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Hassan, Mujtaba

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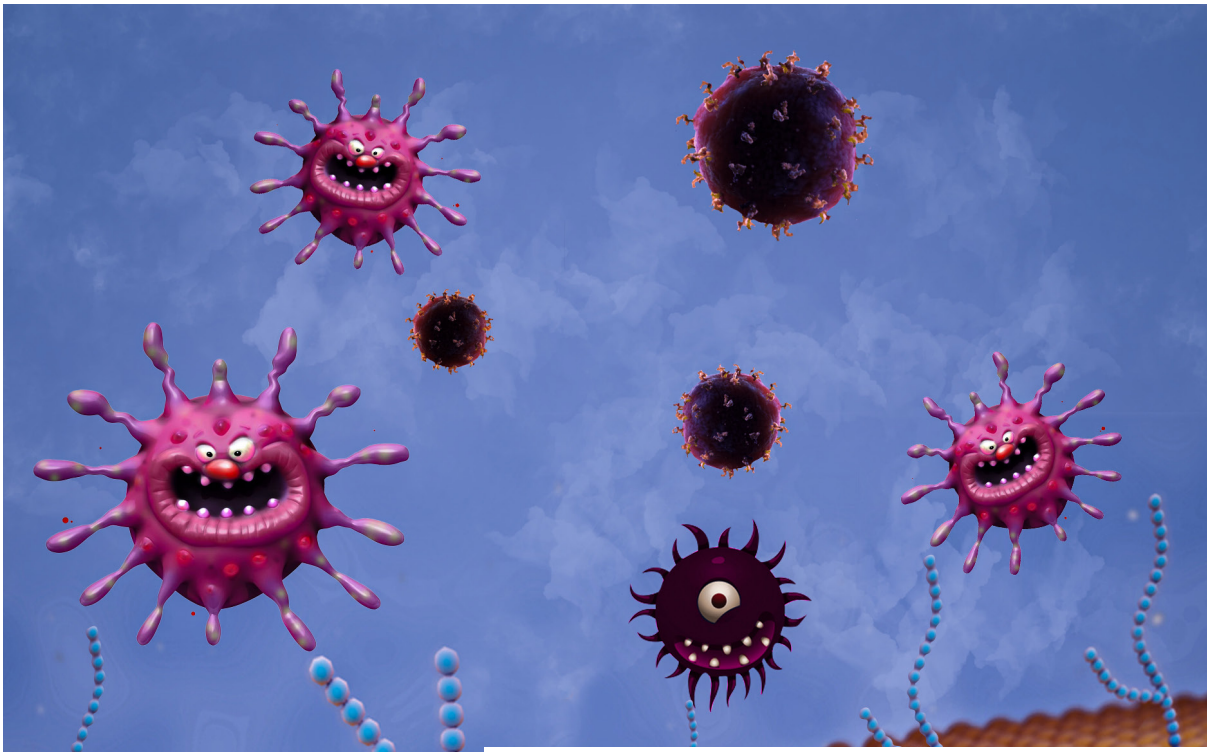

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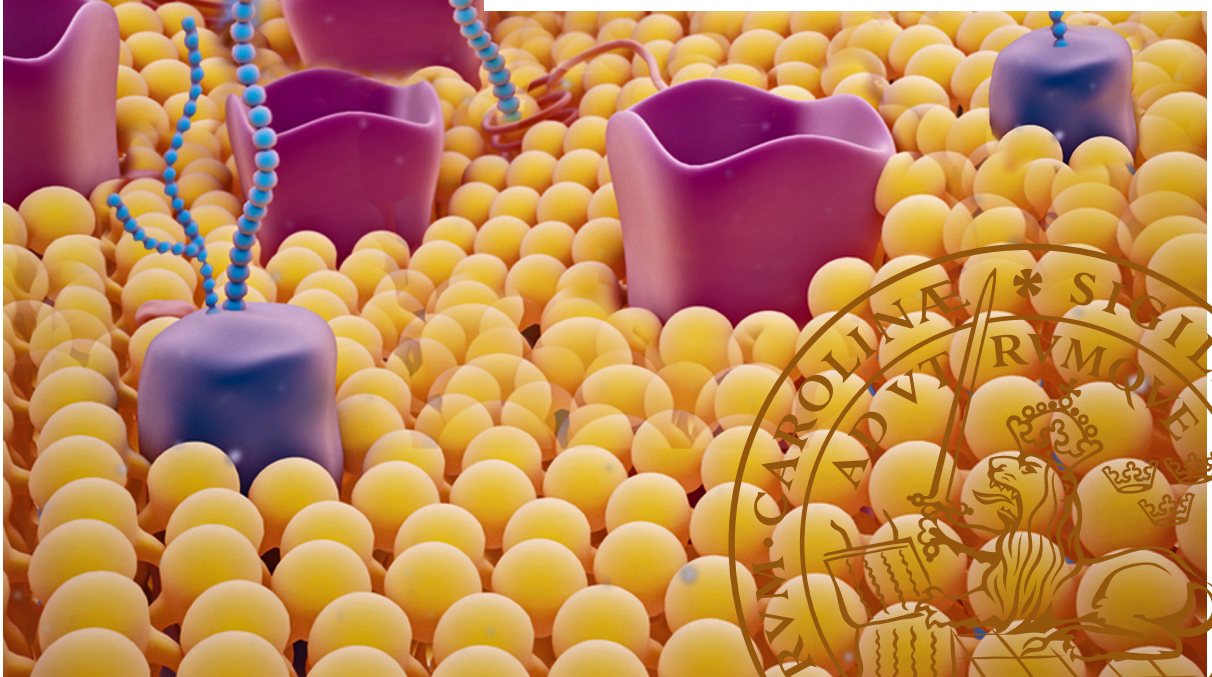

Novel glycomimetic inhibitors and proteolysis-targeting chimeras for human galectins

Mujtaba Hassan

LUND
UNIVERSITY

DOCTORAL DISSERTATION

To be publicly defended with the permission of the Faculty of Science at Lund University, on Thursday, the 24th of November 2022, at 9:00 in lecture hall A at Kemicentrum

Faculty opponent
Prof. Alexander Titz, Helmholtz Institute for Pharmaceutical Research Saarland, Chemical Biology of Carbohydrates, and
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This first part of the thesis describes the design, synthesis, and evaluation of novel glycomimetic inhibitors of human galectin-8, which plays an essential role in pathological lymphangiogenesis, immune system modulation, bone remodeling, and is upregulated in several cancers. The structure-based design of inhibitors of galectin-8 N-terminal domain (galectin-8N) identified a benzimidazole-galactoside with a K_d of 1.8 μM for galectin-8N and 3-fold selectivity over galectin-3, and higher selectivity over the other human galectins. Molecular dynamics simulation showed that the benzimidazole-galactoside binds the non-conserved amino acid Gln47, accounting for the higher selectivity for galectin-8N. Furthermore, the subconjunctival injection of the benzimidazole-galactoside reduced the severity of bacterial keratitis caused by *Pseudomonas aeruginosa* in a mouse model, providing the first evidence that galectin-8 inhibitors can be effective in an actual disease model.

Subsequently, we designed and synthesized a set of C-3 substituted D-galactal derivatives, which led to the discovery of a D-galactal-benzimidazole hybrid with a K_d of 48 μM for galectin-8N and 15-fold selectivity over galectin-3, and higher selectivity over the other human galectins. X-ray structural analysis of the D-galactal-benzimidazole hybrid in complex with galectin-8N followed by molecular dynamics simulation and quantum mechanical calculations showed that the high affinity of the compound for galectin-8N is probably due to the orbital overlap between the LUMO of Arg45 with the electron-rich HOMOs of the olefin and O4 of the D-galactal. A functional assay of the D-galactal-benzimidazole hybrid and the abovementioned benzimidazole-galactoside showed that both compounds reduced the secretion of the proinflammatory interleukin-6 (IL-6) and IL-8 in a dose-dependent manner. Attachment of a *p*-chlorophenyl moiety at C4 of the benzimidazole of the D-galactal-benzimidazole hybrid resulted in the discovery of the most potent selective galectin-8N inhibitor to date with a K_d of 2.9 μM for galectin-8N and 50-fold selectivity over galectin-3, and even higher selectivity over the other human galectins. X-ray structural analysis revealed that the high affinity of the compound for galectin-8N is probably due to the interaction of the *p*-chlorophenyl moiety with Arg59 and/or Tyr141 via cation-π stacking and/or π-π stacking, respectively. This compound represents a promising starting point for the design of ligands that bind galectin-8N with higher affinity and selectivity.

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Keywords: Galectins, Galectin-3, Galectin-8, benzimidazole, affinity, selectivity, X-ray, PROTAC

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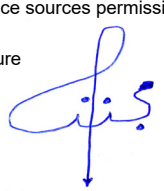
Signature  Date 2022-10-14

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Dedicated to my parents and my wife

Table of Contents

List of publications

This thesis summarizes the following papers, referred to by their Roman numerals. Paper I is reprinted with the permission of Elsevier. Paper II is reprinted with the permission of the American Chemical Society.

I. **Hassan, M.**; van Klaveren, S.; Håkansson, M.; Diehl, C.; Kovačič, R.; Baussière, F.; Sundin, A. P.; Dernovšek, J.; Walse, B.; Zetterberg, F.; Leffler, H.; Anderluh, M.; Tomašič, T.; Jakopin, Ž.; Nilsson, U. J. Benzimidazole–Galactosides Bind Selectively to the Galectin-8 N-Terminal Domain: Structure-Based Design and Optimisation. *Eur. J. Med. Chem.***2021**, *223*, 113664.

Contribution: I conceptualized the study with the co-authors. I built, refined, and deposited one of the X-ray crystal structures in Protein Data Bank. I and Sjors van Klaveren performed the synthesis, purification, and characterization of all compounds. I and Sjors van Klaveren wrote the manuscript, and the co-authors edited it.

II. **Hassan, M**.; Baussière, F.; Guzelj, S.; Sundin, A. P.; Håkansson, M.; Kovačič, R.; Leffler, H.; Tomašič, T.; Anderluh, M.; Jakopin, Ž.; Nilsson, U. J. Structure-Guided Design of D-Galactal Derivatives with High Affinity and Selectivity for the Galectin-8 N-Terminal Domain. *ACS Med. Chem. Lett.* **2021**, *12* (11), 1745–1752.

Contribution: I conceptualized the study with the co-authors. I performed the synthesis, purification, and characterization of most compounds. I built, refined, and deposited all the X-ray crystal structures in Protein Data Bank. I performed the cell assays with Samo Guzelj. I wrote the manuscript, and the co-authors edited it.

III. **Hassan, M.**; Juskaite, R.; Ströhagen, N.; Diel, C.; van Klaveren, S.; Tomašič, T.; Anderluh, M.; Leffler, H.; Logan, D. T.; Jakopin, Ž.; Nilsson, U. J. Targeting an Unexploited Binding Pocket in Galectin-8 N-terminal Domain to Discover Selective High-Affinity Ligands. *Manuscript*.

Contribution: I conceptualized the study with the co-authors. I performed the synthesis, purification, and characterization of most compounds. I wrote the manuscript, and the co-authors edited it.

IV. **Hassan, M.**; Oredsson, S.; Ansar, S.; Bricelj, A.; Tomašič, T.; Izidor, S.; Leffler, H.; Anderluh, A.; Jakopin, Ž.; Nilsson, U. J. Design, Synthesis, and Evaluation of Proteolysis-Targeting Chimeras (PROTACs) for Human Galectins. *Manuscript*.

Contribution: I conceptualized the study with the co-authors. I performed the synthesis, purification, and characterization of all compounds. I performed the biological evaluation with Prof. Stina Oredsson and Dr. Saema Ansar. I wrote the manuscript, and the co-authors edited it.

Publication not included in the thesis:

V. Ramadan, A.; Cao, Z.; **Hassan, M.**; Zetterberg, F.; Nilsson, U. J.; Gadjeva, M.; Rathinam, V.; Panjwani, N. Galectin-8 downmodulates activation of TLR4 pathway and impairs bacterial clearance in a mouse model of Pseudomonas keratitis. *Submitted*.

Popular summary

Discovering a new drug for a given disease is an expensive and time-consuming process that often requires more than a decade of research and over a billion dollars per drug. Drug discovery typically starts with understanding the disease's molecular mechanism and then finding suitable target molecules, known as drug targets, that can be attacked by drugs to treat the disease. The target molecules can be lipids, nucleic acids, or proteins. One way to speed up the drug discovery process is to identify the shape (structure) of the given drug target and assemble (design) a molecule (drug) that fits the shape of the drug target to treat the disease in question. This process (structure-based drug design) resembles putting the missing pieces of a puzzle together or assembling toy blocks. My thesis involves the structure-based design of molecules that block the function of a group of proteins called galectins, which play a role in the development of tumors and inflammation. Since regular cameras cannot be used to capture the shape of proteins, we used another method known as X-ray crystallography to obtain pictures of galectins to identify their shapes. We then used specialized computer software to look at the X-ray structures of galectins to design drug molecules that stop them from causing tumors and inflammations.

The first part of my thesis involved the structure-based design of molecules that block the function of a member of the family of galectins called galectin-8, which plays a role in the development of breast cancer and eye inflammation. This resulted in the discovery of novel molecules that are currently the most potent molecules that inhibit galectin-8 without affecting the functionality of similar galectins. More importantly, these molecules affected the biochemical processes in breast cancer cells and attenuated eye inflammation in laboratory mice.

The second part of the project focused on the structure-based design of molecules that guide the degradation of another galectin called galectin-3, which is involved in the fibrosis of the lung and liver as well as the development of certain tumors. These ensuing results will hopefully open new avenues for drug discovery.

Abstract

Galectins are a family of soluble proteins that bind β-D-galactopyranoside-containing glycoconjugates through their conserved carbohydrate-recognition domains. Galectins have emerged as promising drug targets due to their involvement in various pathological conditions, such as tumor growth and metastasis, autoimmune and inflammatory diseases, as well as metabolic disorders.

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Abbreviations

ACN	Acetonitrile
ALCAM	Activated leukocyte cell adhesion molecule
ATP	Adenosine triphosphate
BLI	Bio-layer interferometry
CRD	Carbohydrate recognition domain
DC_{50}	Half-maximal degradation concentration
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DCM	Dichloromethane
DIPEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	N,N-Dimethylformamide
DSF	Differential scanning fluorimetry
FAC	Frontal affinity chromatography
FimH	Type 1 fimbrin D-mannose specific adhesin
FP	Fluorescence polarization
HIA	Hemagglutinin inhibition assay
HIV	Human immunodeficiency virus
HPLC	High-pressure liquid chromatography
IL	Interleukin
ITC	Isothermal titration calorimetry
K_d	Dissociation constant
kDa	Kilodalton
LacNAc	N-Acetyllactosamine
MD	Molecular dynamics
mRNA	Messenger ribonucleic acid
MST	Microscale thermophoresis
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MW	Microwave
ns	Nanosecond

PA	*Pseudomonas aeruginosa*
PBMC	Peripheral blood mononuclear cell
PhMe	Toluene
PMN	Polymorphonuclear leukocyte
POI	Protein of interest
PROTAC	Proteolysis-targeting chimera
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RAS	Rat sarcoma
RT	Room temperature
SAR	Structure-activity relationship
SEM	Standard error of mean
TDG	Thiodigalactoside
TPSA	Topological polar surface area
UPS	Ubiquitin–proteasome system
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau

1 Introduction

"In this remarkable age of genomics, proteomics, and functional proteomics, I am often asked by my colleagues why glycobiology has apparently lagged so far behind the other fields. The simple answer is that glycoconjugates are much more complex, variegated, and difficult to study than proteins or nucleic acids."

- Professor Saul Roseman[1]

1.1 The sugar code

Carbohydrates are the most abundant class of biomolecules on the planet earth and, as the name indicates, they originate from the stoichiometric proportion of carbon and water $(C_n(H_2O))_m$, where $n \geq m$.[2] The simplest carbohydrates that cannot be hydrolyzed into smaller units are called monosaccharides. There are nine common monosaccharides found in vertebrates, and they include, among others, galactose, the focus of the thesis, in addition to glucose, mannose, and fucose. Monosaccharides exist in solution as an equilibrium mixture of open-chain or ring forms. The cyclization of the open-chain form creates a new asymmetric center, termed the anomeric carbon, at C1 for the aldo sugars or C2 for the keto sugars. According to the stereochemistry of the substituent at the anomeric carbon, carbohydrates can be assigned as α anomers or β anomers. In galactose, the α anomer refers to the anomeric hydroxyl group being axial and the β anomer refers to the anomeric hydroxyl group being equatorial (Figure 1). Monosaccharides can attach to one another via glycosidic bonds giving rise to di- (2 monosaccharides), oligo- (3-10 monosaccharides), and polysaccharides (> 10 monosaccharides).[3] There is a huge diversity of oligosaccharides in nature, and indeed, their diversity exceeds that of any other class of biomolecules. Each hydroxyl group in the monosaccharides can, in principle, serve as an acceptor of the glycosidic bond, and each glycosidic bond is linked to either an α- or β anomeric substituent. Therefore, the combination of monosaccharides can give rise to a large number of linear or branched polysaccharides with diverse geometries as opposed to nucleotides and amino acids with limited linkage sites that attach only in a linear fashion (Figure 1).[4] Other factors contributing to the diversity of glycans are the varying ring sizes of the monosaccharides (e.g. pyranoses and furanoses), the non-template driven

biosynthesis, and the post-biosynthetic modifications of the monosaccharides such as the derivatization of glucose and galactose to 2-amino sugars followed by the N-acetylation which can be viewed as the Umlaut (putting marks over vowel letters) that is found in many languages such as German and Swedish languages.[5,6] With this high permutation of structures, carbohydrates can be considered the third alphabet of life, next to amino acids and nucleotides, where the letters (monosaccharides) combine to form different molecular messages (oligosaccharides) that encode specific biological information (the sugar code).[7]

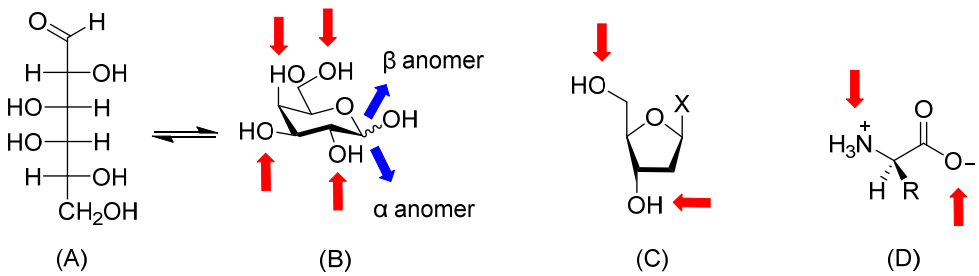


Figure 1: Illustration of the open form of carbohydrates and the linkage points for oligomer formation for carbohydrates, nucleotides, and amino acids. Carbohydrates exist in solution as an equilibrium mixture of open chain (A) and ring (B) forms. Activated carbohydrates via the anomeric positions (α or β) can attach at any of the hydroxyl groups (red arrows), giving rise to a large number of potential oligomers. In contrast, nucleic acids (C) and amino acids (D) can only attach in a linear fashion (red arrows).

