Small-Molecule Galectin Ligands

Structure-Based Optimisation of Affinity and Selectivity

Van Klaveren, Sjors

2024

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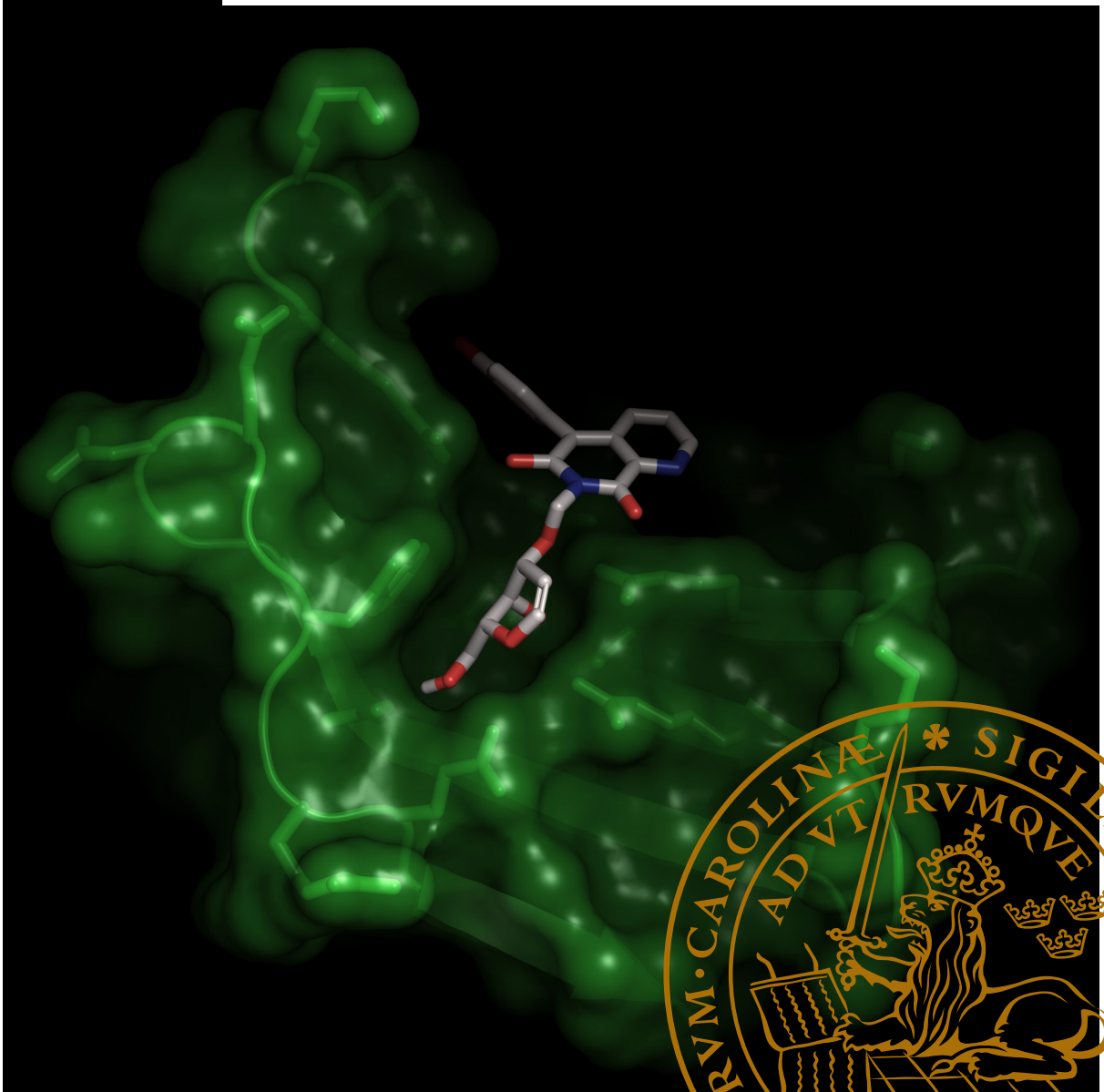
Structure-Based Optimisation of Affinity and Selectivity

