhibitory Potencies of TDG Derivative 9 and Multivalent TDG-Conjugates 11 and 12

| inhibitor | IC ₅₀ (nM) ^a | number of loaded TDG moieties (–) ^b | relative inhibitory potency | relative inhibitory potency per glycan |
|-----------|------------------------------------|--|-----------------------------|--|
| 9 | 9030 ± 27 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 |
| 11 | 19.40 ± 1.09 | 7.0 ± 1.0 | 465.5 ± 27.5 | 66.5 ± 13.4 |
| 12 | 1.88 ± 0.38 | 18.7 ± 1.6 | 4803.2 ± 985.2 | 256.9 ± 74.7 |

^aELISA. ^bTNBSA-assay.

(asymmetric character) of **9** may reduce its affinity when applied as nonconjugated Gal-3 inhibitor. When comparing previous relevant studies, it becomes clear that Gal-3 has a much higher affinity for the symmetrically modified TDG. When both of TDG's galactose moieties carry selected substituents such as C(3)-benzamides^{16,41} or C(3)-triazoles,^{14,17} the Gal-3 affinity is optimal showing apparent K_d values between 22 and 360 nM in a fluorescence polarization assay.

Nevertheless, the conjugation of TDG derivative **9** to nonglycosylated serum protein scaffold gave conjugates **11** and **12**. Their use as inhibitors of Gal-3 binding resulted in extraordinarily high inhibitory potencies and low IC₅₀ values (Table 2). Even though the cargo of compound **11** was only a quantity of seven TDG derivatives, the inhibition strength was increased 465-fold compared to univalent compound **9**. This corresponds to an improvement factor of 66 per loaded TDG. The impact of the multivalent ligand presentation was even more pronounced for glycoconjugate **12** presenting a higher number ($n = 18.7$) of TDG derivatives. Here, the determined IC₅₀ value is reduced by more than 4800-fold compared with that of compound **9**, representing an improvement factor per TDG of 256.

Our findings suggest that multivalent conjugates **11** and **12**, but not monovalent compound **9**, inactivate more Gal-3 molecules than the amount of presented TDG derivatives, as seen before.²² On the one hand, both multivalent inhibitors may induce the formation of Gal-3 complexes, cross-linked by their N-termini.⁴⁴ On the other hand, type-C Gal-3 self-association is most likely. Here, the nonoccupied CRD of Gal-3 molecules interact with already TDG-bound Gal-3 leading to an oligomerization and stacking as reported before.⁴⁵ To the best of our knowledge, the tremendously diminished IC₅₀ value makes multivalent glycoconjugate **12** one of the most effective Gal-3 inhibitors. The multivalent design promotes the cluster glycoside effect resulting in a highly efficient entrapment of Gal-3.^{1,39,46}

Neo-glycoproteins with a cargo of different poly-LacNAc derivatives were recently synthesized and applied as Gal-3 inhibitors.²³ Thus, we may use them as a reference to evaluate the presented results. In particular, those BSA neo-glycoconjugates bearing the LacNAc-LacNAc ($n = 7.5$) or LacdiNAc-LacNAc ($n = 7.4$) glycans are ideal benchmarks because of an equal modification density with regard to conjugate **11**. In that case only moderate inhibition strengths were observed, with IC₅₀ values of 850 nM ([LacNAc-LacNAc] _{$n=7.4$} -BSA) and 1100 nM ([LacdiNAc-LacNAc] _{$n=7.4$} -BSA), respectively.²³ Clearly the TDG ligand has a potency advantage but the implementation of the PEG-spacer may also be a favorable feature in terms of ligand accessibility and flexibility.^{38,47} To evaluate conjugate **12**, previously synthesized neo-glycoproteins were used as ideal references again. Conjugates with LacNAc-LacNAc ($n = 17.8$), LacdiNAc-LacNAc ($n = 18.0$),²³ or derivatized poly-LacNAc hexasaccharides of equal modification density ($n = 16-19$)²⁴ were prepared and thoroughly studied in terms of galectin

interaction. The respective inhibition constants ranged between 60 and 90 nM²³ and 37 and 76 nM.²⁴ Based on the outstanding low IC₅₀ (1.88 nM), the potency of conjugate **12** is at least more than 20-fold elevated in comparison with the most potent reference neo-glycoproteins.