The diversity of carbohydrates in nature is reflected in the diversity of their biological functions, beyond merely being the main energy source of the human diet. For example, carbohydrates are vital to plants as they act as both molecular concrete of cell walls enabling plants to resist gravity, as well as protective materials defending plants against insects and microbes.[2,8] All eukaryotic cells are surrounded by a thick layer, 10-100 Å, of oligosaccharides conjugated to proteins (glycoproteins), or lipids (glycolipids) referred to as the glycocalyx.[9,10] This glycocalyx enables cell-environment interaction, cell adhesion and mediates the recognition of cells by bacteria and viruses.[11] The glycocalyx on the surface of the red blood cells also determines the ABO blood group type (Figure 2).[12]

Due to the abundance and diversity of carbohydrates, several protein folds, termed lectins, have evolved to read the glycan-encoded message and translate it into biological information. The family of lectins includes, among others, C-type lectins, P-type lectins, I-type lectins, and galectins, the focus of this thesis.[2]

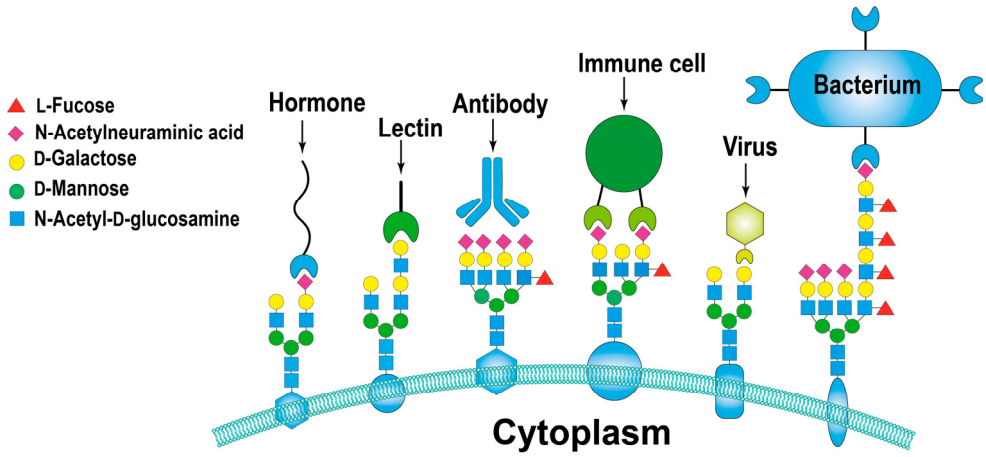


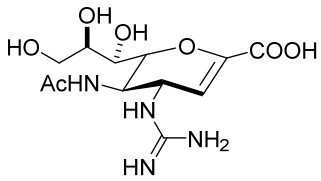
Figure 2: Schematic representation of the diversity of functions of the cell-surface glycoconjugates (glycocalyx). The glycocalyx mediates cell adhesion, cell-environment interaction, as well as recognition by bacteria and viruses.

1.2 Carbohydrates in drug discovery

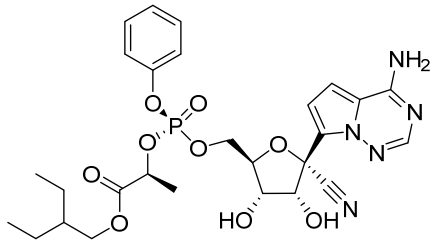
Due to the abovementioned functional diversity of carbohydrates, they are increasingly recognized as hotspots for biomedical intervention. In fact, the utilization of carbohydrates in medicine dates back to more than a century ago, with heparin, a naturally occurring anticoagulant, being the oldest carbohydrate-based drug.[13] Other prime examples of carbohydrate-based drugs are the aminoglycoside antibiotics that have been in use since the early 1940s.[14] Nonetheless, the exploitation of glycans in drug discovery was hindered by the poor understanding of the molecular basis governing glycan functions due to their structural complexity.[15] However, the recent progress in the analytical and biophysical tools allowed for the precise elucidation of glycan structures and revealed the molecular basis for glycan recognition.[16–19] In addition, the advances in carbohydrate chemistry combined with the high throughput screening methods such as glycan microarrays led to the discovery of several glycan receptors.[20,21] This has resulted in a resurgence in the use of carbohydrates in drug discovery. For example, identifying the pentasaccharide sequence responsible for heparin activity led to the discovery of the anticoagulant drug fondaparinux, with better bioavailability and fewer side effects than heparin.[22] Since glycans are involved in the viral invasion of host cells, several carbohydrate-based antiviral drugs such as zanamivir have been developed to stop the viral invasion.[23] Carbohydrates-based drugs can also be used to hijack cell replication since D-ribose and deoxyribose are the building blocks of DNA and RNA. Examples of this include the antiviral drugs ribavirin and

remdesivir, and the anticancer drugs azacitidine and decitabine.[24] Derivatives of D-glucose such as dapagliflozin and canagliflozin are also used to treat type diabetes mellitus.[25] Other examples include the antiplatelet drug ticagrelor[26], the laxative drug lactitol[27], and the anti-Alzheimer's drug sodium oligomannate[28] (Figure 3).

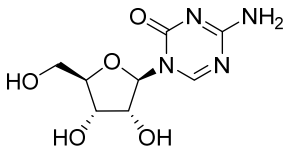
Carbohydrate-based drugs still hold great promise for tackling unmet medical needs. The current research in the development of carbohydrate-based drugs includes, among others, type 1 fimbrin D-mannose specific adhesin (FimH) antagonists for urinary tract infections[29] and Crohn's disease[30,31], sialic acid derivatives as antibacterial agents[32], dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) inhibitors as anti-HIV agents[33], and siglec inhibitors as immunomodulators and anti-inflammatory agents[34]. Of particular note is that the carbohydrate-based FimH antagonist EB8018 (Sibofimloc) was safe and well-tolerated in Phase Ib clinical trials for the treatment of Crohn's disease (ClinicalTrials.gov identifier: NCT02998190) (Figure 3).[35] Developing carbohydrate-based galectin inhibitors is one of the current research hotspots, and we will investigate galectins in more detail throughout the thesis.



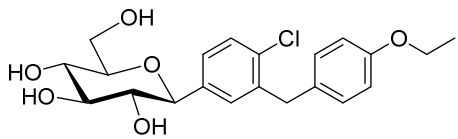
Zanamivir



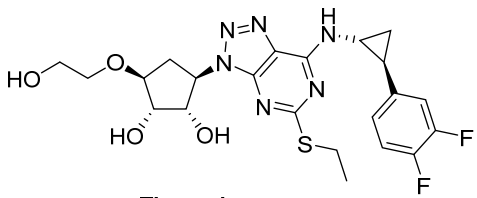
Remdesivir



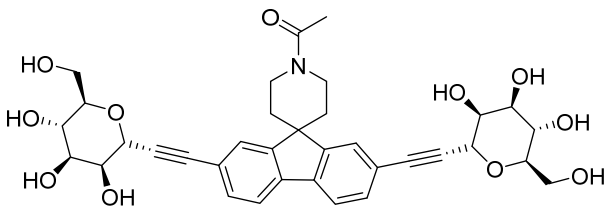
Azacitidine



Gliflozine



Ticagrelor



EB808 (Sibofimloc)

Figure 3: Structures of selected examples of the known carbohydrate-based drugs available for clinical use or currently undergoing clinical trials.

1.3 Galectins: Structure and function

The term galectin was first introduced in 1994 by Barondes and his coworkers to denote the family of proteins that fulfills the two criteria of binding β-D-galactopyranoside-containing glycoconjugates and sharing a significant sequence similarity in the carbohydrate recognition domain (CRD).[36] Galectins are found in the tissues of many members of the animal kingdom, ranging from lower vertebrates (e.g. sponges and nematodes) to mammals (e.g. humans). There are currently 16 known mammalian galectins, which can be classified into three subgroups according to their quaternary structures. The first subgroup consists of the monomeric (prototype) galectins with one CRD that form noncovalent homodimers depending on the concentration and ligand density (1, 2, 5, 7, 10, 11, 13, 14, 15, and 16). The second subgroup encompasses the tandem-repeat galectins with two distinct CRDs at the N- and C-terminal domains joined by a peptide linker of variable lengths (4, 6, 8, 9, and 12). The third subgroup features the lone chimera-type galectin-3 with one CRD at the C-terminal domain in addition to a non-lectin glycine- and proline-rich peptide motif at the N-terminal domain that promotes the formation of high-order oligomers up to pentamers (Figure 4).[37,38] It is worth mentioning that galectins-10, -13, and -16 do not strictly adhere to the definition of galectins as they share the significant sequence similarity in the CRD but do not bind β-D-galactopyranosides.[39–41] Galectins are expressed in all cells; however, their expression varies in different tissues.[42] For example, galectin-1 and galectin-3 are expressed in most tissues[42], while galectin-2 and galectin-4 are expressed primarily in the gastrointestinal tract, with galectin-2 being expressed in the placenta as well[43,44]. Galectin-7 is mainly expressed in the gastrointestinal tract, the skin, and the lining of the mucosal membranes[45], while galectin-8 is expressed in the liver, kidney, cardiac muscle, lung, neuronal tissues, and immune cells[46].

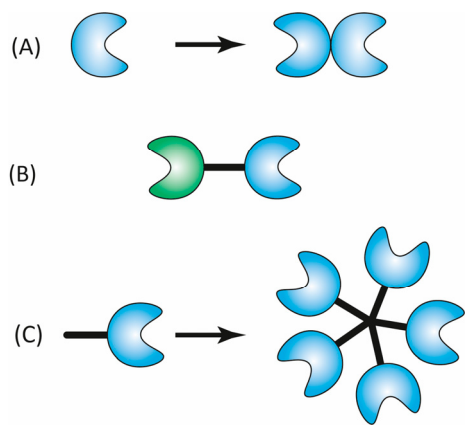


Figure 4: Illustration of the different types of galectins. (A) Prototype galectins with a single CRD which form noncovalent homodimers. (B) Tandem repeat galectins with two distinct CRDs joined by a peptide linker. (C) Chimera-type galectin-3 with one CRD which can form high-order oligomers up to pentamers with its collagen-like tail.

Galectins are synthesized in the cytosolic ribosomes and reside in the cytosol for much of their lifetime. Inside the cell, galectins interact with various cytosolic and nuclear ligands, regulating several cellular activities, such as mRNA splicing, cell division, cell growth, and apoptosis. Galectins are also secreted to the extracellular matrix via a nonclassical Golgi-independent secretory pathway.[47] Outside the cell, galectins are proposed to form dynamic lattices by cross-linking the cell surface β-D-galactopyranoside containing glycolipids and glycoproteins through their CRDs. Thereby, galectins can influence the behavior of the cross-linked glycoconjugates, such as their residence time on the plasma membrane, localization, and intracellular trafficking.[48,49] This results in different cellular responses depending on the type of glycan and galectin involved. For example, galectin-glycoconjugate interactions modulate cell adhesion, migration, proliferation, apoptosis, and immune response. The galectin lattice also regulates metabolic homeostasis through the regulation of nutrient transporters. Thus, galectins are implicated in several pathological conditions, such as tumor growth and metastasis, inflammatory diseases, as well as autoimmune and metabolic disorders.[50] This thesis is focused on developing small-molecule inhibitors for galectin-8 (papers I, II, and III) and galectin-targeted proteolysis-targeting chimeras (PROTACs) (paper IV).

1.4 Galectin-8 as a drug target

Galectin-8 captivated our interest due to its numerous pathological involvements. Firstly, galectin-8 is overexpressed in several cancers, including prostate, lung, kidney, bladder, and breast cancer.[51] Galectin-8 also promotes vascular endothelial growth factor-C (VEGF-C)-mediated pathological lymphangiogenesis, which is implicated in many pathological conditions, including tumor growth and metastasis, organ graft rejection, type 2 diabetes, and corneal inflammation.[52] Furthermore, galectin-8 is involved in several inflammatory and autoimmune disorders as it modulates innate and adaptive immunity through the regulation of T-cell homeostasis.[53,54] Studies on mice also showed that galectin-8 enhances the expression of the osteoclastogenic factor receptor activator of nuclear factor kappa-B ligand (RANKL), which binds to the RANK receptor on osteoclasts and promotes osteoclastogenesis, leading to an increased bone turnover and reduced bone mass.[55,56] Knocking down galectin-8 in the triple-negative breast cancer cells MDA-MB-231 was reported to prevent cell-cell adhesion. Also, knocking down galectin-8 and its glycosylated ligand activated leukocyte cell adhesion molecule (ALCAM) synergistically delayed tumor growth in a murine model of triple-negative breast cancer.[57] Galectin-8 has also been shown to increase the expression of proinflammatory cytokines in cancer cells.[58,59] Besides its pathological involvements, galectin-8 has antibacterial activity due to its ability to bind glycans in damaged vesicles and induce selective autophagy.[60]

Both CRDs (N- and C- terminal domains) are essential for the activity of galectin-8.[61] Therefore, inhibiting one CRD is probably sufficient to block the activity of galectin-8. The N-terminal domain of galectin-8 binds native oligosaccharides with a higher affinity than the C-terminal domain[62,63], and a few reports linked the biological functions of galectin-8 to the unique specificity of its N-terminal domain.[64] Therefore, we focused on developing inhibitors for the N-terminal domain of galectin-8 (galectin-8N).

1.5 Targeted protein degradation

The ubiquitin-proteasome system (UPS) is the main pathway for degrading intracellular proteins in eukaryotic cells.[65] The UPS works by marking proteins for destruction by conjugating them to ubiquitin, a highly conserved 76 amino acid (8.6 kDa) polypeptide, which is then recognized and degraded by the 26S proteasome, a large multisubunit protein complex with six proteolytic sites.[65,66] Conjugating proteins to ubiquitin is carried out by a cascade of enzymes called ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). In short, ubiquitin is activated by conjugating it to an ATP-dependent E1 enzyme via thioether bonds. The activated ubiquitin is then conjugated to an E2 enzyme via *trans*-thioesterification. The E2-ubiquitin complex is then conjugated to an E3 ligase which is a larger protein with a substrate adaptor and accessory proteins. Then depending on the type of E3 ligase, the ubiquitin is either transferred from the E2 ligase to a lysine residue on the surface of the targeted protein directly or passed from E2 to E3 and then to the lysine residue on the targeted protein.[65] The first attempt to hijack the UPS via PROTACs to degrade the protein of interest (POI) was reported in 2001.[67] PROTACs are heterobifunctional molecules composed of a ligand that binds the POI joined by a linker of variable composition to an E3 ligase ligand. Thereby, PROTACs recruit the E3 ligase and form ternary complexes resulting in polyubiquitination and subsequent degradation of the POI by the 26S proteasome.[68]

Traditionally, drug discovery relies on the occupancy-driven pharmacological model, in which the fraction of the bound drug is directly proportional to the ligand's affinity to the target. This requires high-affinity ligands (low nanomolar to picomolar) to elicit the desired effect. Achieving such affinity is sometimes challenging, especially for targets with polar or shallow binding sites. In contrast, PROTACs act through event-driven pharmacology, which requires only the transient formation of the E3 ligase-PROTAC-POI ternary complex, after which the degradation of the POI proceeds regardless of the POI binding. The non-covalently bound PROTAC is released after the destruction of the POI, thus the PROTAC can continue to direct more POIs for degradation (Figure 5). Therefore, high affinity for the target protein is not required for PROTACs, which is best exemplified by the

foretinib-based PROTAC's ability to induce p38α degradation with a half-maximal degradation concentration (DC_{50}) of 210 nM despite having a dissociation constant (K_d) greater than 10 μM for p38α.[69,70] Thus, PROTACs offer a chance to target the undruggable proteome. Indeed, PROTACs have been shown to degrade scaffolding proteins that lack enzymatic activity[71], multicomponent proteins[72], transcription factors, and RAS proteins with no druggable pockets[73]. Moreover, PROTACs have been reported to have a higher target selectivity, reduced off-target toxicity, and a better therapeutic index than traditional inhibitors.[68] Of note, twelve PROTACs have reached clinical trials, with ARV-110 and ARV-471, the first PROTACs to enter clinical trials, successfully progressing to phase II.[74] Therefore, we hypothesized that galectin-targeted PROTACs might offer a chance to exploit the therapeutic potential of the galectins, because their polar and shallow binding pockets usually hinder the development of high-affinity ligands.