SJORS VAN KLAVEREN | FACULTY OF PHARMACY | UNIVERSITY OF LJUBLJANA
CENTRE FOR ANALYSIS AND SYNTHESIS | LUND UNIVERSITY



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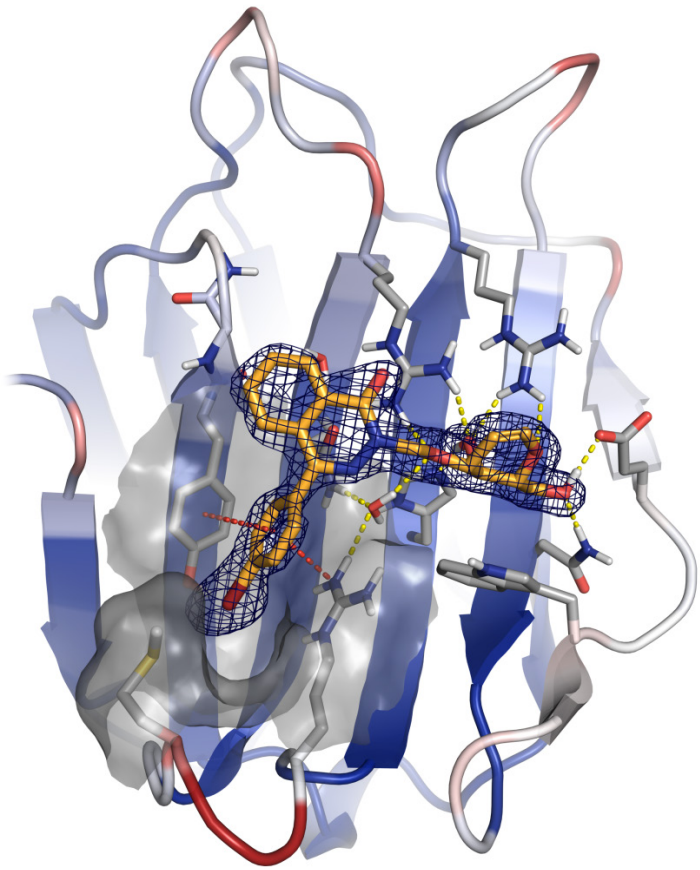

A phthalazinone–galactal ligand binding galectin-8N.

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Faculty of Pharmacy
University of Ljubljana

Centre for Analysis and Synthesis
Department of Chemistry
Faculty of Science

LUND UNIVERSITY

UNIVERSITY OF LJUBLJANA | FFA Faculty of Pharmacy

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Sjors van Klaveren

LUND
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DOCTORAL DISSERTATION

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Organisation: LUND UNIVERSITY
Centre for Analysis and Synthesis, Lund Unversity, Box 124, SE-221 00 Lund, Sweden

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Abstract:

In the body, all cell surfaces are covered with glycans, and many regulatory components have carbohydrates attached to them, which can aid in their function. Galectins are a family of proteins which can bind to specific glycans and oligosaccharides and cross-link them to influence a wide range of cellular processes. The members of the galectin family are structurally similar and can be involved in the same processes. Inside the cell, galectins have been observed assisting in the detection of exposed glycans on damaged intracellular vesicles, initiating autophagy which helps to clear out damaged cells and bacterial infections. Some galectins have a role in promoting angiogenesis and lymphangiogenesis. This is associated with the repair of damaged tissue and with the growth of tumours. Studies have also implicated galectins in autoimmune disorders and neurodegenerative diseases.

The research in this doctoral dissertation focused on the discovery and development of selective, high-affinity ligands for several galectins, with a special focus on galectin-8. Several structurally diverse libraries of compounds were synthesised as part of ligand-based and structure-based approaches. From these libraries, several novel hits for galectin-1, -3, and -8N were discovered and structure–affinity relationships were identified. These hits were optimised to produce galectin ligands with sub-micromolar affinities and analogues with almost complete selectivity for galectin-8N over all other tested galectins. Such highly selective compounds may help to study the biological roles of galectins and the pathologies in which they are involved. This may also, in turn, lead to galectin-8N inhibitors with a pharmaceutical potential.