TDG derivatives have been validated to be valuable inhibitors for galectin research. The aromatic groups on the C3 and C3' positions of TDG tune galectin selectivity and affinity. We herein report on the synthesis of an asymmetrical TDG structure that can be used to yield multivalent compounds through conjugating to a protein scaffold. To obtain the key precursor, a straightforward approach was used to lead to the NHS functionalized-TDG derivative. Subsequent reaction with BSA gave multivalent TDG-glycoconjugates. Weak alkaline pH, adjusted by TEA, was crucial for an effective conjugation. To the best of our knowledge, this is the first example of conjugating a TDG derivative to a nonglycosylated carrier. The multivalent presentation on conjugates **11** and **12** unlocks TDG's full potential. Extraordinarily high multivalency factors were obvious that resulted in one of the most effective inhibition of Gal-3 *in vitro* until now. The result is clearly a combination of the binding properties of the monovalent ligand and the multivalent display by the BSA. As previously noted, potent galectin inhibition cannot be achieved with very weak or nonbinding ligands, conjugated to BSA.³⁴ Furthermore, we note that, while a multivalent scaffold can enhance existing binding potency, the specificity at the multivalent level remains the same.⁴⁸ In other systems, very strong multivalency effects have been reported leading to picomolar inhibition, usually involving the simultaneous binding of ligands to nearby binding sites.⁴⁹ This chelation type mechanism is less likely to contribute to the present system, due to the monovalent nature of the nonaggregated protein. Considering this, other modes of action such as statistical rebinding or aggregation usually lead to smaller effects,⁴⁶ which makes the present results more notable. Furthermore, this work shows that the multivalent inhibitor is able to inhibit far more Gal-3 molecules than its number of attached ligands. This feature is a likely consequence of aggregation phenomena, blocking Gal-3 binding sites, previously observed for Gal-3 and named type-C-self-association.⁴⁵ Systems such as the present, capable of nucleating the process, may lead us to a full understanding of this phenomenon. In the present system, the PEG-spacer likely helps to make the TDGs accessible for the interacting galectins. The multivalent TDG-modified conjugates (**11**, **12**) have the ideal properties for a putative biomedical application because of (i) the serum protein scaffold has the approved quality to be applied to the bloodstream; (ii) the PEG-spacer is biocompatible, sustained, and safe; and (iii) the TDG derivative is considered chemically stable. Hence, cell culture *in vitro* experiments (inhibition of Gal-3 induced angiogenesis) are planned in the due course in order to elucidate the power of the synthesized conjugate. *In vivo* applications may follow. The conjugation of TDGs with different functional groups on C3 and C3' position (e.g., 4-phenoxyphenyl) are planned to

modulate the inhibition potency and tune the galectin specificity on a multivalent level.

METHODS

Preparation of Compound 1 (2,4,6-Tri-O-acetyl-3-deoxy-3-(4-phenyl-1H-1,2,3-triazol)- α -D-galactopyranosyl bromide). Compound 1 (300 mg, 0.80 mmol), sodium L-ascorbate (237.6 mg, 1.2 mmol), CuSO₄·5H₂O (100 mg, 0.40 mmol), Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 4.2 mg, 0.0096 mmol), and phenylacetylene (176.4 μ L, 1.6 mmol) were dissolved in DMF (13.5 mL) and H₂O (1.5 mL). The reaction was performed under microwave irradiation at 80 °C for 40 min. Subsequently, the solvent was evaporated and the residue was dissolved in CH₂Cl₂ (100 mL), washed with H₂O (1 \times 100 mL) and brine (1 \times 100 mL), dried over Na₂SO₄, filtered, and concentrated. The crude product was obtained as a white solid (310 mg) and used for next reaction directly. To a solution of crude (310 mg) in dry CH₂Cl₂ (50 mL) the HBr (33% HBr in acetic acid, 2.0 mL) was dropwise added under N₂ atmosphere. The solution was sealed and stirred overnight at room temperature. A saturated NaHCO₃ solution (50 mL) was added to quench the reaction and then the organic layer was washed with H₂O (1 \times 50 mL) and brine (1 \times 50 mL), dried with Na₂SO₄, and filtered. The residue was purified by silica chromatography (hexanes:EtOAc = 1:1) and gave the product 2 as a light yellow solid (312 mg, two step yield 79%). ¹H NMR (400 MHz, CDCl₃) δ 7.85–7.70 (m, 3H, ar, triazole), δ 7.41 (dd, J = 8.3, 6.8 Hz, 2H, ar), 7.37–7.28 (m, 1H, ar), 6.86 (d, J = 3.8 Hz, 1H, H1), 5.79 (dd, J = 11.4, 3.8 Hz, 1H, H2), 5.63 (dd, J = 3.1, 1.3 Hz, 1H, H4), 5.32 (d, J = 3.0 Hz, 1H, H3), 4.67–4.58 (m, 1H, H5), 4.22 (dd, J = 11.6, 6.3 Hz, 1H, H6a), 4.11 (dd, J = 11.6, 6.3 Hz, 1H, H6b), 2.05, 2.04, and 1.93 (3s, each 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ 170.25, 169.55, 168.94, 128.89, 128.49, 125.70, 119.33, 88.52, 77.17, 71.34, 67.76, 66.85, 60.86, 58.66, 31.40, 29.67, 20.58, 20.45, 20.34.