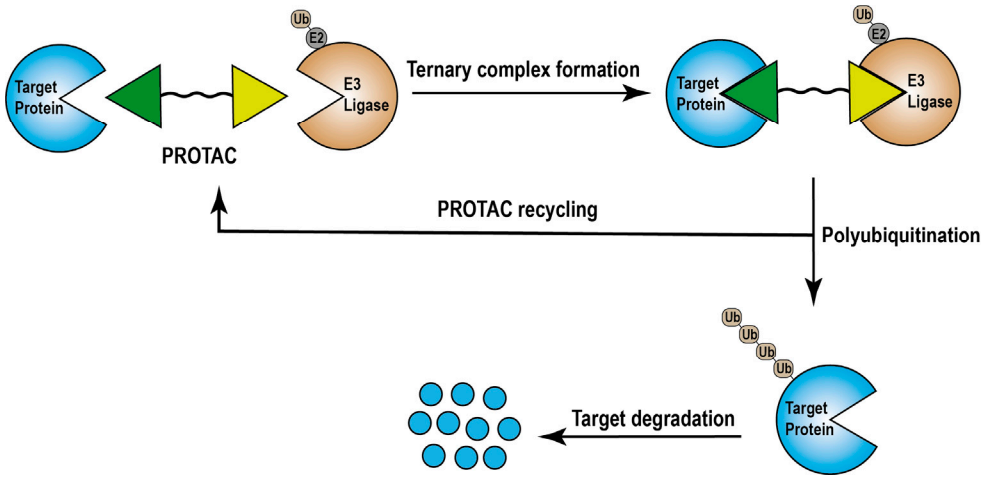


Figure 5: Illustration of the mechanism of the targeted protein degradation by PROTACs.

1.6 Design of galectin inhibitors

As mentioned above, most galectin activities result from binding β-D-galactopyranoside-containing glycoconjugates. Thus, efforts towards developing galectin inhibitors focused on inhibiting the CRD of galectins to compete with the natural ligands. Therefore, we will have a closer look at the galectin CRD to understand the rationale behind the design of galectin inhibitors. The CRD of galectins is a β-sandwich of about 135 amino acids, with one of the sheets, composed of 6 strands, forming the convex side, while the other sheet, consisting of 5 strands, forms the concave side. The concave β-sheet forms the groove in which the carbohydrates bind and is long enough to accommodate a tetrasaccharide. The

CRD can be schematically divided into five subsites (A-E) (Figure 6).[37] N-acetyllactosamine (LacNAc) is a natural ligand fragment for galectins found in many cell-surface glycoconjugates. Taking galectin-3 as an example, the β-galactoside moiety of LacNAc sits in subsite C, which is the most conserved subsite in the CRD. The β-galactoside moiety establishes seven hydrogen bonds, namely HO-4′ with His158, Asn160, and Arg162, HO-6′ with Asn174 and Glu184, and the ring oxygen with Arg162 and Glu184, while H-3′, H-4′, and H-5′ of the galactose engage in a CH-π stacking with Trp181. Subsite D, the second most conserved subsite, harbors the N-acetylglucosamine moiety at C1 of the galactose, with the HO-3 establishing three hydrogen bonds with Arg162 and Glu184 (Figure 6). The other subsites are not as conserved as subsites C and D, and they do not interact with LacNAc, but they contribute to the variation in specificity between galectins for different saccharides.[37] LacNAc forms identical hydrogen-bonding networks with the corresponding amino acids in the other galectins. The observed hydrogen-bonding pattern of the β-galactoside moiety of LacNAc is crucial for the recognition by galectins and is probably indispensable for the design of galectin inhibitors. Therefore, the previous work on galectin inhibitors focused on modifications at the anomeric carbon, HO-2, or HO-3 of the galactose, to access the less conserved CRD subsites to gain binding affinity and/or selectivity.

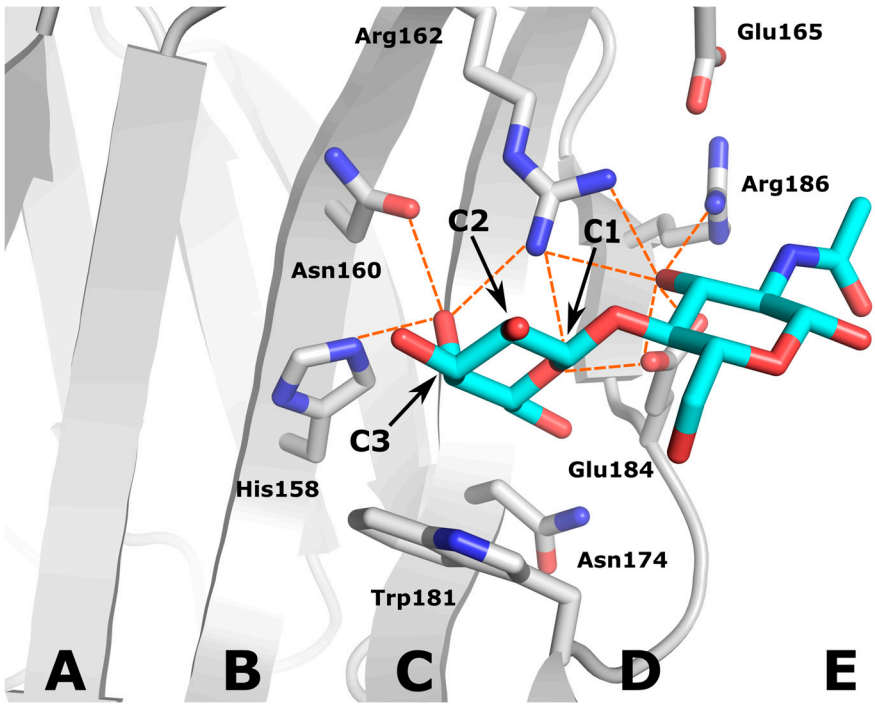


Figure 6: Illustration of subsites A-E of the galectin CRD, exemplified with LacNAc (cyan sticks) bound to the CRD of galectin-3 (PDB ID: 1KJL; grey cartoon representation). Research on galectin inhibitors focused on modifications at C1, C2, and C3 of the galactose moiety.

LacNAc **1** itself has a weak binding affinity for galectins K_d values of about 0.1 mM.[75] Two of the most common reference compounds used in galectin research are methyl β-N-acetyllactosamine (LacNAc-OMe) **2** and thiodigalactoside (TDG) **3** with K_d values of 59 μM and 43 μM for galectin-3, respectively, as determined by fluorescence polarization assay.[76] The journey for developing galectin inhibitors started with the substitution of HO-3′ of LacNAc-OMe **2** with various N-arylamides targeting subsites A and B leading to the discovery of compound **4** bearing 2-carboxynaphth-1-amide with a K_d of 0.32 μM for galectin-3, more than two orders of magnitude improvement over LacNAc-OMe. This improvement in the binding affinity is hypothesized to result from a cation-π stacking with Arg144 in galectin-3.[77,78] Further research identified TDG derivatives with high affinity for galectin-3.[79] This was a milestone in the quest for galectin inhibitors as thioglycosides possess higher chemical and metabolic stability compared to O-glycosides.[80,81] The synthesis of symmetrical fluorinated phenyltriazolyl-TDG derivatives resulted in the discovery of compound **5** (GB0139 formerly known as TD139) with a K_d of 2 nM for galectin-3.[82,83] It is worth mentioning that GB0139 was safe and well-tolerated in phase IIa clinical trials for idiopathic pulmonary fibrosis and it is currently in phase IIb.[84] Attempts to simplify TDG by substituting one of the galactose moieties with a β-thio aglycone to improve its pharmacokinetic properties resulted in the discovery of compound **6** bearing a β-thiotoluene and a K_d value of 5.2 μM for galectin-3.[83] A further modification involved inverting the stereochemistry of the β-thioaryl aglycone and changing the aromatic moiety to 3,4-dichlorophenyl resulted in the discovery of compound **7** with a K_d of 37 nM for galectin-3. The improved affinity caused by the α-3,4-dichlorothiophenyl aglycon is likely due to halogen bonding interaction between the 3-chloro substituent and the backbone carbonyl of Gly182, as well as an S–π interaction between the anomeric sulfur and Trp182.[85] Modification of the aromatic aglycon of compound **7** resulted in the discovery of GB1211 (compound **8**) with a K_d value of 23 nM for galectin-3. GB1211 is the first orally available galectin-3 inhibitor to enter clinical trials, and it is currently in phase IIa for the treatment of cancer and liver cirrhosis. (Figure 7).[86] Other attempts to develop galectin inhibitors included, among other things, mannosides that bound selectively to galectin-9N[87], talosides that bound selectively to galectin-8N and galectin-4C[88], and C-glycosides that bound to galectin-1 and galectin-3 with various selectivity profiles[89–91]. These discoveries paved the way for the research work described in this thesis, which can be viewed as an extension of the previous research with the aim of further understanding the principles that govern the high affinity of the ligands for galectin-8N.

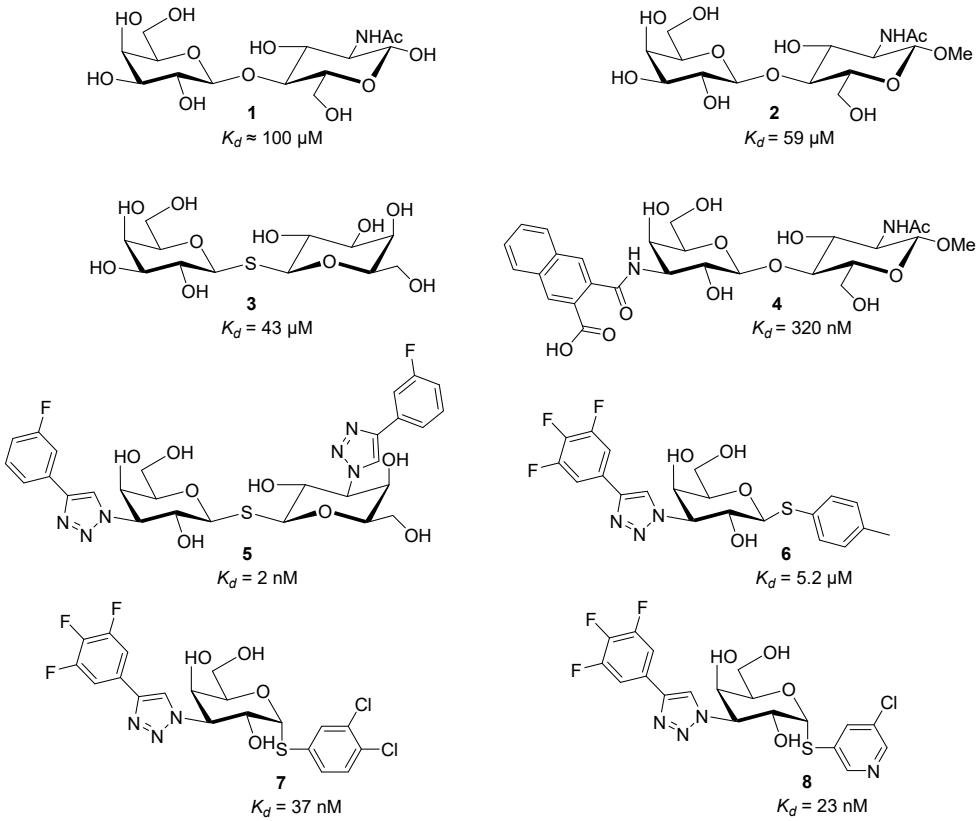


Figure 7: A selected set of previously reported galectin inhibitors and their binding affinities for galectin-3, showing the chronological progress in developing galectin inhibitors.

1.7 Affinity measurement

There are several methods for measuring the binding affinity of ligands to lectins, each with its advantages and disadvantages. The methods commonly used are hemagglutination-inhibition assay (HIA)[92], isothermal titration calorimetry (ITC)[93], and fluorescence polarization assay (FP)[94]. HIA is likely the oldest method to evaluate glycan-lectin interactions, which is based on the observation that erythrocytes agglutinate in the presence of lectins.[95,96] Despite being a rapid and low-cost assay, HIA requires comparatively large amounts of ligand and protein, making it unsuitable for evaluating synthetic ligands obtained by laborious multistep synthesis.[92] ITC is a direct titration method that allows for determining the thermodynamic parameters associated with the ligand-protein interaction (K_d, ΔG, ΔH, and ΔS) in a single experiment. Although ITC is very accurate and gives a

lot of information about ligand-protein interactions, it is time-consuming and requires relatively large amounts of the ligand and the protein; therefore, not suitable for the rapid screening of a large collection of ligands.[93] The method used in this work is FP, which involves exciting a fluorescent probe (a fluorescein-tagged disaccharide in this case) with a plane-polarized light and measuring the degree of polarization remaining in the emitted light. This assay is based on two principles: firstly, the rotational movement of the molecule is responsible for the depolarization of the emitted light, and secondly, when a fluorescent probe is bound to a protein (galectin in this case), the ligand-protein complex rotates slower than the free ligand, due to the larger size of the complex. Low concentrations of the ligands or ligands with weak affinity will leave most of the fluorescent probe bound to the protein, causing it to emit less depolarized light as opposed to high concentrations of the ligands or ligands with high affinity. The polarization is then measured as anisotropy (A) or polarization (P), which is used to calculate the amount of bound and free probe in solution. The amount of free and bound probe is then used to determine the K_d of the ligand to the galectin. FP is a fast and reliable method for determining the K_d values of synthesized ligands without the need for large amounts of ligand and protein and is thus our method of choice.[94] The evaluation of the binding affinity ligands to galectins can also be carried out with other methods such as frontal affinity chromatography (FAC)[97], microscale thermophoresis (MST)[98], bio-layer interferometry (BLI)[99], and differential scanning fluorimetry (DSF)[100].

2 Hypothesis and objectives

Having introduced the biological and pathological implications of galectins, in particular galectin-8, in Chapter 1, we now know the importance of developing potent and selective galectin inhibitors as both tool compounds to further study galectin functions and therapeutic candidates to tackle galectin-related pathological conditions.

The work in this thesis is based on the following hypotheses:

- Selective galectin-8N inhibitors are potential antitumor and anti-inflammatory agents
- The structure-based design will lead to the discovery of galectin-8N inhibitors with improved binding affinity and selectivity over the previously reported inhibitors
- Galectin-targeted PROTACs will lead to the discovery of first-in-class galectin degraders

Therefore, this research aims to:

- Investigate the principles that govern the affinity and selectivity of ligands to galectin-8N
- Design and synthesize galectin-8N inhibitors with improved binding affinity and selectivity over the existing inhibitors
- Evaluate the galectin-8 inhibitors as potential anti-tumor and anti-inflammatory agents
- Design and synthesize PROTACs targeting human galectins to investigate whether galectins are amenable for targeted protein degradation

3 Benzimidazole-galactosides as selective inhibitors of galectin-8 N-terminal domain (paper I)

3.1 Background

The starting point for this project was the previously published quinoline-galactoside **9**, which was the only reported monosaccharide-based ligand that binds galectin-8N with a higher affinity over the other human galectins, with a K_d of 110 μM for galectin-8N, and 3-fold selectivity over galectin-3 and higher selectivity over the other human galectins.[101] It has been shown that the carboxylate moiety and the quinoline nitrogen are essential for the ligand's affinity for galectin-8N and selectivity over the other galectins. Removal of the carboxylate moiety (compound **10**) resulted in a six-fold decrease in the binding affinity for galectin-8N and compromised the selectivity over galectin-3. Furthermore, the removal of the quinoline nitrogen (compound **11**) resulted in about a 30-fold loss in the binding affinity for galectin-8N and reversed the selectivity over galectin-3 (Figure 8).

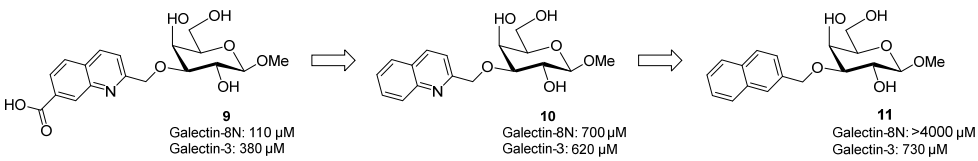


Figure 8: The structures of the previously reported quinoline–galactosides **9** and **10**, and the naphthalene-galactoside **11**.