Key words: Galectin, Galectin inhibitor, Selectivity, Galactal, Phthalazinone

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Guide to this dissertation

Preamble

I am very happy to welcome you to the dissertation I have prepared to demonstrate what we have achieved in my time in the laboratories in Ljubljana, Slovenia and in Lund, Sweden. I have always wanted to write my dissertation with a decent section of informal introduction—of which this preamble is part—to allow all non-expert friends, family, and strangers to understand (1) what it is medicinal chemists do, (2) what drug discovery is all about, (3) what I have been doing in my years abroad. You will find this popular introduction in Chapter 1.

Guide

The introduction to this doctoral dissertation is covered in **Chapter 1**. For non-experts in this specific field of science research, **Chapter 1.2** is a short explanation of how this work is situated in the pharmaceutical research field, and what that field looks like. The first part is a popular introduction with an amusing allegory.

Chapter 1.3 is an extensive coverage of the protein target: galectins, their function and structure; and **Chapter 1.4** describes known galectin inhibitors, their development until now, and how the field has developed alongside.

Chapter 2 presents to you the hypotheses that the research in this doctoral dissertation was set out to address. The method list is included in **Chapter 3**.

The results of this doctoral dissertation are found in **Chapter 4**, included as papers. The manuscript in **Chapter 4.3**, chronologically the last of the research presented in this dissertation, has been submitted to a peer-reviewed international scientific journal but is not yet published.

Chapter 5 discusses the results and relates their significance to the hypotheses, and **Chapter 6** concludes if the hypotheses can be confirmed or refuted based on the research presented in this dissertation. **Chapter 7** gives some concluding remarks.

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Summary

In the body, all cell surfaces are covered with glycans and many regulatory components, such as proteins, have oligosaccharides linked to them. These carbohydrates can aid in the function of proteins and, in specific cases, allow their recognition and organisation. Galectins are a family of relatively small and soluble proteins, which are specialised in binding to glycans and other oligosaccharides containing a β-galactoside. This binding occurs in the carbohydrate recognition domain (CRD), which is slightly variable for each galectin, giving them some degree of selectivity.

Galectins often present multiple CRDs simultaneously, either in a single protein or by oligomerisation. These configurations allow galectins to cross-link between binding partners, contributing to cell-cell and cell-matrix signalling. Functionally, the members of the galectin family can have overlapping and sometimes contradictory roles in a variety of cellular processes. Inside the cell, galectins have been observed assisting in the detection of exposed glycans on damaged intracellular vesicles, initiating autophagy to clear out damaged cells and bacterial infections. Some galectins affect the growth of blood and lymph vessels, associated with the repair of damaged tissue and tumour growth. Galectin-8 is recognised in several cancer types, also attributed to its ability to engage in protein–protein interactions with signalling protein, and in inducing the expression of cytokines, which is correlated with the promotion of tumour growth and metastasis *in vivo*.

The therapeutic potential of targeting the CRD of galectins is receiving a considerable amount of attention. Research into galectin ligands has been proliferating for about 20 years and the most promising small molecule drugs (all targeting galectin-3) have reached clinical trials for the treatment of idiopathic pulmonary fibrosis, liver fibrosis (NASH), and lung cancer. However, the complexities of galectin interactions and their overlapping functions, warrant the need for galectin-specific probes which can be used to better understand their mechanisms. Highly selective galectin-8N ligands can also pave the way for potential therapeutic agents against tumours and inflammatory conditions.

The research in this dissertation focused on the discovery and development of selective, high-affinity ligands for several galectins, with a special focus on galectin-8. For this purpose, several different strategies were pursued. The first strategy was a ligand-based screening of a library constructed around a triazolyl–galactoside scaffold with slight variations. A structure–activity relationship (SAR) indicated that the *meta*-substitution of the carboxylic acid was optimal, which was supported in docking studies performed using this compound library in the binding pocket of galectin-8N. This project, progressing through a scaffold-hopping approach, culminated in the discovery of a benzimidazole–galactoside ligand. Further

introduction of the α-thiodichlorophenyl aglycon on the anomeric position of galactose saw the affinity jump another 100-fold, but with a relative reduction in selectivity over galectin-3.