HRMS (EI, m/z) calculated for C₂₀H₂₂BrN₃O₇H⁺ ([M + H]⁺): 496.0714, found 496.0707.

Preparation of Compound 3 (2,4,6-tri-O-acetyl-3-azido- α -D-galactopyranosyl bromide). Compound 1 (1.2 g, 3.2 mmol) was dissolved in CH₂Cl₂ (50 mL) and EtOAc (5.0 mL) and then titanium tetrabromide (TiBr₄, 2.4 g, 6.4 mmol) was added slowly. The reaction mixture was stirred under sealed conditions overnight at room temperature. NaOAc (2.0 g, 24 mmol) was added to quench the reaction and washed with H₂O (3 \times 50 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated in vacuo. Silica chromatography (Hexanes:EtOAc = 3:1) yielded 7 as a clear oil (855 mg, 67.4%). ¹H NMR (400 MHz, CDCl₃) δ 6.68 (d, J = 3.9 Hz, 1H, H1), 5.48 (dd, J = 3.4, 1.4 Hz, 1H, H4), 4.98–4.89 (m, 1H, H2), 4.39 (td, J = 6.8, 6.1, 1.5, 0.7 Hz, 1H, H5), 4.17 (dd, J = 11.5, 6.1 Hz, 1H, H6a), 4.11 (dd, J = 10.6, 3.3 Hz, 1H, H3), 4.03 (dd, J = 11.5, 6.8 Hz, 1H, H6b), 2.16 (s, 3H, C(O)CH₃), 2.14 (s, 3H, C(O)CH₃), 2.05 (s, 3H, C(O)CH₃).

¹³C NMR (101 MHz, CDCl₃) δ 170.27, 169.75, and 169.58 (C(O)CH₃), 88.19 (C-1), 71.29 (C-5), 69.47 (C-2), 67.05 (C-4), 60.95 (C-6), 58.32 (C-3), 20.69, 20.60, 20.50 (C(O)CH₃).

Preparation of Compound 4 (Tri-isopropylsilyl 3-azido-2,4,6-tri-O-acetyl-1-thio- β -D-galactopyranoside). To a solution of 3 (770 mg, 1.95 mmol) in dry CH₃CN (10 mL) N₂ gas was purged for 10 min, then K₂CO₃ (809 mg, 5.86 mmol) was added followed by tri-isopropylsilylthiol (TIPSSH, 628 μ L, 2.93 mmol), and the reaction was stirred for 3 h at

room temperature. After complete conversion of the starting material according to TLC monitoring, the solvent was evaporated and the residue was dissolved in CH₂Cl₂ (20 mL), washed with H₂O (2 \times 20 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated in vacuo. Silica chromatography (hexanes:EtOAc = 4:1) yielded 4 as a white solid (300 mg, 30%).

¹H NMR (400 MHz, CDCl₃) δ 5.42 (dd, J = 3.5, 1.0 Hz, 1H, H4), 5.20 (t, J = 9.8 Hz, 1H, H2), 4.60 (d, J = 9.5 Hz, 1H, H1), 4.11 (dd, J = 11.5, 5.6 Hz, 1H, H6a), 4.00 (dd, J = 11.6, 7.1 Hz, 1H, H6b), 3.79 (ddd, J = 6.9, 5.5, 1.1 Hz, 1H, H5), 3.53 (dd, J = 10.1, 3.4 Hz, 1H, H3), 2.15, 2.12, 2.02 (3s, 9H, 3 COCH₃), 1.25 (m, 3H, -SiC₃H₃), 1.14–1.07 (m, 18H, -SiC₃H₃C₆H₁₈).