3.2 Structural analysis

Our first objective was to investigate the reason behind the observed structure-activity relationship (SAR) of the quinoline-galactosides. Therefore, we obtained the X-ray crystal structure of the galectin-8N-**9** complex at a resolution of 1.6 Å. The crystal structure showed that the binding mode of the methyl β-D-galactopyranoside in compound **9** is identical to that of the corresponding galactose

in the galectin-8N–lactose complex. The HO-4 of the methyl β-D-galactopyranoside is involved in a hydrogen-bonding network with Arg45, Arg69, and His65, while the HO-6 engages in hydrogen bonding interactions with Asn79 and Glu89. The importance of the quinoline nitrogen stems from its placement in a favorable position for the electron-rich nitrogen to establish an ion-dipole interaction with the guanidinium side chain of Arg45, while the importance of the carboxylate moiety is due to its establishment of water-mediated hydrogen bonds with Arg45 and Gln47 (Figure 9). It is worth mentioning that Gln47 is unique to galectin-8N, and that is probably one reason for the higher selectivity of the quinoline-galactoside **9** for galectin-8N compared to compounds **10** and **11**.[102]

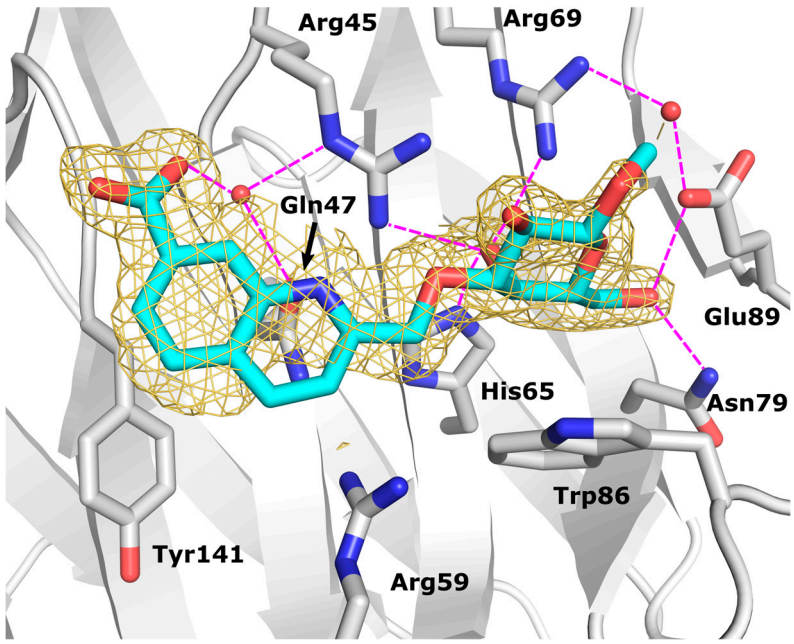
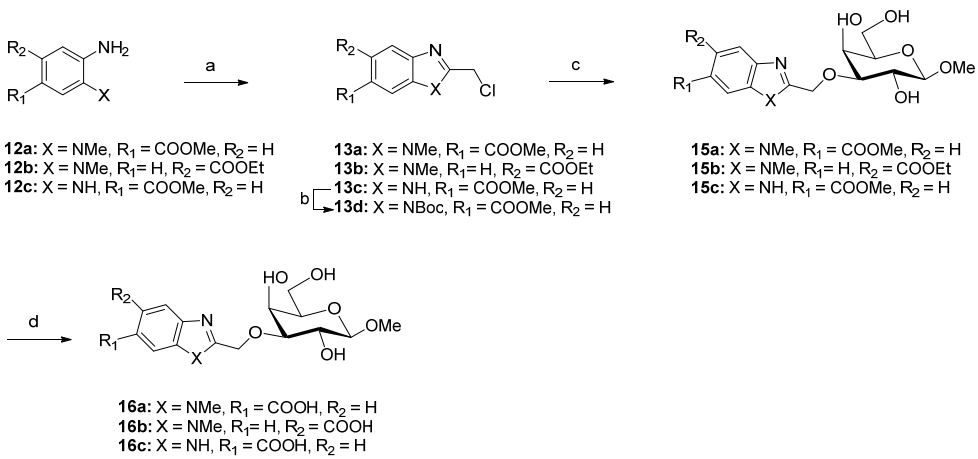


Figure 9: The electron density map (yellow mesh) $2|F_o| - |F_c|\alpha_c$ contoured at 1σ for compound **9** (cyan sticks) in complex with galectin-8N (PDB ID: 7AEN; grey cartoon representation).

3.3 Design, synthesis, and evaluation

We then set to investigate the effect of changing the composition of the heterocyclic ring on the binding affinity for galectin-8N and the selectivity over the other human galectins. Thus, we synthesized a collection of triazole, benzimidazole, benzothiazole, and benzoxazole galactosides as quinoline surrogates. Evaluation of the binding affinity of the compounds for galectin-8N and galectin-3 in a fluorescence polarization assay showed that none of the triazole, benzothiazole, and

benzoxazole galactosides had improved affinity or selectivity for galectin-8N over the previously reported compounds **9** or **10** and their SAR is discussed in detail in paper I. However, the benzimidazole galactosides showed higher selectivity for galectin-8N over galectin-3 than the quinoline galactosides. Therefore, this chapter focuses on the synthesis and evaluation of the benzimidazole-galactosides as galectin-8N inhibitors. The key steps for the synthesis of the benzimidazole galactosides are shown in Scheme 1. In short, the 2-(chloromethyl)benzimidazoles **13a**-**13c** were synthesized by 2-chloro-1,1,1-trimethoxyethane condensation with the corresponding 1,2-diamines **12a**-**12c**. Boc protection of compound **13c** afforded compound **13d**. Compounds **13a**, **13b**, and **13d** were then coupled to methyl β-D-galactopyranoside **14** via the stannylene-mediated regioselective O-3 alkylation to afford the methyl esters **15a**–**15c**. Notably, the stannylene-mediated alkylation did not proceed with the unprotected 1H-benzimidazole, while the alkylation with the Boc-protected benzimidazole resulted in a spontaneous deprotection of the Boc group. The carboxylic acids **16a**-**16c** were then obtained by the alkaline hydrolysis of the esters.[102]



Scheme 1: Synthesis of compounds **15a**-**15c** and **16a**-**16c**: (a) 2-chloro-1,1,1-trimethoxyethane, $BF_3.Et_2O$, DCM, rt, (92-94%); (b) Boc_2O, DMAP, DMF, rt, (82%); (c) i. **14**, Bu_2SnO, MeOH, 70 °C, 2h; ii. N-Bu_4NBr, 1,4-dioxane, 85 °C, overnight, (65-77%); (d) KOH, EtOH, H_2O, 80 °C, (37-56%).

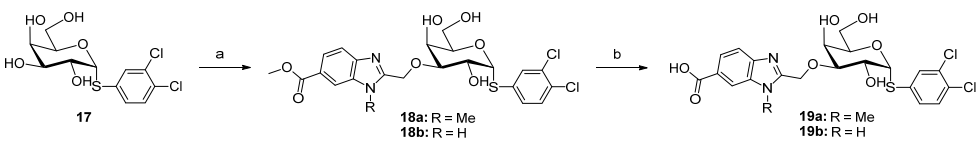
Evaluation of the binding affinities of the compounds for galectin-8N and galectin-3 in a fluorescence polarization assay showed that the esters **15a**–**15c** and the carboxylic acid **16b** possess lower affinities for galectin-8N compared to quinoline-galactoside **9**, with negligible selectivity over galectin-3. In contrast, the carboxylic acids **16a** and **16c** displayed similar affinities for galectin-8N compared to the quinoline-galactoside **9**, with K_d values of 190 μM and 312 μM, respectively. Compound **16a** had 7-fold selectivity over galectin-3, making it the most selective monosaccharide-based galectin-8N inhibitor at that time (Table 1).

Table 1: K_d value of compounds **15a**–**15c** and **16a**–**16c** (μM)[a].

Compound	Galectin-8N	Galectin-3
9	110	380
10	700	620
11	>4000	730
14	6300	4400
15a	1100 ± 120	NB[b]
15b	1600 ± 310	2000 ± 56
15c	1200 ± 210	1600 ± 110
16a	190 ± 24	1400 ± 84
16b	3600 ± 190	1100 ± 140
16c	310 ± 26	590 ± 16

[a]Results represent the mean ± SEM of n = 4 to 8. [b]NB, Non-binding up to the highest tested concentration of 1500 μM.

In Chapter 1, we discussed that attaching the α-3,4-dichlorothiophenyl aglycon improved the binding for galectin-3. The binding affinity of the quinoline-galactosides for galectin-8N also improved by attaching the same aglycon.[101] Based on this, we speculated that the α-3,4-dichlorothiophenyl aglycon might improve the binding affinity of the benzimidazole-galactosides for galectin-8N. Therefore, we synthesized compounds **19a** and **19b** according to Scheme 2.



Scheme 2: Synthesis of compounds **19a**, and **19b**: (a) **13a** or **13d**, Bu_2SnO, *n*-Bu_4NI, PhMe, ACN, MW, 120 °C, 1 h (31–34%); (b) **18a**, KOH, EtOH, H_2O, 80 °C, 6 h (31%). (c) **18b**, LiOH, EtOH, H_2O, 80 °C, overnight (41%).

Evaluation of the binding affinities of compounds **19a** and **19b** on a panel of human galectins in a fluorescence polarization assay showed that both compounds gained about two orders of magnitude of affinity for galectin-8N. Although the selectivity of the benzimidazole-galactosides over galectin-3 was compromised, compound **19a** still had 9-fold selectivity over galectin-9N and higher selectivity over the other human galectins (Table *2*). Compound **19a** was the most potent selective galectin-8N ligand at that time.

Table 2: K_d Values of compounds **19a** and **19b** (μM)[a]

	Galectins (N = N-terminal domain, C = C-terminal domain)							
Compound	1	**3**	4N	4C	**8N**	8C	9N	9C
19a	130 ± 11	**5 ± 1.1**	130 ± 12	120 ± 37	**1.8 ± 0.1**	760 ± 160	16 ± 1.5	45 ± 2
19b	87 ± 6.1	**4.1 ± 0.4**	78 ± 14	160 ± 12	**2.8 ± 0.1**	450 ± 23	13 ± 1.2	18 ± 0.7

[a]Results represent the mean ± SEM of n = 4 to 8.

3.4 Molecular modeling

We then performed molecular dynamics simulations (MD) of compounds **16a** and **19a** with galectin-8N and galectin-3 to investigate the reason for the selectivity of the benzimidazole galactosides for galectin-8N. Simulations of compound **16a** with galectin-8N and galectin-3 were performed for 500 nanoseconds (ns) and 300 ns, respectively, while the simulations of compound **19a** with galectin-8N and galectin-3 were performed for 300 ns. However, only **19a** will be discussed in this chapter, as **16a** is discussed in paper I, and the galactose moiety establishes the same hydrogen-bonding networks in both compounds. The simulations showed that the benzimidazole of compound **19a** established a cation-π stacking with the guanidinium side chain of Arg45 in galectin-8N, or the corresponding Arg144 in galectin-3, but with different orientations. The N-methyl group of the benzimidazole pointed towards Arg45 in galectin-8N, while it pointed towards Asp148 in galectin-3. Thus, the electron-rich nitrogen of the benzimidazole can establish a hydrogen bond with Gln47 in galectin-8N, which is not possible with the corresponding Ala146 or Asp148 in galectin-3. Moreover, the carboxylate moiety is placed in a favorable position to establish a water-mediated hydrogen bond with Gly142. However, compound **19a** is less selective than compound **16a** due to the placement of the *m*-chloro substituent in a favorable position to establish a halogen bond with the backbone carbonyl oxygen of Gly87 and Gly182 in galectin-8N and galectin-3, respectively (Figure 10). The binding of the nitrogen of the benzimidazole to the non-conserved Gln47 is the possible reason for the higher selectivity of the benzimidazole-galactosides for galectin-8N.

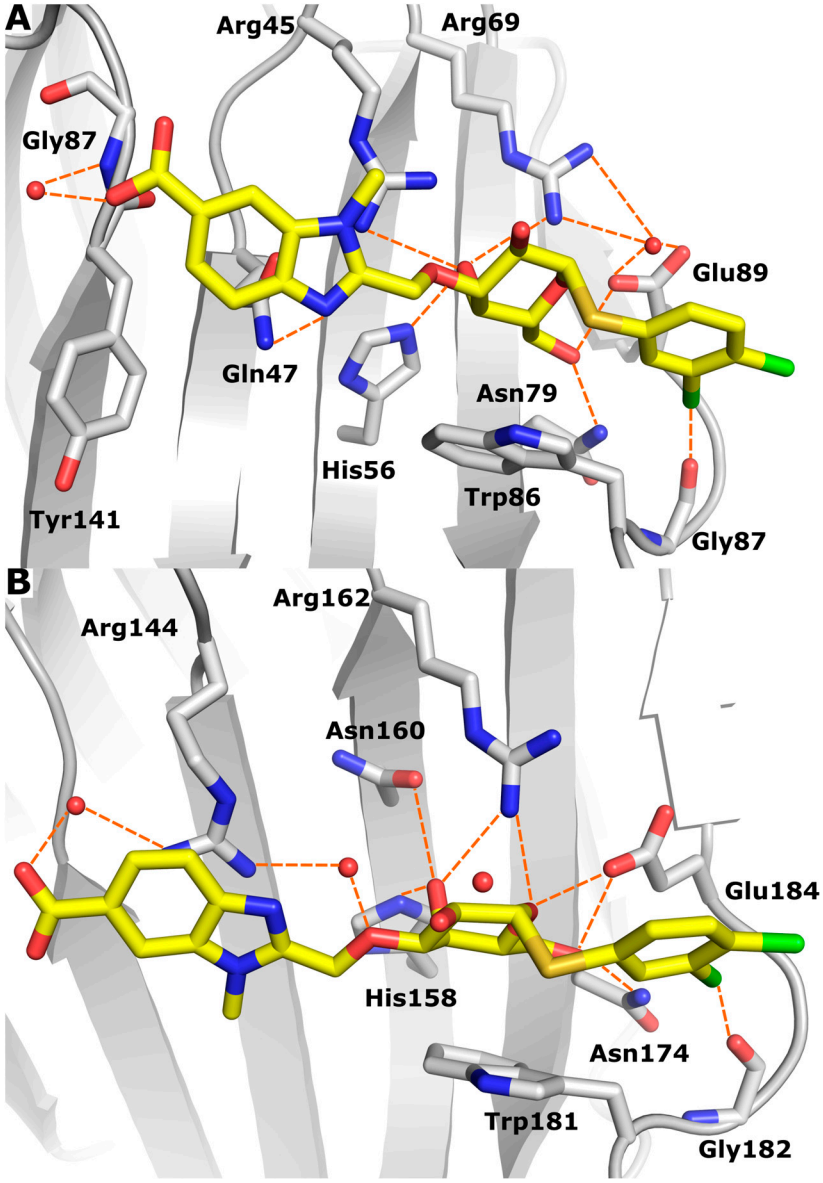


Figure 10: (A) and (B) Molecular dynamics simulation snapshots of representative low-energy conformations of **19a** (in yellow sticks) in complex with galectin-8N and galectin-3, respectively.

3.5 In vivo evaluation

To explore the therapeutic potential of galectin-8 inhibitors, compound **19a** was evaluated by the Panjwani Lab (Tufts University School of Medicine, Boston, MA) for its ability to reduce bacterial keratitis caused by *Pseudomonas aeruginosa* (PA) in a mouse model. Two groups of mice were infected by PA. One of the groups received a subconjunctival injection of compound **19a** (5 mg/ml), while the other group received a subconjunctival injection of a vehicle (PBS + 23% DMSO). The group that received compound **19a** had significantly less PA keratitis than the control group, as detected by the reduced corneal opacity, bacterial load, polymorphonuclear (PMN) infiltration, and reduction of the expression level of IL1β (Figure 11). They also found that galectin-8 knock-out mice were resistant to PA keratitis, while galectin-1, -3, and -9 knock-out mice were susceptible to PA keratitis. It was also shown that exogenous galectin-8 exacerbates PA keratitis by downmodulating TLR-4 pathway, and reducing the PA killing capacity of the neutrophils. Taken together, compound **19a** likely reduces PA keratitis in the mouse model via inhibiting galectin-8. This is the first-ever proof of concept that synthetic-molecule galectin-8 inhibitors are pharmacologically active in a disease model (paper V).

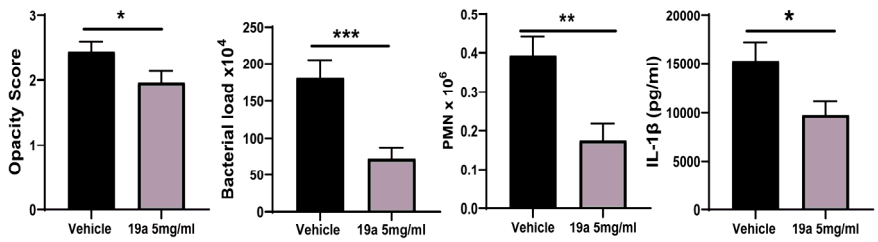


Figure 11: The effect of compound **19a** on the severity of PA keratitis in a mouse model. Data are plotted as Mean ± SEM of 3 independent experiments. Statistical levels of significance were analyzed by the student t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6 Conclusion

We have obtained the X-ray crystal structure of the quinoline-galactoside **9** with galectin-8N. Based on this structure, we have designed, synthesized, and evaluated a set of quinoline surrogates leading to the discovery of the benzimidazole-galactoside **16a** with a K_d 190 μM for galectin-8N and 7-fold selectivity over galectin-3. Proceeding with this result, we have designed and synthesized compound **19a** bearing an α-3,4-dichlorothiophenyl aglycon. Evaluation of the

binding affinity of compound **19a** in a fluorescence polarization assay showed that the compound gained about two orders of magnitude binding affinity for galectin-8N with a K_d of 1.8 μM. Although the introduction of the α-3,4-dichlorothiophenyl aglycon compromised the selectivity of compound **19a** over galectin-3, it still maintained selectivity over the other galectins. MD simulations suggested that the interaction between the electron-rich nitrogen of the benzimidazole with the non-conserved Gln47 in galectin-8N accounts for the selectivity of the benzimidazole–galactosides. We also showed that the subconjunctival injection of compound **19a** reduces PA-induced keratitis in a mouse model. This finding might pave the way for discovering a novel drug to treat PA keratitis and possibly other diseases involving galectin-8.

4 D-Galactal derivatives with high affinity and selectivity for galectin-8 N-terminal domain (paper II)

4.1 Background

This work was based on our observation that D-galactal **20** binds galectin-8N with about 5-fold higher affinity and higher selectivity over galectin-3 than methyl β-D-galactopyranoside **14**. Based on the results we obtained in Chapter 3, we hypothesized that synthesizing C-3 substituted quinoline and benzimidazole D-galactal derivatives might lead to the discovery of ligands that bind galectin-8N with a higher affinity and/or selectivity (Figure 12).