A structure-based investigation was performed on galectin-1, galectin-3, and galectin-8N. This project aimed to reveal specific interactions that are available in the different CRDs, leading to ligands with increased affinities for any single one, or all three, of these galectins. A focused virtual library of triazolyl-1-thio galactosides with diversely decorated phenyl groups was designed, of which 24 were synthesised with relative ease and in a short time. Several of these compounds showed binding affinities well below 1 mM for one or more of the target proteins, from which SARs were constructed. Affinity trends were observed for galectin-3 and some favoured motifs were revealed for all three galectins. An acetamide-based derivative was further optimised in two different, previously established, approaches. The two resulting structures showed promising potential as pan-galectin inhibitors with low to sub-micromolar affinities.

The final project presented in this dissertation covers the structure-guided discovery and optimisation of a class of galectin-8N selective ligands based on the phthalazinone–D-galactal scaffold. After an exploratory investigation to establish a reliable synthetic route, a group of phthalazinone-derived ligands were synthesised to highlight the individual contribution of the scaffold components in a comprehensive SAR. The binding interactions of the main hit, the 4-phenyl phthalazinone–D-galactal, were further analysed in an innovative molecular dynamics meta-analysis. This revealed two possible binding modes, depending on an induced-fit of the ligand interacting with a flexible loop that is specific for galectin-8N.

In a succeeding sub-project, the 4-phenyl group on the phthalazinone scaffold was further decorated with groups of increasingly larger size. Because these ligands engage in a sub-pocket that is only present in galectin-8N, they can evoke a selectivity over other galectins. A convincing SAR could link the size of the atom or group attached at the *para*-position of the 4-phenyl group to their determined binding affinity. The 4-(*p*-bromophenyl)phthalazinone derivative showed an almost complete selectivity profile for galectin-8N over all other tested human galectins. An X-ray crystal structure of the protein–ligand complex was solved at a resolution of 1.22 Å (PDB ID: 8CM8), which revealed the phthalazinone–galactal ligand with its *p*-bromophenyl group positioned in the specific sub-pocket of galectin-8N. The remarkable selectivity for galectin-8N suggests that the D-galactal–(*p*-bromo phenyl)phthalazinone could be used as a molecular tool for studying galectin-8 biology and associated pathologies and may lead to the discovery of galectin-8N-selective ligands with pharmaceutical potential.

Abbreviations

ACN	Acetonitrile
AcOEt	Ethyl acetate
AcOH	Acetic acid
CRD	Carbohydrate recognition domain
DC50	Half-maximal degradation concentration
DCM	Dichloromethane
DIPEA	*N*,*N*-Diisopropylethylamine (Hünig's base)
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DSF	Differential scanning fluorimetry
EtOH	Ethanol
FP	Fluorescence polarisation
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IC50	The half-maximal inhibitory concentration
IR	Infrared
ITC	Isothermal titration calorimetry
K_d	Dissociation constant
kDa	Kilodalton
MD	Molecular dynamics
MeOH	Methanol
MS	Mass spectrometry
MW	Microwave
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PDB	Protein data bank
RT	Room temperature
SAR	Structure-activity relationship
SEM	Standard error of the mean
TDG	Thiodigalactoside
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TMS	Tetramethyl silane
Tol	Toluene
TPSA	Topological polar surface area
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Dissertation Outline

This dissertation is set up in the form of chapters. The initial chapter is an introduction to the research field, the target protein, and an overview of existing small-molecule galectin ligands. The hypotheses are stated in chapter 2, and chapter 4 present the research work. The relation of the research to the hypotheses is discussed together in chapters 5 and 6.

To allow *any* reader to learn from this dissertation, the introductory chapters follow an increasing level of pre-requisite knowledge and advanced terminology in the field. **Chapter 1.2** describes medicinal chemistry in brief and gives a short overview of the drug discovery pipeline. This background is essential for understanding the outline of the dissertation given here.