¹³C NMR (101 MHz, CDCl₃) δ 170.57, 170.25, 169.39 (3 COCH₃), 80.37 (C-1), 75.47 (C-5), 62.87 (C-3), 72.17 (C-2), 68.12 (C-4), 62.13 (C-6), 20.84, 20.76, 20.51 (3COCH₃), 18.52, 18.22 (6 -SiCHCH₃), 12.76 (3 -SiCH).

HRMS (EI, m/z): calculated for C₂₁H₃₇N₃O₇SSiNa⁺ ([M + Na]⁺): 526.2014, found 526.2011.

Preparation of Compound 5 (3-azido-3'-phenyl-2,2',4,4',6,6'-hexa-O-acetyl β -D-thiodigalactoside). The solution of 2 (190 mg, 0.38 mmol) in dry CH₃CN (10 mL) was added by 4 (193 mg, 0.38 mmol), N₂ gas was purged for 10 min through the solution, and tetra-*n*-butylammoniumfluoride (TBAF, 1 M in THF, 460 μ L) was added. Following complete conversion of the starting material after 5 min according to TLC analysis, the solvent was evaporated and silica chromatography (hexanes:EtOAc = 1:1 \rightarrow 1:2) gave compound 5 (160 mg, 54%) as a white solid.

¹H NMR (400 MHz, CDCl₃) δ = 7.80 (s, 1H, triazole), 7.77–7.71 (m, 2H, ar), 7.43–7.28 (m, 3H, ar), 5.75–5.68 (m, 1H, H-2'), 5.61 (d, 1H, J = 3.2 Hz, H-4'), 5.47 (d, 1H, $J_{4,3}$ = 3.4 Hz, $J_{4,5}$ = 1.1 Hz, H-4), 5.21 (dd, 1H, $J_{3,2'}$ = 8.6 Hz, $J_{3,4'}$ = 2.5 Hz, H-3'), 5.17 (m, 1H, H-2), 4.98 (1H, d, $J_{1,2'}$ = 9.8 Hz, H-1'), 4.84 (1H, $J_{1,2}$ = 10.0 Hz, H-1), 4.11 (m, 5H, H-5', H-6ab, H-6a'b'), 3.89 (1H, td, $J_{5,4}$ = 1.2 Hz, $J_{5,6ab}$ = 6.4 Hz, H-5), 3.67 (1H, $J_{3,2}$ = 10.1 Hz, $J_{3,4}$ = 3.4 Hz, H-3), 2.15, 2.13, 2.08, 2.05, 2.04, and 2.02 (6s, total 18H, C(O)CH₃).

¹³C NMR (101 MHz, CDCl₃) δ 170.34, 170.25, 169.86, 169.54, 169.37, 168.70, 147.92, 129.96, 128.90, 128.47, 125.68, 118.26, 82.11, 81.51, 77.34, 77.02, 76.70, 75.53, 68.71, 68.43, 67.66, 66.33, 62.94, 62.78, 61.56, 61.38, 20.76, 20.68, 20.62, 20.61, 20.47, 20.38.

HRMS (EI, m/z) calculated for C₃₂H₃₈N₆O₁₄SH⁺ ([M + H]⁺): 763.2239, found 763.2277.

Preparation of Compound 6 (3-(4-phenyl-1H-1,2,3-triazol)-3'-azido-thiodigalactoside). NaOMe (40 mg, 2.5 mmol) was added in the solution of compound 5 (120 mg, 0.16 mmol) in CH₃OH (5.0 mL) and the mixture was stirred for 6 h at room temperature. The solution was neutralized with DOWEX-H⁺ resin, filtered, and evaporated. Crude 6 was obtained as a white solid and used in the next step without further purification.