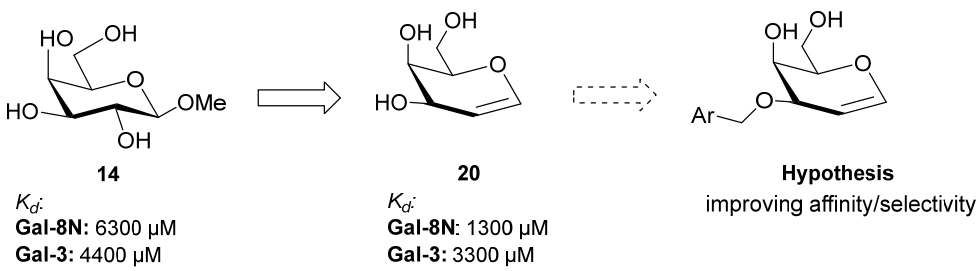
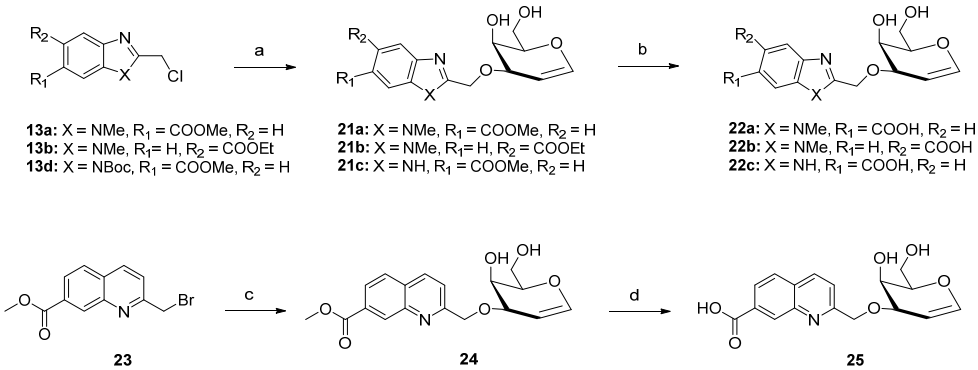


Figure 12: Structures and K_d values of methyl β-D-galactopyranoside **14**, D-galactal **20,** and the hypothesis for improving the affinity of ligands for galectin-8N and/or selectivity over the other human galectins.

4.2 Synthesis and evaluation

The compounds were synthesized according to Scheme 3. The alkyl halides **13a**-**13d** and **23** were synthesized as described in the literature[101,102], then coupled to D-galactal **20** via the stannylene-mediated regioselective O-3 alkylation to afford the esters **21a**-**21c** and **24**.[101,102] The subsequent alkaline hydrolysis afforded the carboxylic acids **22a**-**22c** and **25**.



Scheme 3: Synthesis of compounds **21a**-**21c**, **22a**-**22c**, **24** and **25**: (a) D-Galactal **20**, Bu_2SnO, *n*-Bu_4NI, PhMe, ACN, MW, 120 °C, 30 minutes (44-67%). (b) KOH, EtOH, H_2O, 80 °C, 6 h (41-76%). (c) D-galactal **20**, Bu_2SnO, *n*-Bu_4Br, DIPEA, 80 °C, 90 minutes, (50%). (d) KOH, EtOH, H_2O, 80 °C, 6 h, (83%).

The binding affinities of the benzimidazoles **21a**–**21c** and **22a**–**22c**, and the quinolines **24** and **25** for galectins -1, -2, -3, -4, -7, -8C, -8N, -9C, and -9N were determined in the fluorescence polarization assay. All synthesized D-galactal derivatives had higher affinities for galectin-8N than D-galactal **20** and 2-7-fold higher affinities for galectin-8N than their galactose counterparts. Compound **22c** exhibited the highest gain in binding affinity compared to its galactoside counterpart **16c** with a K_d of 46 μM. Except for compounds **21b** and **22b**, the carboxylic acid derivatives had 4-8-fold higher affinities for galectin-8N than their ester counterparts. In terms of selectivity, except for compounds **21b** and **22b**, the D-galactal derivatives were more than 2-fold selective for galectin-8N over the other human galectins, with compound **22a** being the most selective galectin-8N ligand in the set with 15-fold selectivity over galectin-3, 27-fold selectivity over galectin-1, and even higher selectivity over the other human galectins (Table ***3***).

Table 3: K_d values of compounds **21a**–**21c**, **22a**–**22c**, **24** and **25** (μM)[a]

	Galectins (N = N-terminal domain, C = C-terminal domain)									
Compound	1	2	**3**	4N	4C	7	**8N**	8C	9N	9C
20[103]	1600 ± 140	NA[b]	**3300 ± 650**	2400 ± 13	5300	NA[b]	**1300 ± 72**	4100 ± 470	NB[c]	4800
21a	2200 ± 138	NA[b]	**1300 ± 130**	1700 ± 60	2600 ± 230	NA[b]	**400 ± 34**	3000 ± 380	NB[c]	NA[b]
21b	840 ± 20	NA[b]	**680 ± 37**	NA[b]	NA[b]	NA[b]	**690 ± 53**	1500 ± 180	NA[b]	NA[b]
21c	NB[c]	NA[b]	**1200 ± 287**	3200	2500	NA[b]	**180 ± 19**	3000 ± 300	NB[c]	NB[c]
22a	1300 ± 130	1400 ± 24	**690 ± 30**	1400 ± 30	1700 ± 150	NB[c]	**48 ± 4**	4000 ± 500	1400 ± 57	NB[c]
22b	1100 ± 87	NA[b]	**770 ± 129**	3600	2500 ± 300	NA[b]	**810 ± 54**	3000 ± 30	NB[c]	NB[c]
22c	NB[c]	990 ± 170	**550 ± 32**	1400 ± 230	1900 ± 400	NB[c]	**46 ± 4**	NA[b]	NB[c]	2500
24	NB[c]	NB[c]	**1700 ± 40**	1300 ± 12	3700 ± 250	NA[b]	**230 ± 16**	NB[c]	1000 ± 51	NB[c]
25	3600	1100 ± 200	**590 ± 78**	NB[c]	2800 ± 770	NB[c]	**48 ± 6**	4700 ± 22	1800 ± 160	NB[c]

[a]Results represent the mean ± SEM of n = 4 to 8. [b]NA, Not available. [c]NB, Non-binding up to the highest tested concentration of 1500 μM.

4.3 Structural analysis

We then obtained the X-ray structures of galectin-8N-**22a** and galectin-8N-**25** complexes at 1.52 Å and 2.1 Å resolutions, respectively, to investigate the reason for the high affinity of the D-galactal derivatives for galectin-8N. The crystal structure of galectin-8N-**25** complex is discussed in detail in paper II, and we will focus on the crystal structure of galectin-8N-**22a** complex. The D-galactal ring is placed in the same subsite and establishes an identical hydrogen bonding network as the D-galactose ring discussed in Chapter 3. The benzimidazole moiety is placed in a favorable position to establish a cation-π stacking with Arg45, while the electron-rich nitrogen of the benzimidazole engages in a dipole-dipole interaction with Gln 47 and Arg59. The carboxylate moiety of the benzimidazole establishes a water-mediated hydrogen bond with Gly142. The crystal structure did not clarify the reason for the superiority of the D-galactal derivatives over the D-galactose derivatives. Notably, the endocyclic double bond of the D-galactal ring is placed within 3.5 Å to the electropositive side chain of Arg45. Which is why we speculated

that the double bond might account for the higher affinity of the compound for galectin-8N through a cation-π interaction with Arg45. To that end, we first performed a 300 ns MD simulation on the galectin-8N-**22a** complex, then selected a representative snapshot at 265 ns and calculated the single-point molecular orbitals between **22a** and the neighboring amino acid side chains with Jaguar. This showed that the LUMO of Arg45 interacts with the HOMO of O4 and the HOMO of the endocyclic double bond in the D-galactal ring. This orbital overlap may contribute to the high affinity of the D-galactal derivatives for galectin-8N (Figure 13). These interactions may be less productive in the other galectins accounting for the high selectivity of the D-galactal derivatives for galectin-8N.

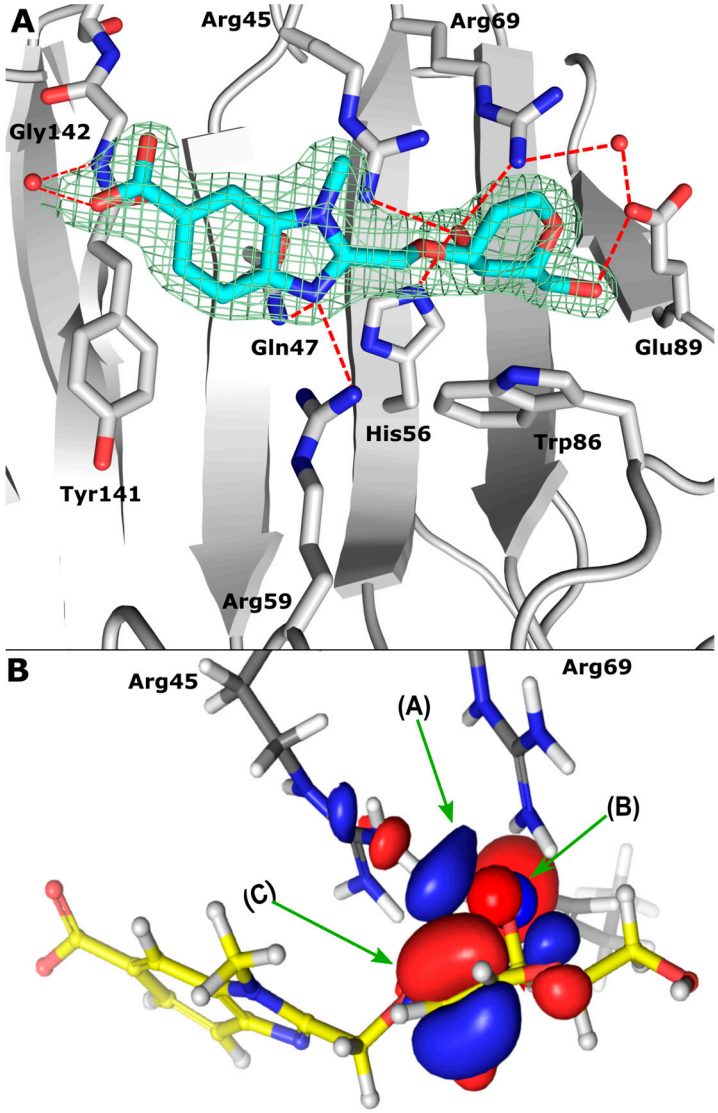


Figure 13: (A) Electron densities $2|F_o| - |F_c|\alpha_c$ contoured at 1σ for compound **22a** (cyan sticks) in complex with galectin-8N (grey cartoon representation) at 1.52 Å (PDB ID: 7P1M). (B) Quantum mechanical calculations on a representative MD snapshot (265 ns) of the galectin-8N–**22a** complex (yellow sticks) using Jaguar (Schrodinger suite). The calculations revealed that the LUMO of Arg45 of galectin-8N (A, depicted in blue) interacts with the HOMO of O4 (B, depicted in red) and the HOMO of the endocyclic double bond (C, depicted in red) of the D-galactal ring.

4.4 Biological evaluation

We then evaluated the cytotoxicity of compounds **19a** (from chapter 3), **22a**, and **22c** via a (3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay in K562, and MDA-MB-231 cancer cell lines, as well as human peripheral blood mononuclear cells (PBMCs), and none of the compounds decreased the viability of any of the cells at concentrations ranging from 1 µM to 100 µM. As mentioned in Chapter 1, galectin-8 was reported to increase the secretion of proinflammatory cytokines in cancer cells. Therefore, we assessed compounds **19a** and **22a** for their effect on the secretion of the proinflammatory cytokines in triple-negative breast cancer cells MDA-MB-231. The result showed that both compounds reduced the secretion of interleukin-6 (IL-6) and IL-8 in MDA-MB-231 cells in a dose-dependent manner at concentrations of 10 and 100 µM (Figure 14). This effect is of utmost importance as IL-6 protects MDA-MB-231 cells from the cytotoxicity and apoptosis induced by chemotherapeutic agents, while IL-8 promotes the migration and metastasis of MDA-MB-231 cells.

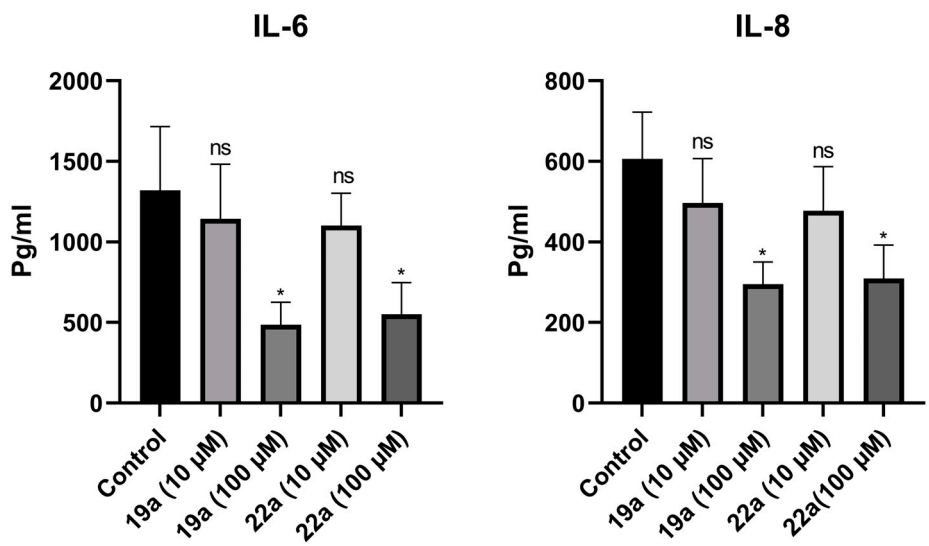


Figure 14: Effect of compounds **19a** and **22a** on the secretion of IL-6 and IL-8 expressed in pg/mL. The effects were measured after incubating the cells with the compounds at 10 µM and 100 µM concentrations. Compounds **19a** and **22a** reduce the secretion of IL-6 and IL-8 in a dose-dependent manner at concentrations of 10 µM and 100 µM concentrations. The results shown are means ± SEM of three independent experiments. ns, not significant ($p > 0.05$); * $p < 0.05$ versus untreated controls.

4.5 Conclusion

Based on our observation that D-galactal **20** binds galectin-8N with a higher affinity than methyl β-D-galactopyranoside **14**, we designed and synthesized a set of C-3 substituted quinoline and benzimidazole-D-galactal derivatives. This has led to the discovery of the D-galactal-benzimidazole hybrid **22a** a K_d of 48 μM for galectin-8N and 15-fold selectivity over galectin-3, and higher selectivity over the other human galectins. We then obtained the X-ray crystal structure of galectin-8N-**22a** complex at 1.52 Å resolution. Performing MD simulation on the obtained crystal structure followed by quantum mechanical calculations revealed an orbital overlap between the LUMO of Arg45 with the electron-rich HOMOs of the olefin and O4 of the D-galactal. This overlap might contribute to the high affinity of compound **22a** for galectin-8N. Compounds **19a** and **22a** showed no cytotoxicity for healthy human cells and selected cancer cell lines. A functional assay revealed that compounds **19a** and **22a** reduced the secretion of the proinflammatory cytokines IL-6 and IL-8 in MDA-MB-231 cells in a dose-dependent manner. It is worth mentioning that compound **22a** was the most selective synthetic galectin-8N inhibitor at that time.

5 Targeting an unexploited binding pocket in the galectin-8 N-terminal domain (paper III)

5.1 Background

In this work, we aimed to improve the binding affinity of the D-galactal-benzimidazole hybrids discussed in Chapter 4 for galectin-8N. We first investigated the effect of attaching an aromatic moiety at N1 of the benzimidazole of the D-galactal-benzimidazole hybrid, but this did not improve the binding affinity for galectin-8N, and the result is discussed in paper III. Hence, we shifted our attention to targeting an unexploited binding pocket in galectin-8N to improve the binding affinity of the D-galactal-benzimidazole hybrids. From the X-ray crystal structure of galectin-8N-**22a** complex, we observed that C4 of the benzimidazole is facing a hydrophobic pocket surrounded by Tyr141 and Arg59.[103] Thus, we hypothesized that attaching an aromatic ring at C4 of the benzimidazole might improve the binding affinity for galectin-8N (Figure 15). Accordingly, a set of D-galactal-benzimidazole hybrids were synthesized, and their SAR is discussed in the paper. This chapter will be focused on the best-performing compound in the series, compound **30,** carrying a *p*-chlorophenyl substituent at C4 of the benzimidazole.

