**Structure-Guided Design of \( \beta \)-Galactal Derivatives with High Affinity and Selectivity for the Galectin-8 N-Terminal Domain**

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**ABSTRACT:** Galectin-8 is a carbohydrate-binding protein that plays a crucial role in tumor progression and metastasis, antibacterial autophagy, modulation of the immune system, and bone remodeling. The design, synthesis, and protein affinity evaluation of a set of C-3 substituted benzimidazole and quinoline \( \beta \)-galactal derivatives identified a \( \beta \)-galactal-benzimidazole hybrid as a selective ligand for the galectin-8 N-terminal domain (galectin-8N), with a \( K_d \) of 48 \( \mu \)M and 15-fold selectivity over galectin-3 and even better selectivity over the other mammalian galectins. X-ray structural analysis of galectin-8N in complex with one benzimidazole- and one quinoline-galactal derivative at 1.52 and 2.1 Å together with molecular dynamics simulations and quantum mechanical calculations of galectin-8N in complex with the benzimidazole derivative revealed orbital overlap between a NH LUMO of Arg45 with electron rich HOMOs of the \( \beta \)-galactal and Olefin and O4 of the \( \beta \)-galactal. Such overlap is hypothesized to contribute to the high affinity of the \( \beta \)-galactal-derived ligands for galectin-8N. A (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay evaluation of the \( \beta \)-galactal-benzimidazole hybrid and an analogous galactoside derivative on a panel of cell lines with MTS assay showed no effect on cell viability up to 100 \( \mu \)M concentration. A subsequent functional assay using the MDA-MB-231 cell line demonstrated that the \( \beta \)-galactal-benzimidazole hybrid and the analogous galactoside derivative reduced the secretion of the proinflammatory cytokines interleukin-6 (IL-6) and IL-8 in a dose-dependent manner. Therefore, these compounds represent potential probes for galectin-8N pharmacology investigations and possibly promising leads for the design and synthesis of potent and selective galectin-8 inhibitors as potential antitumor and anti-inflammatory agents.

**KEYWORDS:** Galectin-8N, \( \beta \)-galactal, benzimidazole, selectivity, X-ray crystallography, cytokine secretion

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logical conditions such as tumor growth and metastasis, solid organ graft rejection, and corneal inflammation. Notably, galectin-8 is upregulated in several cancer tissues including breast, prostate, bladder, kidney, and lung tissues. In addition, galectin-8 also plays important roles in autoimmune and inflammatory disorders, antibacterial autophagy, osteoporosis, and bone loss. Its involvement in several pathological conditions attests to the importance of selective galectin-8 inhibitors as research tools and potential antitumor and anti-inflammatory drugs.

The two CRDs of galectin-8 share the same amino acids forming the galactose-binding site, namely, Trp86, His65, Asn67, Arg69, Asn79, and Glu89. However, there are major differences between the two CRDs, such as the presence of Arg45, Arg59, Tyr141, and Gln47 in the N-terminal CRD (galectin-8N) and the presence of Ser255, Asn257, and Arg348 in the C-terminal CRD (galectin-8C). The Arg59 side chain is responsible for the preferential binding of galectin-8N to anionic glycans, such as 3′- O-sulfate/3′-O-sialylated lactosides, via salt bridges. Moreover, galectin-8 is upregulated in several cancer tissues including biomaterials and tissue engineering.8 In addition, the substantial similarity of di-mammalian galectins has always been a challenging task, due to the tricyclic carbohydrate-benzene hybrid 1 (Kd = 1.8 μM) previously described.29 The D-galactal 3 represents the minimum active fragment of compound 2.

Moreover, the chemistry of the 1,2-glycals is well-established with a plethora of reactions that can be conducted at the different carbons of D-galactal.22-27 Therefore, D-galactal (3) represents a promising galactose mimic for the iterative design and optimization of galectin-8N ligands. Herein, we report the design, synthesis, and biological evaluation of C-3 substituted D-galactal derivatives as galectin-8N ligands with high selectivity over the other mammalian galectins.