Preparation of Compound 7. To a solution 3,6,9,12-tetraoxapentadec-14-yn-1-ol of (200 mg, 0.86 mmol) in 5 mL of THF was added sodium (0.6 mg, 0.025 mmol). When the sodium was dissolved, *tert*-butyl acrylate (0.125 mL, 0.86 mmol) was added. The solution was stirred for 20 h at room temperature and H₂O (1 mL) was added to quench the reaction. After removal of the solvent, the residue was suspended in brine and extracted three times with ethyl acetate. The combined organic layers were dried over Na₂SO₄ before the solvent was removed. The resulting oil was purified

by silica chromatography (hexanes:EtOAc = 1:1 → 1:2) to give compound **7** (120 mg, 39%) as a colorless oil.

¹H NMR (400 MHz, CDCl₃) δ 4.17 (d, *J* = 2.4 Hz, 2H), 3.73–3.53 (m, 18H), 2.46 (t, *J* = 6.6 Hz, 2H), 2.40 (t, *J* = 2.4 Hz, 1H), 1.41 (s, 9H).

¹³C NMR (101 MHz, CDCl₃) δ 170.82, 80.42, 74.43, 70.57, 70.56, 70.53, 70.46, 70.36, 70.33, 69.07, 69.07, 66.85, 58.34, 36.24, 28.08, 28.05.

Preparation of Compound 8. Compound **7** (33 mg, 0.092 mmol) and compound **6** crude (48 mg) were dissolved into CH₃CN (2.0 mL) and then CuI (18 mg, 0.093 mmol) was added into the solution. The resulting mixture was heated under microwave irradiation to 80 °C for 90 min. After complete conversion of the starting material according to TLC monitoring, the mixture was concentrated in vacuo, and then H₂O (1.0 mL) was added. A clear solution was obtained after centrifuge, which was purified by size-exclusion chromatography (Bio-Gel P2 fine; column 2.5 cm × 120 cm; flow rate 0.3 mL/min; elution with H₂O/*n*-Butanol = 95/5). The fractions containing the product were pooled and freeze-dried to give compound **8** (52 mg, 0.060 mmol, 64%) as a white fluffy solid.

¹H NMR (500 MHz, D₂O) δ 8.55 (s, 1H), 8.26 (s, 1H), 7.87 (d, *J* = 7.7 Hz, 2H), 7.55 (t, *J* = 7.6 Hz, 2H), 7.48 (t, *J* = 7.5 Hz, 1H), 6.81–6.69 (m, 2H), 5.14 (dd, *J* = 9.8, 7.4 Hz, 2H), 5.02 (td, *J* = 13.7, 10.5, 2.9 Hz, 2H), 4.80–4.73 (m, 2H), 4.74 (s, 2H), 4.46 (dt, *J* = 27.5, 10.3 Hz, 3H), 4.26 (dd, *J* = 22.4, 3.0 Hz, 2H), 4.06 (td, *J* = 8.5, 4.4 Hz, 3H), 3.89–3.76 (m, 4H), 3.75 (t, *J* = 2.4 Hz, 2H), 3.66 (dq, *J* = 7.8, 4.3, 3.5 Hz, 16H), 3.40–3.26 (m, 2H), 3.00–2.88 (m, 4H), 2.56 (t, *J* = 6.0 Hz, 2H), 1.45 (s, 9H).

¹³C NMR (126 MHz, D₂O, extracted from HSQC) δ 121.43, 124.35, 125.65, 126.36, 125.65, 129.20, 129.23, 128.75, 84.13, 84.12, 66.86, 66.97, 63.11, 66.85, 67.89, 79.54, 79.55, 61.03, 66.44, 61.05, 61.06, 69.53, 69.52, 69.50, 70.21, 42.99, 42.99, 35.06, 27.18, 27.17, 27.18.

HRMS (EI, *m/z*) calculated for C₃₈H₃₈N₆O₁₅SNa⁺ ([*M* + Na]⁺): 893.3579, found 893.3595.

Preparation of Compound 9. Compound **8** (52 mg, 0.060 mmol) was added into TFA/CH₂Cl₂ (10 mL, 1:1) and the solution was stirred for 2 h at room temperature. After being fully evaporated, the residue was purified by size-exclusion chromatography (Bio-Gel P2 fine; column 2.5 cm × 120 cm; flow rate 0.3 mL/min; elution with H₂O/*n*-Butanol = 95/5). The fractions containing the product were pooled and freeze-dried to give compound **9** (48 mg, 0.059 mmol, 98%) as a white solid.