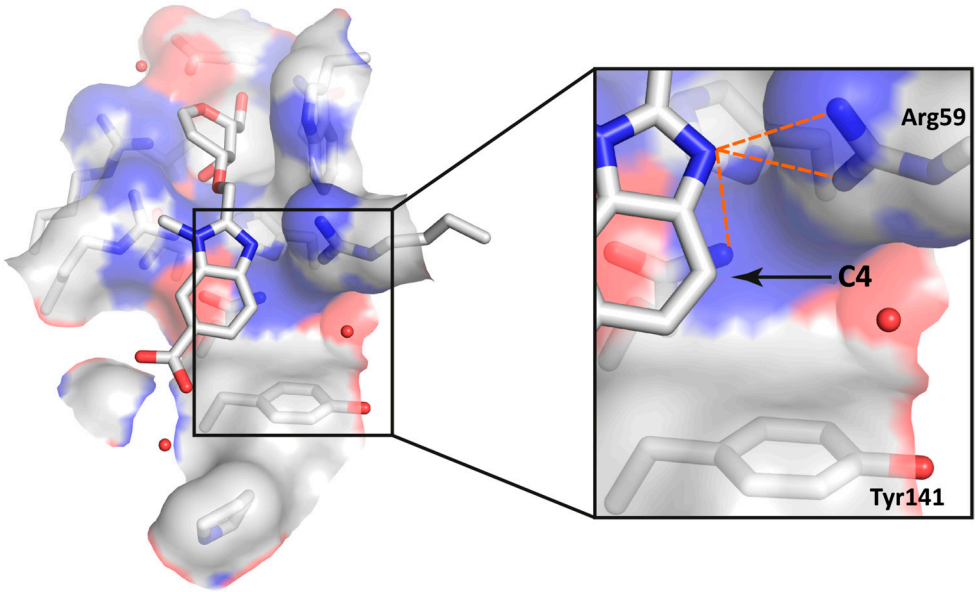
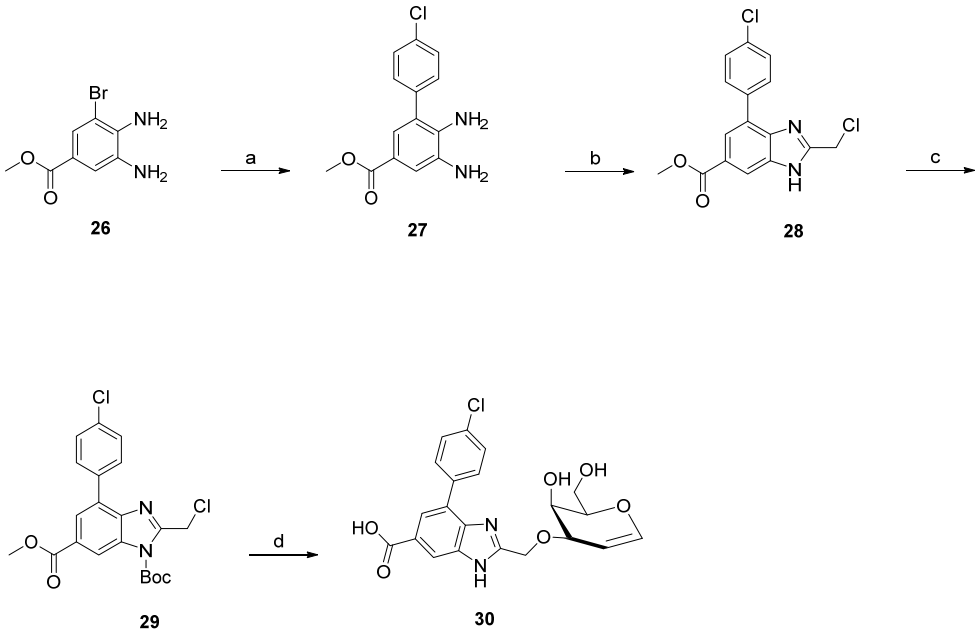


Figure 15: The x-ray crystal structure of galectin-8N-**22a** complex showing that C4 of the benzimidazole faces an unexploited binding pocket surrounded by Arg59 and Tyr141 (PDB code: 7P1M).

5.2 Synthesis and evaluation

The synthesis of compound **30** started with Suzuki-Miyaura coupling of 4-chlorophenylboronic acid with the bromophenylene diamine **26** to afford compound **27,** which was converted to the benzimidazole **28** by condensation with 2-chloro-1,1,1-trimethoxyethane, followed by Boc protection to afford compound **29**. Compound **29** was then coupled to D-galactal via the stannylene-mediated regioselective O-3 alkylation to afford the methyl ester, which was used without purification in the subsequent alkaline hydrolysis to afford compound **30** (Scheme 4).



Scheme 4: Synthesis of compound **30**. (a) 4-chlorophenylboronic acid, K_2CO_3, $Pd(dppf)Cl_2$, 1,4-dioxane: H_2O (2:1), MW, 120 °C, 30 mins (81%). (b) 2-chloro-1,1,1-trimethoxyethane, $BF_3.Et_2O$, DCM, rt, overnight (94%). (c) Boc_2O, DMAP, DMF, rt, 2h (91%). (d) i. D-Galactal **20**, Bu_2SnO, MeOH, 70 °C, 2h; ii. N-Bu_4NBr, 1,4-dioxane, 85 °C, overnight; iii. KOH, EtOH, H_2O, 80 °C, overnight (24%).

Evaluation of the binding affinity of compound **30** for galectin-8N in a fluorescence polarization assay showed that the *p*-chlorophenyl moiety resulted in about 16-fold increase in the binding affinity for galectin-8N compared to **22a** with a K_d of 2.9 µM. Moreover, compound **30** exhibited 50-fold selectivity over galectin-3 and even higher selectivity over the other human galectins, making it the most potent selective galectin-8N inhibitor to date (Table 4).

Table 4: K_d values of compound **30** (µM)[a]

	Galectin-X (N – N-terminal domain, C – C-terminal domain)							
Compound	1	**3**	4N	4C	**8N**	8C	9N	9C
30	NB[b]	**150 ± 17**	560 ± 29	NB[c]	**2.9 ± 0.4**	2000	1850	NB[a]

[a] Results represent the mean ± SEM of n= 4 to 8. [b]NB, Non-binding up to the highest tested concentration of 1500 µM. Compound **30** represents the most potent selective galectin-8N inhibitor to date.

5.3 Structural analysis

We then obtained the X-ray crystal structure of galectin-8N-**30** complex at 1.3 Å resolution. This showed that the D-galactal moiety of compound **30** identically binds galectin-8N as previously seen with compound **22a**. However, the benzimidazole moiety has moved toward Arg59 and possibly no longer establishes a cation-π stacking with Arg45. Expectedly, the *p*-chlorophenyl at C4 of the benzimidazole is sandwiched between Arg59 and Tyr141. Thus, the increase in the binding affinity caused by the attachment of the *p*-chlorophenyl moiety is likely due to π-π stacking with Tyr141 and/or cation-π stacking with Arg59 (Figure 16). This crystal structure and the observed SAR discussed in the manuscript confirm that targeting this pocket is a valid strategy to improve the affinity for galectin-8N, thus confirming our hypothesis.

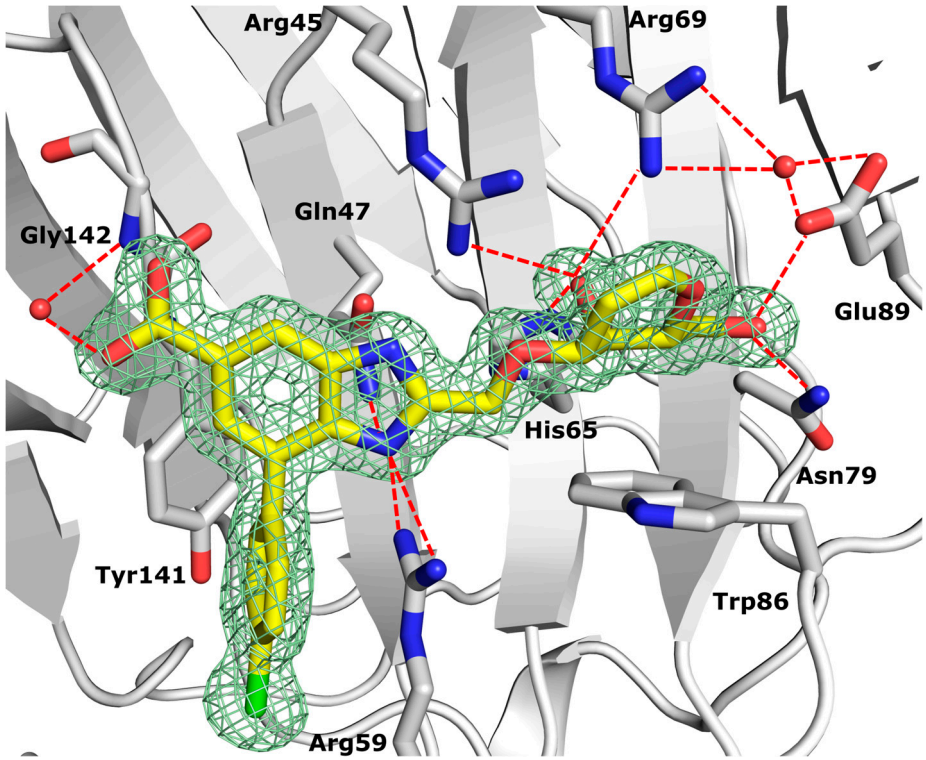


Figure 16: Electron densities $2|F_0| - |F_c|\alpha_c$ contoured at 1σ for compound **30** (yellow sticks) in complex with galectin-8N (grey cartoon representation) (1.3 Å).

5.4 Conclusion

We have designed and synthesized a set of D-galactal-benzimidazole hybrids functionalized at C4 of the benzimidazole with aromatic moieties to explore a yet unexploited binding pocket in galectin-8N. This has led to the discovery of compound **30**, featuring a *p*-chlorophenyl substituent at C4 of the benzimidazole, which represents the most potent selective galectin-8N inhibitor to date. We then obtained the crystal structure of the galectin-8N-**30** complex at a resolution of 1.3 Å. The crystal structure revealed that the high affinity of compound **30** for galectin-8N is probably due to the interaction of the *p*-chlorophenyl moiety with Arg59 and/or Tyr141 via cation-π stacking and/or π-π stacking, respectively. Thus compound **30** represents a promising lead compound for the design and synthesis of galectin-8N inhibitors with higher affinity and selectivity.

6 Targeting human galectins with PROTACs (paper IV)

6.1 Background

In this project, we aimed to investigate whether galectins are amenable to targeted protein degradation by PROTACs. Therefore, we designed, synthesized, and evaluated disaccharide-based PROTACs targeting human galectins. However, a patent was published while working on the project showing that monosaccharide-based PROTACs induced galectin-3 degradation in THP-1 cell line.[104] Our disaccharide PROTACs were designed based on compound **5** (GB0139) mentioned in chapter 1, with the thiodiogalactoside carrying a single trifluorinated 3-(4-aryl-1,2,3-triazol-1-yl) moiety on one galactose moiety, while the other galactose moiety was used to attach the E3 ligase ligands (Figure 17). We used a von Hippel–Lindau (VHL) E3 ligase ligand in our design because the selected cancer cell lines co-expresses galectin-3 and VHL E3 ligase.[70,105]

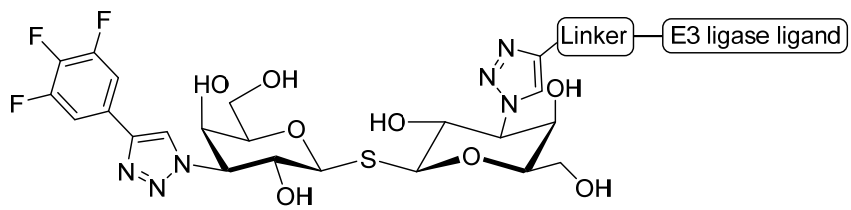
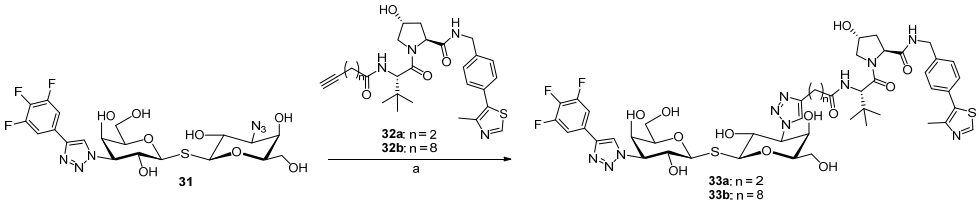


Figure 17: Design of the galectin-targeting disaccharide PROTACs.

6.2 Synthesis and affinity evaluation

The synthesis of the azide **31** is described in the literature, [83] and the synthesis of the alkynes **32a** and **32b** is described in the manuscript. Copper-catalyzed azide-alkyne cycloaddition afforded the disaccharide PROTACs **33a** and **33b** (Scheme 5).



Scheme 5: Synthesis of compounds **33a** and **33b**. (a): CuI, DIPEA, DMF, 50 ºC, overnight (16-36%)

The binding affinities of compounds **33a** and **33b** for human galectin-3 were evaluated in a fluorescence polarization assay. Both compounds displayed lower affinities for galectin-3 than compound **5** (K_d = 2 nM), with K_d values of 9 nM and 57 nM for **33a** and **33b**, respectively (Table 5).

Table 5*:* K_d values of compounds **33a** and **33b** (nM)[a].

Compound	Galectin-3
33a	9 ± 2
33b	57 ± 6

[a] Results represent the mean ± SEM of n= 4 to 8.

6.3 Evaluation of galectin-3 degradation

The ability of compounds **33a** and **33b** to induce galectin-3 degradation was evaluated in JIMT-1 and MDA-MB-231 breast cancer cell lines. Galectin-3 degradation was assessed with Western blotting at **33a** and **33b** concentration of 25 µM for 24 and 48 hours. Both compounds failed to induce galectin-3 degradation at the tested conditions. The failure of the compounds to induce galectin-3 degradation can be due to the low cellular permeability of the compounds due to their high topological polar surface area (TPSA). Another possible reason for the lack of effect is the hook effect due to the saturation of galectin-3 and the E3 ligase caused by the high concentration used in the assay. Therefore, further investigation is needed to determine the reason for the failure of the PROTACs to induce galectin-3 degradation.

6.4 Conclusion

We have designed and synthesized the galectin-targeting PROTACs **33a** and **33b**. Both compounds displayed nanomolar binding affinities for galectin-3 as determined by fluorescence polarization assay. However, both compounds failed to induce galectin-3 degradation in JIMT-1 and MDA-MB-231 breast cancer cell lines. Thus, the reason behind the lack of activity of the PROTACs should be investigated.

7 Concluding remarks and future perspectives

The first objective of the thesis was to discover selective inhibitors for human galectin-8 as potential antitumor and anti-inflammatory agents. Therefore, we obtained the crystal structure of galectin-8N in complex with the previously published quinoline-galactoside **9**. The structure-based design of galectin-8N inhibitors based on the crystal structure has led to the discovery of the benzimidazole-galactoside **16a** with a K_d of 190 μM for galectin-8N and 7-fold selectivity over galectin-3. Introducing the α-3,4-dichlorothiophenyl moiety to the benzimidazole-galactosides increased the binding affinity for galectin-8N by about two orders of magnitude, resulting in compound **19a** with a K_d of 1.8 μM for galectin-8N and 3-fold selectivity over galectin-3, and higher selectivity over the other human galectins. MD simulations showed that the selectivity of the benzimidazole-galactosides is likely due to binding the non-conserved Gln47 in galectin-8N. The subconjunctival injection of compound **19a** reduced the severity of the PA keratitis in a mouse model. This *in vivo* study provided the first proof of concept that galectin-8 inhibitors can be effective in an actual disease model.

The synthesis of O-3 substituted D-galactal derivatives resulted in the discovery of the D-galactal-benzimidazole hybrid **22a** with a K_d of 48 μM for galectin-8N and 15-fold selectivity over galectin-3 and higher selectivity over the other human galectins. We then obtained the X-ray crystal structure of galectin-8N-**22a** complex at 1.52 Å resolution. Performing MD simulation on the crystal structure followed by quantum mechanical calculations showed that the high affinity of the compound for galectin-8N can be attributed to the orbital overlap between the LUMO of Arg45 with electron-rich HOMOs of the olefin and O4 of the D-galactal. The D-galactal-benzimidazole hybrid **22a** and the parent benzimidazole galactoside **19a** reduced the secretion of the proinflammatory IL-6 and IL-8 in the triple-negative breast cancer cells MDA-MB-231. Attachment of a *p*-chlorophenyl moiety at C4 of the benzimidazole of the D-galactal-benzimidazole hybrid resulted in the discovery of compound **30** that represents the most potent selective galectin-8N inhibitor to date with a K_d of 2.9 μM for galectin-8N and 50-fold selectivity over galectin-3 and even higher selectivity over the other human galectins. X-ray structural analysis showed that the increase in the binding affinity caused by the *p*-chlorophenyl moiety is likely due to π-π stacking with Tyr141 and/or cation-π stacking with Arg59 in galectin-

8N. Thus, compound **30** represents a promising starting point for developing ligands that bind galectin-8N with higher affinity and selectivity.

In the future, analogues of compound **30** bearing different aromatic moieties can be synthesized to explore the SAR of the binding pocket. Benzimidazole-galactosides bearing aromatic groups at C4 of the benzimidazole can also be synthesized to investigate if the effect seen on D-galactal derivatives can be translated to galactosides as well. A modular synthesis of the benzimidazole-galactosides and/or D-galactal hybrids should be developed to accelerate the synthesis of the analogues. Furthermore, the mechanism of the reduction of the proinflammatory cytokines should be confirmed. The best-performing compounds should also be evaluated for their pharmacokinetic properties and their activity in an animal model.

The last part of the thesis aimed to investigate whether galectins are amenable to targeted protein degradation by PROTACs. We synthesized the galectin-targeted PROTACs **33a** and **33b** bearing the VHL E3 ligase ligand. Evaluation of the binding affinity of the galectin-targeted PROTACs in a fluorescence polarization assay showed that both compounds possess nanomolar affinities galectin-3. However, none of the compounds did induce galectin-3 degradation in JIMT-1 and MDA-MB-231 breast cancer cell lines.

In the future, the reason for the failure of the PROTACs to induce galectin-3 degradation should be investigated. The binding affinities of compounds **33a** and **33b** for the other human galectins as well as their ability to induce their degradation should be evaluated. An important future investigation is to synthesize monosaccharide-based galectin-targeting PROTACs with presumably improved cell permeability and to evaluate them for their ability to induce targeted degradation of the galectins.

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References

(1) Roseman, S. Reflections on Glycobiology. *J. Biol. Chem.* **2001**, *276* (45), 41527–41542.