**Results and Discussion.** **Inhibitor Design and Synthesis.** Based on the reported binding affinities of the benzimidazole and quinoline-galactosides to galectin-8N, we hypothesized that their D-galactal featuring counterpart might result in ligands that bind galectin-8N with improved affinity. Hence, D-galactal was alkylated with benzimidazolyl- and quinolylmethyl moieties at O3. The benzimidazolylmethyl chlorides 4a−4c and the quinolylmethyl chloride 7 were synthesized as previously reported.19 Alkylation of the D-galactal 3 with the benzimidazolylmethyl chlorides 4a−4c and the quinolylmethyl chloride 7 via stannylene-mediated 3-O-alkylation of the D-galactal 3 afforded the benzimidazole methyl esters 5a−5c and the quinoline methyl ester 8 (Scheme 1).25,26 The subsequent alkaline hydrolysis of the esters produced the benzimidazole carboxylates 6a−6c and the quinoline carboxylate 9 (Scheme 1).

**Galectin Affinity Evaluation.** The binding affinities of the benzimidazoles 5a−5c and 6a−6c and the quinolines 8 and 9 for galectins-1, -3, -4, -7, -8C, -8N, -9C, and -9N were determined in a competitive fluorescence polarization assay as previously described.30−31 The D-galactal 3 had a 5-fold higher affinity for galectin-8N compared to the methyl β-D-galactopyranoside (Table 1), which supports our hypothesis that the endocyclic double bond of D-galactal 3 plays a role in increasing the binding affinity to galectin-8N. All synthesized D-galactal derivatives had higher binding affinities for galectin-8N than D-galactal 3. Except for compounds 5b and 6b, the carboxylic acid derivatives had a 4−8-fold higher affinity for galectin-8N compared to their ester counterparts. In addition, all D-galactal derivatives showed a 2−7-fold higher affinity for galectin-8N compared to the previously published benzimidazole and quinoline methyl β-D-galactosides.19 Compounds 6a, 6c, and 9 had nearly identical Kd values for galectin-8N, with compound 6c exhibiting the highest gain in binding affinity compared to its galactoside equivalent with a Kd of 46 μM. In terms of selectivity, except for compounds 5b and 6b, the D-galactal derivatives were more than 2-fold more selective for galectin-8N over other mammalian galectins, with compound...
6a being the most selective galectin-8N ligand to date with 15-fold selectivity over galectin-3, 27-fold selectivity over galectin-1, and even higher selectivity over the other mammalian galectins (Table 1).

**Structural Analysis.** To investigate the binding modes of compounds 6a and 9 for galectin-8N, we solved their X-ray crystal structures in complex with galectin-8N according to the previously published protocol.20 Galectin-8N was first cocrystallized with lactose, and then the ligands were soaked into galectin-8N-lactose crystals, followed by collection of X-ray diffraction data at 1.52 and 2.1 Å resolution for the galectin-8N–6a complex and galectin-8N–9 complex, respectively. Both complexes showed that the O4 and O6 of the D-galactal moiety in both complexes is placed in proximity of the D-galactal ring interacts with guanidinium side chains of Arg45 and Arg69 (Figure 2). As for the galectin-8N–6a complex, the five-membered ring of the benzimidazole ring is placed in a favorable position to engage in cation−π stacking with Arg45 while the basic nitrogen of the benzimidazole engages in a dipole−dipole interaction with Gln47 and Arg59. The carboxylate moiety of the benzimidazole establishes a water-mediated hydrogen bond with Gly142. As for the galectin-8N–9 complex, the quinoline ring is placed in a favorable position to establish an interaction with Arg45 while the carboxylate moiety establishes a water-mediated hydrogen bond with Gly142. Altogether, the MD essentially reproduced the X-ray structure and the 200 ns simulations produced conformational data at 1.52 and 2.1 Å resolution for the galectin-8N–6a complex (Figure 2A). The benzimidazole of 6a was during the simulations rotated 180 degrees relative to the benzimidazole of 6b, placing the carboxylate similar to the position in the X-ray structure and to the carboxylate of 6b. Consequently, the 6a benzimidazole N3 hydrogen bound to Gln47 as well as formed intermittent polar interactions with Asn79 and Glu89, while the O3 engages in a hydrogen bonding interaction with Arg45 (Figure 2).