Chapter 1.3 brings the reader up to speed on the field of the target proteins, galectins, and **Chapter 1.4** describes the former results of searching for galectin ligands. Especially the diverse functionality of galectins is brought to light, along with the pathologies that are affected by galectins. A brief overview of the overarching structural features of existing galectin inhibitors is given, and supplemented by a description of our current understanding of protein–ligand interactions in relation to the structural features of galectins. Galectin-8 receives special attention as the primary target for the research described in this thesis.

The description of the hypotheses is found in **Chapter 2**. The list of methods is included in **Chapter 3**.

Chapter 4.1 describes the research carried out within the framework of **Hypothesis 1**. For the probing of the carbohydrate recognition domain of galectins, a library of 3-triazolyl galactosides presenting a benzoic ester or -acid group in variable configurations was synthesised and their binding affinities were investigated. The structure–activity relationships (SARs) derived from this library were used to further optimise a galactoside carrying a privileged heterocyclic scaffold. The synthesis, analysis and optimisation of these compounds were published as a scientific research paper entitled *Benzimidazole–Galactosides Bind Selectively to the Galectin-8 N-Terminal Domain: Structure-Based Design and Optimisation*, *Eur. J. Med. Chem.* **2021**, *223*, 113664.

Chapter 4.2 describes the creation of a 3-triazolyl-1-thiogalactoside library that was synthesised for screening for affinity in galectin-1, -3, and -8N, which is within the scope of **Hypothesis 3**. The results of this screening and the following optimisation are described in a paper entitled *Design and Synthesis of Novel 3-Triazolyl-1-Thiogalactosides as Galectin-1, -3 and -8 Inhibitors*, *RSC Adv.* **2022**, *12* (29), 18973–18984.

The study of phthalazinone heterocyclic structures as ligands of galectin-8N is the premise of **Hypothesis 2.** The conception of phthalazinone-based ligands is first laid out in **Chapter 4.3**, with an extensive study of the sub-scaffold contributions, molecular dynamics and binding pose analysis to determine the origin of the affinity and selectivity of phthalazinone ligands for galectin-8N. These results were published in a scientific research paper entitled *Selective Galectin-8N Ligands: The Design and Synthesis of Phthalazinone-D-Galactals*, *ChemMedChem* **2022**, *17* (6), e202100575.

The discovery of phthalazinone–galactals is expanded upon in **Chapter 4.4**, which describes an effort to optimise the phthalazinone scaffold by introducing different halogens on the 4-phenyl group of the phthalazinone scaffold. The resulting ligands had their physicochemical and preliminary *in vitro* pharmacokinetic properties assessed. X-ray crystallography was used to resolve the binding pose of the bromo-analogue in a galectin-8N crystal structure. At the time of writing, a manuscript describing these findings has been submitted to a peer-reviewed journal.

Chapter 5 is an in-depth discussion of the results and how they pertain to each of the hypotheses which, in **Chapter 6**, will be conclusively disputed or confirmed. Concluding remarks follow in **Chapter 7**.

1.2 Popular introduction

A light-hearted comparison

I would like you to imagine a shape-sorter box. Yes, a children's toy. It is the one where you have to fit little wooden shapes into matching holes. Supposedly, this is a good toy for kids to explore sizes, visualise shapes, and develop problem-solving skills. Well, it may sound strange, but as a medicinal chemist, I sometimes feel a little like a kid playing with a fancy, grown-up version of that toy.

Instead of wooden shapes and a box full of holes, we are working with small molecules and proteins. You see, proteins have a precise contour that often has pockets similar to the holes in the shape box. This pocket can only fit molecules with a certain shape – kind of like how the star-shaped hole on the shape-sorter toy can only fit the star-shaped block.

A shape-sorter box toy.[1]

The shape of a protein pocket is of course more complex than those found on the toy. One difference is that the surface of the pocket has an electronic potential. Some parts of the surface are positively charged, whereas others are negative or neutral. What you must know about this charge is that, similar to a magnet, opposite sides attract.

As a medicinal chemist, I want to make molecules that have the right shape and the positive and negative charges in the right places to match the surface of a protein pocket. It is like making the right block yourself so that it can fit precisely in the box. That is the chemistry part.