(2) Kaltner, H.; Abad-Rodríguez, J.; Corfield, A. P.; Kopitz, J.; Gabius, H.-J. The Sugar Code: Letters and Vocabulary, Writers, Editors and Readers and Biosignificance of Functional Glycan–Lectin Pairing. *Biochem. J.* **2019**, *476* (18), 2623–2655.

(3) Ouellette, R. J.; Rawn, J. D. Carbohydrates. In *Principles of Organic Chemistry*; Ouellette, R. J., Rawn, J. D., Eds.; Elsevier: Boston, 2015; pp 343–370.

(4) Laine, R. A. The Information-Storing Potential of the Sugar Code. In *Glycosciences*; Gabius, H.-J., Gabius, S., Eds.; Wiley-VCH Verlag GmbH: Weinheim, Germany, 1996; pp 1–14.

(5) Gabius, H.-J. Glycans: Bioactive Signals Decoded by Lectins. *Biochem. Soc. Trans.* **2008**, *36* (6), 1491–1496.

(6) Gabius, H.-J. The Sugar Code: Why Glycans Are so Important. *Biosystems* **2018**, *164*, 102–111.

(7) Gabius, H.-J.; Roth, J. An Introduction to the Sugar Code. *Histochem. Cell Biol.* **2017**, *147* (2), 111–117.

(8) Trouvelot, S.; Héloir, M.-C.; Poinssot, B.; Gauthier, A.; Paris, F.; Guillier, C.; Combier, M.; Trdá, L.; Daire, X.; Adrian, M. Carbohydrates in Plant Immunity and Plant Protection: Roles and Potential Application as Foliar Sprays. *Front. Plant Sci.* **2014**, *5*.

(9) VanTeeffelen, J. W.; Brands, J.; Stroes, E. S.; Vink, H. Endothelial Glycocalyx: Sweet Shield of Blood VesselsB. *Trends Cardiovasc. Med.* **2007**, *17* (3), 5.

(10) Nieuwdorp, M.; Meuwese, M. C.; Mooij, H. L.; Ince, C.; Broekhuizen, L. N.; Kastelein, J. J. P.; Stroes, E. S. G.; Vink, H. Measuring Endothelial Glycocalyx Dimensions in Humans: A Potential Novel Tool to Monitor Vascular Vulnerability. *J. Appl. Physiol.* **2008**, *104* (3), 845–852.

(11) Möckl, L. The Emerging Role of the Mammalian Glycocalyx in Functional Membrane Organization and Immune System Regulation. *Front. Cell Dev. Biol.* **2020**, *8*, 253.

(12) Daniels, G.; Reid, M. Blood Groups: The Past 50 Years. *Transfusion*. **2010**, *50*, 281–289.

(13) Linhardt, R. J. 2003 Claude S. Hudson Award Address in Carbohydrate Chemistry. Heparin: Structure and Activity. *J. Med. Chem.* **2003**, *46* (13), 2551–2564.

(14) Krause, K. M.; Serio, A. W.; Kane, T. R.; Connolly, L. E. Aminoglycosides: An Overview. *Cold Spring Harb. Perspect. Med.* **2016**, *6* (6), a027029.

(15) Nilsson, J.; Halim, A.; Grahn, A.; Larson, G. Targeting the Glycoproteome. *Glycoconj. J.* **2013**, *30* (2), 119–136.

(16) Everest-Dass, A. V.; Moh, E. S. X.; Ashwood, C.; Shathili, A. M. M.; Packer, N. H. Human Disease Glycomics: Technology Advances Enabling Protein Glycosylation Analysis – Part 1. *Expert Rev. Proteomics* **2018**, *15* (2), 165–182.

(17) Kailemia, M. J.; Xu, G.; Wong, M.; Li, Q.; Goonatilleke, E.; Leon, F.; Lebrilla, C. B. Recent Advances in the Mass Spectrometry Methods for Glycomics and Cancer. *Anal. Chem.* **2018**, *90* (1), 208–224.

(18) Agirre, J.; Davies, G. J.; Wilson, K. S.; Cowtan, K. D. Carbohydrate Structure: The Rocky Road to Automation. *Curr. Opin. Struct. Biol.* **2017**, *44*, 39–47.

(19) Sirohi, D.; Chen, Z.; Sun, L.; Klose, T.; Pierson, T. C.; Rossmann, M. G.; Kuhn, R. J. The 3.8 Å Resolution Cryo-EM Structure of Zika Virus. *Science* **2016**, *352* (6284), 467–470.

(20) Krasnova, L.; Wong, C.-H. Oligosaccharide Synthesis and Translational Innovation. *J. Am. Chem. Soc.* **2019**, *141* (9), 3735–3754.

(21) Puvirajesinghe, T.; Turnbull, Jeremy. Glycoarray Technologies: Deciphering Interactions from Proteins to Live Cell Responses. *Microarrays* **2016**, *5* (1), 3.

(22) Bauer, K. A.; Hawkins, D. W.; Peters, P. C.; Petitou, M.; Herbert, J.-M.; Boeckel, C. A. A.; Meuleman, D. G. Fondaparinux, a Synthetic Pentasaccharide: The First in a New Class of Antithrombotic Agents - The Selective Factor Xa Inhibitors. *Cardiovasc. Drug Rev.* **2006**, *20* (1), 37–52.

(23) Unione, L.; Gimeno, A.; Valverde, P.; Calloni, I.; Coelho, H.; Mirabella, S.; Poveda, A.; Arda, A.; Jimenez-Barbero, J. Glycans in Infectious Diseases. A Molecular Recognition Perspective. *Curr. Med. Chem.* **2017**, *24* (36).

(24) Kataev, V. E.; Garifullin, B. F. Antiviral Nucleoside Analogs. *Chem. Heterocycl. Compd.* **2021**, *57* (4), 326–341.

(25) Xu, B.; Li, S.; Kang, B.; Zhou, J. The Current Role of Sodium-Glucose Cotransporter 2 Inhibitors in Type 2 Diabetes Mellitus Management. *Cardiovasc. Diabetol.* **2022**, *21* (1), 83.

(26) Deeks, E. D. Ticagrelor: A Review of Its Use in the Management of Acute Coronary Syndromes. *Drugs* **2011**, *71* (7), 909–933.

(27) Martínez-Monteagudo, S. I.; Enteshari, M.; Metzger, L. Lactitol: Production, Properties, and Applications. *Trends Food Sci. Technol.* **2019**, *83*, 181–191.

(28) Syed, Y. Y. Sodium Oligomannate: First Approval. *Drugs* **2020**, *80* (4), 441–444.

(29) Schönemann, W.; Cramer, J.; Mühlethaler, T.; Fiege, B.; Silbermann, M.; Rabbani, S.; Dätwyler, P.; Zihlmann, P.; Jakob, R. P.; Sager, C. P.; Smieško, M.; Schwardt, O.; Maier, T.; Ernst, B. Improvement of Aglycone Π-Stacking Yields Nanomolar to Sub-nanomolar FimH Antagonists. *ChemMedChem* **2019**, *14* (7), 749–757.

(30) Chevalier, G.; Laveissière, A.; Desachy, G.; Barnich, N.; Sivignon, A.; Maresca, M.; Nicoletti, C.; Di Pasquale, E.; Martinez-Medina, M.; Simpson, K. W.; Yajnik, V.; Sokol, H.; Adegbamigbe, T.; Ahmad, T.; Arnott, I.; Bouhnik, Y.; Carbonnel, F.; Colombel, J.-F.; Doherty, G.; Cummings, J. R. F.; Hébuterne, X.; Herfarth, H.; Kevans, D.; de Chambrun, G. P.; Nachury, M.; Nancey, S.; Roblin, X.; Tremelling, M. A. W.; Plassais, J.; Strozzi, F.; Cervino, A.; Morra, R.; Bonny, C. Blockage of Bacterial FimH Prevents Mucosal Inflammation Associated with Crohn's Disease. *Microbiome* **2021**, *9* (1), 176.

(31) Reinisch, W.; Hébuterne, X.; Buisson, A.; Schreiber, S.; Desreumaux, P.; Primas, C.; Paillarse, J.; Chevalier, G.; Bonny, C. Safety, Pharmacokinetic, and Pharmacodynamic Study of Sibofimloc, a Novel FimH Blocker in Patients with Active Crohn's Disease. *J. Gastroenterol. Hepatol.* **2022**, *37* (5), 832–840.

(32) Bozzola, T.; Scalise, M.; Larsson, C. U.; Newton-Vesty, M. C.; Rovegno, C.; Mitra, A.; Cramer, J.; Wahlgren, W. Y.; Radhakrishnan Santhakumari, P.; Johnsson, R. E.; Schwardt, O.; Ernst, B.; Friemann, R.; Dobson, R. C. J.; Indiveri, C.; Schelin, J.; Nilsson, U. J.; Ellervik, U. Sialic Acid Derivatives Inhibit SiaT Transporters and Delay Bacterial Growth. *ACS Chem. Biol.* **2022**, *17* (7), 1890–1900.

(33) Ordanini, S.; Varga, N.; Porkolab, V.; Thépaut, M.; Belvisi, L.; Bertaglia, A.; Palmioli, A.; Berzi, A.; Trabattoni, D.; Clerici, M.; Fieschi, F.; Bernardi, A. Designing Nanomolar Antagonists of DC-SIGN-Mediated HIV Infection: Ligand Presentation Using Molecular Rods. *Chem. Commun.* **2015**, *51* (18), 3816–3819.

(34) Lenza, M. P.; Atxabal, U.; Oyenarte, I.; Jiménez-Barbero, J.; Ereño-Orbea, J. Current Status on Therapeutic Molecules Targeting Siglec Receptors. *Cells* **2020**, *9* (12), 2691.

(35) Reinisch, W.; Hébuterne, X.; Buisson, A.; Schreiber, S.; Desreumaux, P.; Paillarse, J. M.; Bonny, C. P568 An Open-Label, Multicenter, Phase Ib, Pharmacokinetic (Pk) and Safety Study of a Fimh Blocker, Sibofimloc (TAK-018/EB8018), in Patients with Crohn's Disease (CD). *J. Crohns Colitis* **2020**, *14*, S479–S480.

(36) Barondes, S. H.; Castronovo, V.; Cooper, D. N. W.; Cummings, R. D.; Drickamer, K.; Felzi, T.; Gitt, M. A.; Hirabayashi, J.; Hughes, C.; Kasai, K.; Leffler, H.; Liu, F.-T.; Lotan, R.; Mercurio, A. M.; Monsigny, M.; Pillai, S.; Poirer, F.; Raz, A.; Rigby, P. W. J.; Rini, J. M.; Wang, J. L. Galectins: A Family of Animal β-Galactoside-Binding Lectins. *Cell* **1994**, *76* (4), 597–598.

(37) Leffler, H.; Carlsson, S.; Hedlund, M.; Qian, Y.; Poirier, F. Introduction to Galectins. *Glycoconj. J.* **2002**, *19*, 433–440.

(38) Kaminker, J. D.; Timoshenko, A. V. Expression, Regulation, and Functions of the Galectin-16 Gene in Human Cells and Tissues. *Biomolecules* **2021**, *11* (12), 1909.

(39) Su, J.; Wang, Y.; Si, Y.; Gao, J.; Song, C.; Cui, L.; Wu, R.; Tai, G.; Zhou, Y. Galectin-13, a Different Prototype Galectin, Does Not Bind β-Galacto-Sides and Forms Dimers via Intermolecular Disulfide Bridges between Cys-136 and Cys-138. *Sci. Rep.* **2018**, *8* (1), 980. https://doi.org/10.1038/s41598-018-19465-0.

(40) Si, Y.; Yao, Y.; Jaramillo Ayala, G.; Li, X.; Han, Q.; Zhang, W.; Xu, X.; Tai, G.; Mayo, K. H.; Zhou, Y.; Su, J. Human Galectin-16 Has a Pseudo Ligand Binding Site and Plays a Role in Regulating c-Rel-Mediated Lymphocyte Activity. *Biochim. Biophys. Acta BBA - Gen. Subj.* **2021**, *1865* (1), 129755.

(41) Su, J.; Gao, J.; Si, Y.; Cui, L.; Song, C.; Wang, Y.; Wu, R.; Tai, G.; Zhou, Y. Galectin-10: A New Structural Type of Prototype Galectin Dimer and Effects on Saccharide Ligand Binding. *Glycobiology* **2018**, *28* (3), 159–168.

(42) Cooper, D. Galectinomics: Finding Themes in Complexity. *Biochim. Biophys. Acta Gen. Subj.* **2002**, *1572* (2–3), 209–231.

(43) Hutter, S.; Martin, N.; von Schönfeldt, V.; Messner, J.; Kuhn, C.; Hofmann, S.; Andergassen, U.; Knabl, J.; Jeschke, U. Galectin 2 (Gal-2) Expression Is Downregulated on Protein and MRNA Level in Placentas of Preeclamptic (PE) Patients. *Placenta* **2015**, *36* (4), 438–445.

(44) Froehlich, R.; Hambruch, N.; Haeger, J.-D.; Dilly, M.; Kaltner, H.; Gabius, H.-J.; Pfarrer, C. Galectin Fingerprinting Detects Differences in Expression Profiles between Bovine Endometrium and Placentomes as Well as Early and Late Gestational Stages. *Placenta* **2012**, *33* (3), 195–201.

(45) Magnaldo, T.; Fowlis, D.; Darmon, M. Galectin-7, a Marker of All Types of Stratified Epithelia. *Differentiation* **1998**, *63* (3), 159–168.

(46) Hadari, Y. R.; Paz, K.; Dekel, R.; Mestrovic, T.; Accili, D.; Zick, Y. Galectin-8: A New Rat Lectin, Related to Galectin-4. *J. Biol. Chem.* **1995**, *270* (7), 3447–3453.

(47) Cummings, R. D.; Liu, F.-T.; Rabinovich, G. A.; Stowell, S. R.; Vasta, G. R. Galectins. In *Essentials of Glycobiology*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2015; pp 469–480.

(48) Boscher, C.; Dennis, J. W.; Nabi, I. R. Glycosylation, Galectins and Cellular Signaling. *Curr. Opin. Cell Biol.* **2011**, *23* (4), 383–392.

(49) Lajoie, P.; Goetz, J. G.; Dennis, J. W.; Nabi, I. R. Lattices, Rafts, and Scaffolds: Domain Regulation of Receptor Signaling at the Plasma Membrane. *J. Cell Biol.* **2009**, *185* (3), 381–385.

(50) Nabi, I. R.; Shankar, J.; Dennis, J. W. The Galectin Lattice at a Glance. *J. Cell Sci.* **2015**, *128* (13), 2213–2219.

(51) Vinik, Y.; Shatz-Azoulay, H.; Zick, Y. Molecular Mechanisms Underlying the Role of Galectin-8 as a Regulator of Cancer Growth and Metastasis. *Trends Glycosci. Glycotechnol.* **2018**, *30* (172), SE119–SE128.

(52) Chen, W.-S.; Cao, Z.; Sugaya, S.; Lopez, M. J.; Sendra, V. G.; Laver, N.; Leffler, H.; Nilsson, U. J.; Fu, J.; Song, J.; Xia, L.; Hamrah, P.; Panjwani, N. Pathological Lymphangiogenesis Is Modulated by Galectin-8-Dependent Crosstalk between Podoplanin and Integrin-Associated VEGFR-3. *Nat. Commun.* **2016**, *7* (1), 11302.

(53) Tribulatti, M. V.; Carabelli, J.; Prato, C. A.; Campetella, O. Galectin-8 in the Onset of the Immune Response and Inflammation. *Glycobiology* **2020**, *30* (3), 134–142.

(54) Sampson, J. F.; Suryawanshi, A.; Chen, W.; Rabinovich, G. A.; Panjwani, N. Galectin-8 Promotes Regulatory T-cell Differentiation by Modulating IL-2 and TGFβ Signaling. *Immunol. Cell Biol.* **2016**, *94* (2), 213–219.

(55) Vinik, Y.; Shatz-Azoulay, H.; Hiram-Bab, S.; Kandel, L.; Gabet, Y.; Rivkin, G.; Zick, Y. Ablation of the Mammalian Lectin Galectin-8 Induces Bone Defects in Mice. *FASEB J.* **2018**, *32* (5), 2366–2380.

(56) Vinik, Y.; Shatz-Azoulay, H.; Vivanti, A.; Hever, N.; Levy, Y.; Karmona, R.; Brumfeld, V.; Baraghithy, S.; Attar-Lamdar, M.; Boura-Halfon, S.; Bab, I.; Zick, Y. The Mammalian Lectin Galectin-8 Induces RANKL Expression, Osteoclastogenesis, and Bone Mass Reduction in Mice. *eLife* **2015**, *4*, e05914.

(57) Ferragut, F.; Cagnoni, A. J.; Colombo, L. L.; Sánchez Terrero, C.; Wolfenstein-Todel, C.; Troncoso, M. F.; Vanzulli, S. I.; Rabinovich, G. A.; Mariño, K. V.; Elola, M. T. Dual Knockdown of Galectin-8 and Its Glycosylated Ligand, the Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166), Synergistically Delays in Vivo Breast Cancer Growth. *Biochim. Biophys. Acta Mol. Cell Res.* **2019**, *1866* (8), 1338–1352.

(58) Shatz-Azoulay, H.; Vinik, Y.; Isaac, R.; Kohler, U.; Lev, S.; Zick, Y. The Animal Lectin Galectin-8 Promotes Cytokine Expression and Metastatic Tumor Growth in Mice. *Sci. Rep.* **2020**, *10* (1), 7375.

(59) Patel, B.; Kishor, C.; Houston, Todd. A.; Shatz-Azoulay, H.; Zick, Y.; Vinik, Y.; Blanchard, H. Rational Design and Synthesis of Methyl-β- D -Galactomalonyl Phenyl Esters as Potent Galectin-8 *N* Antagonists. *J. Med. Chem.* **2020**, *63* (20), 11573–11584.