**Cytotoxicity Evaluation.** We have investigated the cytotoxicity of the compounds 1, 6a, and 6c via a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) (MTS) assay in K562 and MDA-MB-231 cancer cell lines to establish the direct antitumor activity of compounds, as well as human peripheral blood mononuclear cells (PBMCs), to evaluate their safety. None of the compounds decreased the viability of cancer cell lines at concentrations ranging from 1 to 100 μM, thus demonstrating a lack of antitumor activity. However, the lack of direct cytotoxicity of compounds 1, 6a, and 6c in PBMCs at concentrations up to 100 μM also renders these compounds suitable as tool compounds to study the biological roles of galectin-8 on the selected cell lines as well as provides a good
starting point for the design and synthesis of potent and selective galectin-8N inhibitors.

Assessment of Cytokine Secretion Profile.

Triple-negative breast cancer accounts for 15−20% of all breast cancers, with an increased risk of metastasis and a high mortality rate. It is characterized by the absence of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, making it resistant to the clinically available medications for breast cancer. Importantly, galectin-8 is upregulated in several tumor cells including the triple-negative breast cancer cells MDA-MB-231. A recent study has shown that knocking down galectin-8 in MDA-MB-231 cells prevents cell-cell adhesion while knocking down galectin-8 and its glycosylated ligand activated leukocyte cell adhesion molecule (ALCAM) synergistically delays the tumor growth in vivo. It has been established that galectin-8 upregulates the expression of proinflammatory cytokines in different cell lines, including osteoblasts, E0771 breast cancer cells, and breast cancer cells. MDAX-MB-231, as well as other breast cancer cell lines. MDAX-MB-231, as well as other breast cancer cell lines, in vitro. Galectin-8 is characterized by the absence of estrogen receptor 2, making it resistant to the clinically available medications for breast cancer. A recent study has shown that knocking down galectin-8 in MDA-MB-231 cells prevents cell-cell adhesion while knocking down galectin-8 and its glycosylated ligand activated leukocyte cell adhesion molecule (ALCAM) synergistically delays the tumor growth in vivo. It has been established that galectin-8 upregulates the expression of proinflammatory cytokines in different cell lines, including osteoblasts, E0771 breast cancer cells, and breast cancer cells. MDAX-MB-231, as well as other breast cancer cell lines.