When a molecule fits and sits inside the pocket, we say that it is '*binding*' that protein. The binding of a molecule to a protein can change how that protein works. This is the basis of all chemical signals that are sent throughout the body and between cells. So, with the right molecule made, we can block these natural signals, or create new signals to change what goes on inside the body. That is the medicinal part.

It's like a sophisticated game of shape sorting, and it's all in the name of discovering new drugs to help people. Who knew medicinal chemistry could be so much fun?

Our research

In this thesis, we have been searching for molecules that fit in the binding pocket of proteins called galectins. We have created screening libraries to discover which molecular shapes might give the best binding affinity (ligand-based discovery). We have also used protein crystallography and computer-based methods to reveal what the binding pocket looks like and tried to design molecules with the optimal shape to match (structure-based discovery). Merging these techniques and their results, we have identified potent inhibitors for galectin-8, which also were impressively selective over other members of the galectin family. This discovery can help further research into understanding the role of galectin-8 in the human body.

1.2.1 Medicinal Chemistry

Chemistry is the study of molecules, understanding their behaviour, and knowing how to synthesise them. In medicinal chemistry (also called pharmaceutical chemistry) we work on the identification and development of new molecules that can interact with biological entities, such as proteins, to change their behaviour.

Proteins are the fundamental workforce of biology and have a great variety of functions in the body. They can provide structural support for cells, transmit signals inside and between cells, and transport molecules around the body. Enzymes, for example, are proteins that can modify molecules into new products and metabolise nutrients. The structure of a protein explains its function, and vice versa: a protein that transports chloride ions from outside the cell to its interior is indeed shaped like a tunnel through the cell membrane. Another clear shape–function link is found in the binding pocket of proteins. Small molecules or other proteins can 'bind' a protein if it has a suitable pocket containing the right amino acids for a favourable interaction between the two binding partners. Changing a single amino acid in the binding pocket of a protein can effectively change its binding properties, and consequently change, or even completely inhibit, their function. Similarly, changing the structure of a small-molecule drug can improve its binding to interfere with the protein function. A ligand that activates or enhances the natural function of a protein is called an agonist. An antagonist is a ligand that interferes with a protein, for example, if binding of the ligand to the protein prevents normal signals from being transmitted throughout the body.

Small-molecule drugs are the active ingredient in many common medicines. They are compounds with a low molecular weight that often bind specific protein targets and are used to treat a wide range of diseases. Traditionally, medicinal chemists prefer smaller molecules because their size makes it easier to predict their behaviour in the body. When a small molecule binds to a protein, it interacts with the protein's binding pocket (**Figure 1**) through a combination of non-covalent interactions, such

as hydrogen bonding, electrostatic interactions, and Van der Waals forces. In specific cases, ligands can be made to react with their protein target to form a covalent bond. Understanding how small molecules bind to proteins and modulate their function is a key focus of medicinal chemistry research. Tuning the strength and specificity of the binding interactions enhances the potency and efficacy of the drug while minimising off-target effects. Off-target binding can lead to side effects, so the selectivity of inhibitors is very important for the safety of a drug.

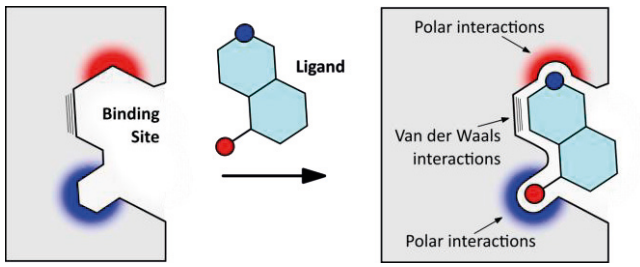


Figure 1. A drug binding to a protein target. The polar features of the ligand and the binding site, being electron-rich (red) and electron-poor (blue), attract each other. Non-polar regions interact through weaker Van der Waals forces.