(60) Thurston, T. L. M.; Wandel, M. P.; von Muhlinen, N.; Foeglein, Á.; Randow, F. Galectin 8 Targets Damaged Vesicles for Autophagy to Defend Cells against Bacterial Invasion. *Nature* **2012**, *482* (7385), 414–418.

(61) Levy, Y.; Auslender, S.; Eisenstein, M.; Vidavski, R. R.; Ronen, D.; Bershadsky, A. D.; Zick, Y. It Depends on the Hinge: A Structure-Functional Analysis of Galectin-8, a Tandem-Repeat Type Lectin. *Glycobiology* **2006**, *16* (6), 463–476.

(62) Carlsson, S.; Öberg, C. T.; Carlsson, M. C.; Sundin, A.; Nilsson, U. J.; Smith, D.; Cummings, R. D.; Almkvist, J.; Karlsson, A.; Leffler, H. Affinity of Galectin-8 and Its Carbohydrate Recognition Domains for Ligands in Solution and at the Cell Surface. *Glycobiology* **2007**, *17* (6), 663–676.

(63) Cagnoni, A. J.; Troncoso, M. F.; Rabinovich, G. A.; Mariño, K. V.; Elola, M. T. Full-Length Galectin-8 and Separate Carbohydrate Recognition Domains: The Whole Is Greater than the Sum of Its Parts? *Biochem. Soc. Trans.* **2020**, *48* (3), 1255–1268.

(64) Carlsson, S.; Carlsson, M. C.; Leffler, H. Intracellular Sorting of Galectin-8 Based on Carbohydrate Fine Specificity. *Glycobiology* **2007**, *17* (9), 906–912.

(65) Nandi, D.; Tahiliani, P.; Kumar, A.; Chandu, D. The Ubiquitin-Proteasome System. *J Biosci* **2006**, 19.

(66) Livneh, I.; Cohen-Kaplan, V.; Cohen-Rosenzweig, C.; Avni, N.; Ciechanover, A. The Life Cycle of the 26S Proteasome: From Birth, through Regulation and Function, and onto Its Death. *Cell Res.* **2016**, *26* (8), 869–885.

(67) Sakamoto, K. M.; Kim, K. B.; Kumagai, A.; Mercurio, F.; Crews, C. M.; Deshaies, R. J. Protacs: Chimeric Molecules That Target Proteins to the Skp1–Cullin–F Box Complex for Ubiquitination and Degradation. *Proc. Natl. Acad. Sci.* **2001**, *98* (15), 8554–8559.

(68) Neklesa, T. K.; Winkler, J. D.; Crews, C. M. Targeted Protein Degradation by PROTACs. *Pharmacol. Ther.* **2017**, *174*, 138–144.

(69) Smith, B. E.; Wang, S. L.; Jaime-Figueroa, S.; Harbin, A.; Wang, J.; Hamman, B. D.; Crews, C. M. Differential PROTAC Substrate Specificity Dictated by Orientation of Recruited E3 Ligase. *Nat. Commun.* **2019**, *10* (1), 131.

(70) Bondeson, D. P.; Smith, B. E.; Burslem, G. M.; Buhimschi, A. D.; Hines, J.; Jaime-Figueroa, S.; Wang, J.; Hamman, B. D.; Ishchenko, A.; Crews, C. M. Lessons in PROTAC Design from Selective Degradation with a Promiscuous Warhead. *Cell Chem. Biol.* **2018**, *25* (1), 78-87.e5.

(71) Hines, J.; Gough, J. D.; Corson, T. W.; Crews, C. M. Posttranslational Protein Knockdown Coupled to Receptor Tyrosine Kinase Activation with PhosphoPROTACs. *Proc. Natl. Acad. Sci.* **2013**, *110* (22), 8942–8947.

(72) Farnaby, W.; Koegl, M.; Roy, M. J.; Whitworth, C.; Diers, E.; Trainor, N.; Zollman, D.; Steurer, S.; Karolyi-Oezguer, J.; Riedmueller, C.; Gmaschitz, T.; Wachter, J.; Dank, C.; Galant, M.; Sharps, B.; Rumpel, K.; Traxler, E.; Gerstberger, T.; Schnitzer, R.; Petermann, O.; Greb, P.; Weinstabl, H.; Bader, G.; Zoephel, A.; Weiss-Puxbaum, A.; Ehrenhöfer-Wölfer, K.; Wöhrle, S.; Boehmelt, G.; Rinnenthal, J.; Arnhof, H.; Wiechens, N.; Wu, M.-Y.; Owen-Hughes, T.; Ettmayer, P.; Pearson, M.; McConnell, D. B.; Ciulli, A. BAF Complex Vulnerabilities in Cancer Demonstrated via Structure-Based PROTAC Design. *Nat. Chem. Biol.* **2019**, *15* (7), 672–680.

(73) Bai, L.; Zhou, H.; Xu, R.; Zhao, Y.; Chinnaswamy, K.; McEachern, D.; Chen, J.; Yang, C.-Y.; Liu, Z.; Wang, M.; Liu, L.; Jiang, H.; Wen, B.; Kumar, P.; Meagher, J. L.; Sun, D.; Stuckey, J. A.; Wang, S. A Potent and Selective Small-Molecule Degrader of STAT3 Achieves Complete Tumor Regression In Vivo. *Cancer Cell* **2019**, *36* (5), 498-511.e17.

(74) Békés, M.; Langley, D. R.; Crews, C. M. PROTAC Targeted Protein Degraders: The Past Is Prologue. *Nat. Rev. Drug Discov.* **2022**, *21* (3), 181–200.

(75) Johannes, L.; Jacob, R.; Leffler, H. Galectins at a Glance. *J. Cell Sci.* **2018**, *131* (9), jcs208884.

(76) Cumpstey, I.; Salomonsson, E.; Sundin, A.; Leffler, H.; Nilsson, U. J. Studies of Arginine–Arene Interactions through Synthesis and Evaluation of a Series of Galectin-Binding Aromatic Lactose Esters. *ChemBioChem* **2007**, *8* (12), 1389–1398.

(77) Sörme, P.; Qian, Y.; Nyholm, P.-G.; Leffler, H.; Nilsson, U. J. Low Micromolar Inhibitors of Galectin-3 Based on 3′-Derivatization of N-Acetyllactosamine. *ChemBioChem* **2002**, *3* (2–3), 183–189.

(78) Sörme, P.; Arnoux, P.; Kahl-Knutsson, B.; Leffler, H.; Rini, J. M.; Nilsson, U. J. Structural and Thermodynamic Studies on Cation−Π Interactions in Lectin−Ligand Complexes: High-Affinity Galectin-3 Inhibitors through Fine-Tuning of an Arginine−Arene Interaction. *J. Am. Chem. Soc.* **2005**, *127* (6), 1737–1743.

(79) Delaine, T.; Cumpstey, I.; Ingrassia, L.; Mercier, M. L.; Okechukwu, P.; Leffler, H.; Kiss, R.; Nilsson, U. J. Galectin-Inhibitory Thiodigalactoside Ester Derivatives Have Antimigratory Effects in Cultured Lung and Prostate Cancer Cells. *J. Med. Chem.* **2008**, *51* (24), 8109–8114.

(80) Witczak, Z. J.; Culhane, J. M. Thiosugars: New Perspectives Regarding Availability and Potential Biochemical and Medicinal Applications. *Appl. Microbiol. Biotechnol.* **2005**, *69*, 237–244.

(81) Pachamuthu, K.; Schmidt, R. R. Synthetic Routes to Thiooligosaccharides and Thioglycopeptides. *Chem. Rev.* **2006**, *106*, 160–187.

(82) Delaine, T.; Collins, P.; MacKinnon, A.; Sharma, G.; Stegmayr, J.; Rajput, V. K.; Mandal, S.; Cumpstey, I.; Larumbe, A.; Salameh, B. A.; Kahl-Knutsson, B.; Hattum, H. van; Scherpenzeel, M. van; Pieters, R. J.; Sethi, T.; Schambye, H.; Oredsson, S.; Leffler, H.; Blanchard, H.; Nilsson, U. J. Galectin-3-Binding Glycomimetics That Strongly Reduce Bleomycin-Induced Lung Fibrosis and Modulate Intracellular Glycan Recognition. *ChemBioChem* **2016**, *17*, 1759.

(83) Peterson, K.; Kumar, R.; Stenström, O.; Verma, P.; Verma, P. R.; Håkansson, M.; Kahl-Knutsson, B.; Zetterberg, F.; Leffler, H.; Akke, M.; Logan, D. T.; Nilsson, U. J. Systematic Tuning of Fluoro-Galectin-3 Interactions Provides Thiodigalactoside Derivatives with Single-Digit NM Affinity and High Selectivity. *J. Med. Chem.* **2018**, *61* (3), 1164–1175.

(84) Hirani, N.; MacKinnon, A. C.; Nicol, L.; Ford, P.; Schambye, H.; Pedersen, A.; Nilsson, U. J.; Leffler, H.; Sethi, T.; Tantawi, S.; Gravelle, L.; Slack, R. J.; Mills, R.; Karmakar, U.; Humphries, D.; Zetterberg, F.; Keeling, L.; Paul, L.; Molyneaux, P. L.; Li, F.; Funston, W.; Forrest, I. A.; Simpson, A. J.; Gibbons, M. A.; Maher, T. M. Target Inhibition of Galectin-3 by Inhaled TD139 in Patients with Idiopathic Pulmonary Fibrosis. *Eur. Respir. J.* **2021**, *57* (5), 2002559.

(85) Zetterberg, F. R.; Peterson, K.; Johnsson, R. E.; Brimert, T.; Håkansson, M.; Logan, D. T.; Leffler, H.; Nilsson, U. J. Monosaccharide Derivatives with Low-Nanomolar Lectin Affinity and High Selectivity Based on Combined Fluorine-Amide, Phenyl-Arginine, Sulfur-π, and Halogen Bond Interactions. *ChemMedChem* **2018**, *13* (2), 133–137.

(86) Zetterberg, F. R.; MacKinnon, A.; Brimert, T.; Gravelle, L.; Johnsson, R. E.; Kahl-Knutson, B.; Leffler, H.; Nilsson, U. J.; Pedersen, A.; Peterson, K.; Roper, J. A.; Schambye, H.; Slack, R. J.; Tantawi, S. Discovery and Optimization of the First Highly Effective and Orally Available Galectin-3 Inhibitors for Treatment of Fibrotic Disease. *J. Med. Chem.* **2022**.

(87) Tejler, J.; Skogman, F.; Leffler, H.; Nilsson, U. J. Synthesis of Galactose-Mimicking 1H-(1,2,3-Triazol-1-Yl)-Mannosides as Selective Galectin-3 and 9N Inhibitors. *Carbohydr. Res.* **2007**, *342* (12–13), 1869–1875.

(88) Öberg, C. T.; Blanchard, H.; Leffler, H.; Nilsson, U. J. Protein Subtype-Targeting through Ligand Epimerization: Talose-Selectivity of Galectin-4 and Galectin-8. *Bioorg. Med. Chem. Lett.* **2008**, *18* (13), 3691–3694.

(89) Dahlqvist, A.; Furevi, A.; Warlin, N.; Leffler, H.; Nilsson, U. J. Stereo- and Regioselective Hydroboration of 1- *Exo* -Methylene Pyranoses: Discovery of Aryltriazolylmethyl C-Galactopyranosides as Selective Galectin-1 Inhibitors. *Beilstein J. Org. Chem.* **2019**, *15*, 1046–1060.

(90) Dahlqvist, A.; Leffler, H.; Nilsson, U. J. C1-Galactopyranosyl Heterocycle Structure Guides Selectivity: Triazoles Prefer Galectin-1 and Oxazoles Prefer Galectin-3. *ACS Omega* **2019**, *4* (4), 7047–7053.

(91) Dahlqvist, A.; Mandal, S.; Peterson, K.; Håkansson, M.; Logan, D. T.; Zetterberg, F. R.; Leffler, H.; Nilsson, U. J. 3-Substituted 1-Naphthamidomethyl-C-Galactosyls Interact with Two Unique Sub-Sites for High-Affinity and High-Selectivity Inhibition of Galectin-3. *Molecules* **2019**, *24* (24), 4554.

(92) Sano, K.; Ogawa, H. Hemagglutination (Inhibition) Assay. In *Lectins*; Methods in Molecular Biology; Humana Press: New York, NY, 2014; Vol. 1200, pp 47–52.

(93) Takeda, Y.; Matsuo, I. Isothermal Calorimetric Analysis of Lectin–Sugar Interaction. In *Lectins*; Methods in Molecular Biology; Humana Press: New York, NY, 2014; Vol. 1200.

(94) Sörme, P.; Kahl-Knutsson, B.; Huflejt, M.; Nilsson, U. J.; Leffler, H. Fluorescence Polarization as an Analytical Tool to Evaluate Galectin–Ligand Interactions. *Anal. Biochem.* **2004**, *334* (1), 36–47.

(95) Sumner, J. B.; Howell, S. F. Identification of Hemagglutinin of Jack Bean with Concanavalin A. *J. Bacteriol.* **1936**, *32* (2), 227–237.

(96) Hirst, G. K. The Quantitative Determination of Influenza Virus and Antibodies by Means of Red Cell Agglutination. *J. Exp. Med.* **1942**, *75* (1), 49–64.

(97) Kasai, K. Frontal Affinity Chromatography: An Excellent Method of Analyzing Weak Biomolecular Interactions Based on a Unique Principle. *Biochim. Biophys. Acta Gen. Subj.* **2021**, *1865* (1), 129761.

(98) Jerabek-Willemsen, M.; Wienken, C. J.; Braun, D.; Baaske, P.; Duhr, S. Molecular Interaction Studies Using Microscale Thermophoresis. *ASSAY Drug Dev. Technol.* **2011**, *9* (4), 342–353.

(99) Laigre, E.; Goyard, D.; Tiertant, C.; Dejeu, J.; Renaudet, O. The Study of Multivalent Carbohydrate–Protein Interactions by Bio-Layer Interferometry. *Org. Biomol. Chem.* **2018**, *16* (46), 8899–8903.

(100) Gao, K.; Oerlemans, R.; Groves, M. R. Theory and Applications of Differential Scanning Fluorimetry in Early-Stage Drug Discovery. *Biophys. Rev.* **2020**, *12* (1), 85–104.

(101) Pal, K. B.; Mahanti, M.; Huang, X.; Persson, S.; Sundin, A. P.; Zetterberg, F. R.; Oredsson, S.; Leffler, H.; Nilsson, U. J. Quinoline–Galactose Hybrids Bind Selectively with High Affinity to a Galectin-8 N-Terminal Domain. *Org. Biomol. Chem.* **2018**, *16* (34), 6295–6305.

(102) Hassan, M.; van Klaveren, S.; Håkansson, M.; Diehl, C.; Kovačič, R.; Baussière, F.; Sundin, A. P.; Dernovšek, J.; Walse, B.; Zetterberg, F.; Leffler, H.; Anderluh, M.; Tomašič, T.; Jakopin, Ž.; Nilsson, U. J. Benzimidazole–Galactosides Bind Selectively to the Galectin-8 N-Terminal Domain: Structure-Based Design and Optimisation. *Eur. J. Med. Chem.* **2021**, *223*, 113664.

(103) Hassan, M.; Baussière, F.; Guzelj, S.; Sundin, A. P.; Håkansson, M.; Kovačič, R.; Leffler, H.; Tomašič, T.; Anderluh, M.; Jakopin, Ž.; Nilsson, U. J. Structure-Guided Design of D-Galactal Derivatives with High Affinity and Selectivity for the Galectin-8 N-Terminal Domain. *ACS Med. Chem. Lett.* **2021**, *12* (11), 1745–1752.

(104) Liu, C.; Wang, W.; Ellsworth, B. A.; Balog, J. A.; Regueiro-Ren, A. Galectin-3 Inhibitors. WO 2021/2429S2 Al.

(105) Rouillard, A. D.; Gundersen, G. W.; Fernandez, N. F.; Wang, Z.; Monteiro, C. D.; McDermott, M. G.; Ma'ayan, A. The Harmonizome: A Collection of Processed Datasets Gathered to Serve and Mine Knowledge about Genes and Proteins. *Database* **2016**, *2016*, baw100.

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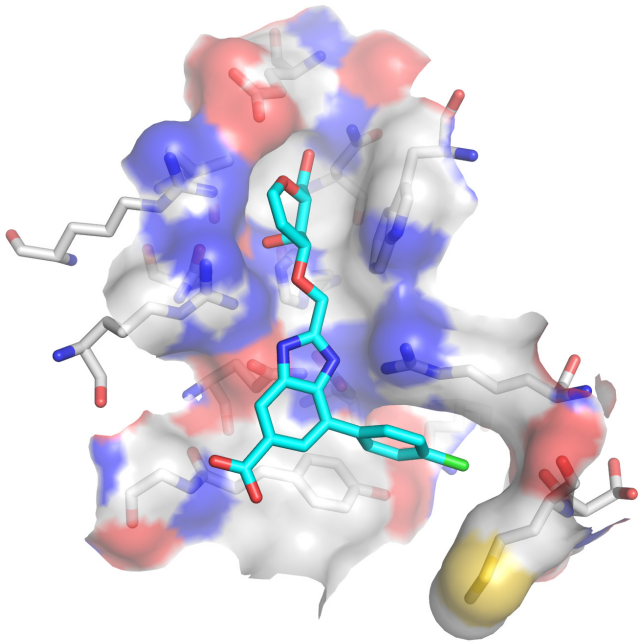

Targeting an unexploited binding pocket in the galectin-8 N-terminal domain leads to the discovery of the most potent selective ligand to date (cyan sticks).

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