¹H NMR (400 MHz, D₂O) δ 8.40 (s, 1H), 8.11 (s, 1H), 7.75–7.68 (m, 2H), 7.44–7.37 (m, 2H), 7.36–7.28 (m, 1H), 4.99 (dd, *J* = 9.8, 6.1 Hz, 2H), 4.87 (ddd, *J* = 11.9, 10.7, 3.0 Hz, 2H), 4.59 (s, 2H), 4.31 (dt, *J* = 20.7, 10.2 Hz, 2H), 4.16–4.06 (m, 2H), 3.91 (td, *J* = 7.6, 4.4 Hz, 2H), 3.74–3.60 (m, 5H), 3.60–3.48 (m, 17H), 2.31 (t, *J* = 6.7 Hz, 2H).

¹³C NMR (101 MHz, D₂O) δ 180.11, 163.09, 162.74, 147.43, 143.78, 129.19, 128.82, 125.65, 124.36, 121.38, 117.70, 114.80, 84.10, 79.48, 69.48, 69.45, 69.43, 69.17, 68.85, 67.98, 67.93, 67.84, 66.94, 66.83, 66.77, 66.74, 63.04, 61.00, 37.52.

HRMS (EI, *m/z*): calculated for C₃₄H₅₀N₆O₁₅SH⁺ ([*M* + H]⁺): 815.3128, found 815.3135.

Preparation of Compound 10. Compound **9** (22 mg, 0.027 mmol) was dissolved in anhydrous DMF (0.5 mL) and DiPEA (4.69 μL, 0.027 mmol) was added, followed by addition of TSTU (8.1 mg, 0.027 mmol). The resulting mixture was stirred for 30 min, and then TLC and HPLC showed that the

starting material was fully converted. To avoid hydrolysis, the crude **10** was used for labeling the protein directly.

Preparation of Multivalent TDG-Conjugates 11 and 12. A volume of 150 μL BSA (60 μM in 35 mM HEPES buffer, pH 7.0) was mixed with 5 μL of the coupling agent (compound **10** crude, 54 mM in DMF) was added into the solution and the reaction mixture was incubated at 4 °C. After 24 and 48 h, additional volumes of 5 μL compound **10** crude were added. As to synthesis of conjugate **12**, the pH of the BSA solution was elevated to pH 9.0 using TEA before adding the conjugation agent. Conjugates **11** and **12** were isolated and rinsed with H₂O using VivaSpin 500 centrifugal concentrators (Sartorius Stedim Biotech, Goettingen, Germany) with an MWCO of 10 kDa. The protein concentration was determined by Bradford reagent (Carl Roth, Karlsruhe, Germany) according to manufacturer's instruction.

Galectin Preparation. Constructs for human His₆-tagged galectin-1 (Gal-1) and His₆-tagged galectin-3 (Gal-3) were used from previous investigations. With regard to galectin-1, the full-length sequence was cloned into His₆-tag providing pETDuet-1 (Novagen, Darmstadt, Germany) using restriction sites *Bam*HI and *Sgs*I, followed by the introduction of the C2S mutation to increase construct stability.⁵⁰ The full-length sequence of galectin-3 was cloned into pETDuet-1 (Novagen, Darmstadt, Germany) using restriction sites *Sgs*I and *Eco*RI.⁴² The expression of both galectins was performed in recombinant *E. coli* Rosetta (DE3) pLysS cells, which were cultivated in 1 L TB medium (5 L baffled flask) containing appropriate antibiotics (80 rpm, 100 μg/mL ampicillin, 34 μg/mL chloramphenicol). After an optical density (OD_{600 nm}) of 0.5–0.8 was reached the temperature was decreased from 37 to 25 °C and isopropyl-1-thio-β-D-galactopyranoside (IPTG, 0.5 mM) was added for inducing protein expression. After 24 h post-induction, the cells were harvested by centrifugation (7000 rpm, 30 min, 4 °C) and stored at –20 °C. With regard to galectin purification, bacteria were suspended in lysis buffer (50 mM HEPES, 500 mM NaCl, 20 mM imidazole, pH 7.5) and sonicated on ice (two cycles, 30 s each). After removal of cell debris by centrifugation (15 000 rpm, 30 min, 4 °C), supernatant was filtered through 0.8 μm syringe filter. HisTrap HP 5 mL columns were used (GE Healthcare) according to the manufacturer's instructions for galectin enrichment. Elution of His₆-tagged galectins was achieved by augmenting the imidazole concentration in one step to 500 mM. Isolated galectins were dialyzed against phosphate buffered saline (PBS, 50 mM NaH₂PO₄, 150 mM NaCl, pH 7.5) supplemented with 2 mM ethylenediaminetetraacetic acid (EPBS) using SnakeSkin Dialysis Tubing (10 kDa MWCO, ThermoFisher Scientific). Durability of Gal-1 was increased by supplementing storage buffer with 20% (v/v) glycerol.