### Table 1. $K_d$ Values of Compounds 5a−5c, 6a−6c, 8, and 9 (μM)

| compd | 1  | 2  | 3  | 4N | 4C | 7  | 8N | 8C | 9N | 9C |
|-------|----|----|----|----|----|----|----|----|----|----|
| 2$^{1,2}$ | 420 | NA$^{b}$ | 4100 | >1000 | >1000 | NA$^{b}$ | 180 | NA$^{b}$ | >1000 | >1000 |
| 3 | 1600 ± 140 | 13000 | 3300 ± 650 | 2400 ± 13 | 5300 | NA$^{b}$ | 1300 ± 72 | 4100 ± 470 | NA$^{b}$ | 6300 | >30000 | 3300 | 8600 ± 7309 |
| methyl β-D-galactoside$^{[3,13,32]}$ | >10000 | 13000 | 1700 ± 60 | 2600 ± 230 | NA$^{a}$ | 400 ± 34 | 3000 ± 380 | NB$^{c}$ | NA$^{b}$ | 1500 ± 180 | NA$^{b}$ | 1600 ± 255 |
| 5a | 2200 ± 138 | NA$^{b}$ | 1300 ± 130 | 2000 ± 130 | NA$^{a}$ | 4100 ± 270 | 180 ± 19 | 4000 ± 500 | 1400 ± 57 | NB$^{c}$ |
| 5b | 840 ± 20 | NA$^{a}$ | 1200 ± 287 | 3200 | 2500 | NA$^{b}$ | 180 ± 19 | 3000 ± 300 | NB$^{c}$ | NB$^{c}$ |
| 5c | NB$^{b}$ | NA$^{b}$ | 680 ± 37 | 2500 | NA$^{b}$ | 48 ± 4 | 4000 ± 500 | 1400 ± 57 | NB$^{c}$ | NB$^{c}$ |
| 6a | 1300 ± 130 | 1400 ± 24 | 690 ± 30 | 1700 ± 150 | NA$^{b}$ | 810 ± 54 | 3000 ± 300 | NB$^{c}$ | NB$^{c}$ | NA$^{b}$ | 1400 ± 57 | NB$^{c}$ |
| 6b | 1100 ± 87 | NA$^{a}$ | 770 ± 129 | 3600 | 2500 ± 300 | NA$^{b}$ | 48 ± 4 | 4000 ± 500 | 1400 ± 57 | NB$^{c}$ | NB$^{c}$ |
| 6c | NB$^{b}$ | 990 ± 170 | 550 ± 32 | 1400 ± 230 | NA$^{b}$ | 46 ± 4 | NA$^{b}$ | 1400 ± 57 | NB$^{c}$ | 1000 ± 51 | NB$^{c}$ |
| 8 | NB$^{b}$ | NB$^{b}$ | 1700 ± 40 | 1300 ± 12 | 3700 ± 250 | NA$^{b}$ | 230 ± 16 | NB$^{c}$ | 1000 ± 51 | NB$^{c}$ |
| 9 | 3600 | 1100 ± 200 | 590 ± 78 | 2800 ± 770 | NA$^{b}$ | 48 ± 6 | 4700 ± 22 | 1800 ± 160 | NB$^{c}$ | NB$^{c}$ |

$a$Results represent the mean ± SEM of $n = 4$−8. $b$Not available. $c$Nonbinding up to the highest tested concentration of 1500 μM.
D122-Luc Lewis lung carcinoma cells in mice as well as vascular endothelial cells. A recent study has shown that treatment of SUM159 breast cancer cells with exogenous galectin-8 stimulated the secretion of IL-6, IL-8 and IL-1β, while cotreatment with galectin-8 antagonists blocked this effect. Since MDA-MB-231 cells express galectin-8 endogenously, we reasoned that inhibition of endogenous galectin-8 might reduce proinflammatory cytokine secretion in these cells. To this end, we treated MDA-MB-231 cells with compounds 6a and 1 at two different concentrations (10 and 100 μM). Both compounds markedly decreased the secretion of IL-6 and IL-8 in a dose-dependent manner compared to the untreated cells. At 100 μM, both compounds diminished the secretion of IL-6 by about 65% while reducing the secretion of IL-8 by about 55%. Both compounds were still active at 10 μM, resulting in about 15% reduction in IL-6 secretion and 20% reduction in IL-8 secretion, albeit the effect was not significant (Figure 5). The observed effect is not a result of direct cytotoxicity, as both compounds had no effect on the viability of the cells at the tested concentrations. While the affinity of compound 1 for galectin-8N is about 25-fold higher than that of compound 6a, their effects on the cytokines secretion at the tested concentrations are similar. On the other hand, compound 6a binds galectin-8N with higher selectivity compared to compound 1. Since MDA-MB-231 cells express galectins-1, -3, and -9, compound 1 could potentially bind these intracellular galectins, reducing its available concentration at the binding site of galectin-8N and/or resulting in antagonizing effects on the secretion of cytokines. Moreover, different time-dependent effects could also be ascribed to a different cellular uptake of the compounds. The possibility that both compounds affect cytokine secretion via binding another target with similar K_d values also must be taken into consideration. This necessitates further investigation of the underlying molecular mechanism and the involvement of galectin-8 in cytokine secretion from MDA-MB-231 cells.