Galectin Binding In Vitro. F16 Maxisorp NUNC-Immuno Modules (Thermo Scientific, Roskilde, Denmark) were immobilized with appropriate amounts of **11**, **12**, or non-modified BSA (0.1 μM in PBS, 50 μL, 5 pmol per well, 18 h). Immobilization and all further steps were performed at room temperature. After three washing steps using PBS supplemented with 0.05% (v/v) Tween 20 (PBST), residual unoccupied binding sites were blocked with PBS containing 2% (w/v) BSA. An additional 3-fold PBST washing step followed. Gal-1 and Gal-3 were added at different concentrations (1 5000 nM, 50 μL, 1 h). An additional 3-fold PBST washing step followed. The addition of peroxidase conjugated anti-His₆-IgG2a from mouse (Roche Diagnostics, 1:4000 in

PBS, 50 μL , 12.5 mU/mL, 1 h) enabled the detection of His₆-tagged galectins. An additional 3-fold PBST washing step followed. Reaction of IgG-conjugated peroxidase was initiated by addition of 3,3',5,5'-tetramethylbenzidine (TMB) One (Kem-En-Tec, Taastrup, Denmark) substrate solution (50 μL). The reaction was stopped by addition of 3 M HCl (50 μL). Spectra Max Plus (Molecular Devices, Biberach, Germany) plate reader was utilized to measure the optical density at 450 nm, which is a measure for galectin binding. Blank values (no Gal-1 and/or Gal-3) were subtracted from all measurement values. SigmaPlot 10 was used to match the data points by nonlinear regression and to extract the respective kinetic parameters (eq 1).

$$Y = \frac{B_{\max} \cdot X}{K_d + X} \quad (1)$$

where Y is binding signal; B_{\max} is maximal binding signal; X is galectin concentration; K_d is half-maximal apparent affinity constant [nM].

Galectin Inhibition *In Vitro*. F16 Maxisorp NUNC-Immuno Modules (Thermo Scientific, Roskilde, Denmark) were immobilized with appropriate amounts of asialofetuin as standard glycoprotein ligand (0.1 μM in PBS, 50 μL , 5 pmol per well, 18 h). Immobilization and all further steps were performed at room temperature. After three washing steps using PBS supplemented with 0.05% (v/v) Tween 20 (PBST), residual unoccupied binding sites were blocked with PBS containing 2% (w/v) BSA. An additional 3-fold PBST washing step followed. As a next step, monovalent inhibitor **9** and multivalent inhibitors **11** and **12** (5 μL each) of different concentration were given in the wells and Gal-3 (45 μL , 5.56 μM) was added for 1 h. Signals for Gal-3 binding to ASF decreased as more inhibitor was present. Blank values (no Gal-3) were subtracted from all measurement values showing that almost complete inhibition of Gal-3 molecules was reached. SigmaPlot 10 was used to match the data points by nonlinear regression and extract the respective kinetic parameters, illustrated by the Hill Equation (three parameters, eq 2).

$$Y = \frac{\text{top}^b \cdot X^b}{\text{IC}_{50}^b + X^b} \quad (2)$$

where Y is binding signal; top is binding signal without inhibitor; X is inhibitor concentration; IC_{50} is inhibitor concentration for half-maximal (50%) inhibition [nM].

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00047.

Analytical methods, synthetic details, MS spectra, and NMR data (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Lothar Elling and Dominic Laaf gratefully acknowledge the financial support by German Research Foundation (DFG, project EL 135/12-1). Hao Zhang gratefully acknowledge the financial support by a scholarship (file No. 201306210049) from the China Scholarship Council (<http://www.csc.edu.cn/>).

■ ABBREVIATIONS

TDG, thiodigalactoside; NHS, *N*-hydroxysuccinimide; Gal-3, galectin-3; LacNAc, *N*-acetylglucosamine; LacdiNAc, *N*',*N*'-diacetylglucosamine

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