It should be noted that IL-6 and IL-8 play important roles in breast cancer pathophysiology. For example, Wang et al. have shown that IL-6 protects MDA-MB-231 cells from the cytotoxicity and apoptosis induced by chemotherapeutic effects of compound 1 for galectin-8N (A, depicted in red) interacts with the HOMO of the D-galactal ring (C, depicted in red) of compound 6a. These interactions are unique and account for the higher affinity of 6a for galectin-8N. (C) Quantum mechanical calculations on a representative MD snapshot (202 ns) of the galectin-8N−3 (in yellow sticks) complex using Jaguar (Schrodinger suite). The LUMO of Arg45 of galectin-8N (A, depicted in red) interacts with the HOMO of the D-galactal 3 olefin (B, depicted in blue).
agents such as doxorubicin and paclitaxel via increased expression of HIF-α.38 Similarly, IL-8 is overexpressed in all ER-negative breast cancer cells including MDA-MB-231. Recent studies have shown that IL-8 promotes the migration and metastasis of MDA-MB-231 cells through the induction of the extracellular traps formation, as well as the activation of PI3K-Akt signaling pathway and epithelial–mesenchymal transition.39 A previous study has also shown that inhibiting both IL-6 and IL-8 in MDA-MB-231 cells inhibits cell viability, colony formation, as well as cell migration.40 Therefore, the inhibitory effects of compounds 1 and 6a are of significant importance since treatment for the triple-negative breast cancer is limited to cytotoxic agents with limited durable response rates due to chemoresistance that accounts for 90% of drug failures.

Conclusion. In conclusion, we have designed and synthesized 6-galactal derivatives carrying benzimidazolyl- and quinolyl-methyl moieties at O3 that display higher affinity and selectivity for galectin-8N compared to the galactose derivatives. Compound 6a is the most selective galectin-8N ligand to date with 15-fold selectivity over galectin-3. The quantum mechanical calculations have revealed that the interaction of the LUMO of Arg45 with the HOMO of O4 and the HOMO of the olefin in the galactal ring are responsible for the high affinity and selectivity of the compounds for galectin-8N. Compounds 1, 6a, and 6c were directly cytotoxic to neither cancer cell lines nor healthy cells. Finally, compounds 1 and 6a have also reduced the secretion of IL-6 and IL-8 in MDA-MB-231 cells in a dose-dependent manner, making them promising starting points toward galectin-8N-inhibitory lead compounds.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00371.

Experimental procedures and physical data for new compounds, procedures for X-ray crystallography and atomic structure determination, biological assay conditions, and molecular modeling procedures (PDF)
Author Contributions

Mu.H. and U.J.N. conceptualized the study and designed the ligands. Mu.H. and F.B. synthesized and characterized the compounds. H.L. measured binding affinities and Mu.H. and F.B. interpreted the data. Ma.H., and R.K. performed the crystallization and data collection. Mu.H. refined and analyzed the diffraction data. Ma. H. supervised the X-ray diffraction data refinement and analysis. A.P.S. performed the QM calculations. Mu.H., S.G., M.A., and Z.J. planned the cell studies. Mu.H. conducted the cytotoxicity assays and the assessment of cytokine secretion profile experiments, while S.G. did the flow cytometric analysis. U.J.N, Z.J., and T.T supervised and provided support throughout the project. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): H.L. and U.J.N. are shareholders in Galecto Biotech Inc., a company developing galectin inhibitors. The other authors have no conflicts to declare.

Crystal structures and the diffraction data have been deposited in the Protein Data Bank under accession IDs 7P1M (6a complex) and 7P11 (9 complex).

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ABBREVIATIONS

CRD, carbohydrate recognition domain; N, N-terminal domain; C, C-terminal domain; VEGF-C, vascular endothelial growth factor C; MW, microwave; SEM, standard error of the mean; MD, molecular dynamics; ns, nanosecond; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; DMF, N,N-dimethylformamide; HPLC, high performance liquid chromatography; DMSO, dimethyl sulfoxide; TCEP, (tris(2-carboxyethyl)phosphine); Tris, (tris-(2-carboxyethyl)phosphine); PEG, polyethylene glycol; MME, monomethoxy ethers; PDB, protein data bank; DIPEA, N,N-diisopropylethylamine; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; PBMC, peripheral blood mononuclear cell.

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