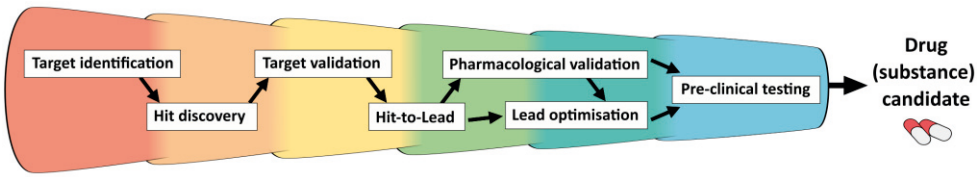
There is a lot of space for creativity in the design of small-molecule ligands, but also many challenges. Limited, first, by the chemical building blocks that are available and, second, by the chemical reactions that can be used to combine and modify these building blocks, a medicinal chemist has to solve multiple puzzles at the same time. Fortunately, a wide toolbox of techniques is available to help. Techniques based on biophysics, structural biology, and computational chemistry can help to understand how (well) a small molecule binds to the target protein to support the drug discovery and development pipeline (**Figure 2**).[2,3]

If the role of a protein is well understood, creating a drug molecule to interfere with that role likely gives a predictable outcome. There are many cases, however, where the function of a protein is still unknown. Medicinal chemists can once again jump into the picture: if a molecule is created that can selectively occupy the binding pocket of that protein, thereby preventing natural binding processes from occurring, this could reveal what its biological role is. When applied in this way, inhibitors are regarded as 'molecular probes' rather than drugs and are applied to the study of biological processes on the molecular level.

The small-molecule inhibitors discovered as a part of this research are, primarily, considered to be used as molecular probes to investigate the natural function of galectin-8. If their binding, selectivity, and safety profiles are very promising, these molecules can also be considered in the hit-to-lead phase of the drug discovery pipeline.

1.2.2 The Drug Discovery Pipeline

The drug discovery pipeline describes a multi-stage process in which potential drug candidates are identified and developed (**Figure 2**). The earliest stages involve the identification of potential targets and molecules ('hits') that can show promising binding activity to these targets.[4] More advanced stages cover the optimisation of these hit compounds through chemical modification, as well as testing their efficacy and safety in a variety of biochemical and biological assays.



	Biochemistry and **Structural biology**	**Medicinal chemistry**	**Biophysics**	**Chemical biology**	**Bioinformatics** and **Computational chemistry**
Target identification	Disease models		Assay development	Ligand-based discovery	Homology modelling
Hit discovery		De novo design	Affinity testing High-throughput screening		Virtual screening Docking
Target validation	Biomarkers	Chemical probes		Knock-out models	
Hit-to-lead		Library synthesis Scaffold hopping		Structure–activity relationships	Molecular dynamics simulations
Pharmacological validation	Cell-based studies			*In vitro* ADME Toxicology	
Lead optimisation	Co-crystallisation	Drug optimisation	X-ray structure analysis		
Pre-clinical testing	*In vivo* ADME			Pharmacokinetics	

Figure 2. The drug discovery pipeline and an overview of different techniques and methods in the pipeline according to their subfield in medicinal chemistry.

In the early stages of drug discovery, every piece of information can be valuable in supporting the selection of a hit. For example, structural biology can be used to identify positive contributions of the molecule to the binding parameters and, through the use of computational methods, to search for and explain novel hit compounds. Biophysical analysis of discovered hits is also important to steer early drug discovery and is extensively used in the pharmaceutical industry to identify potential problems with physicochemical properties, such as solubility.[5]

The focus of this project is on galectins, a family of proteins that play a critical role in a variety of biological processes. Although their structures have been extensively studied, additional research is needed to understand their myriad functions. It is through combining the different techniques from the subfields of drug discovery (**Figure 2**) that it becomes possible to identify new hit compounds for galectins, investigate how they bind, and reveal their pharmaceutical potential. In turn, these compounds may help future research to better understand the role of galectins,

which may then enable the development of new effective pharmaceuticals. This exemplifies how research in drug discovery, as in all science, is reliant on what techniques and knowledge have been established before them.

1.3 Galectins

Lectins are proteins that bind sugars. Galectins, specifically, have an affinity for the D-galactose monosaccharide and for polysaccharides that contain β-D-galactose.[6] Thus, the name is short for galactose-binding-lectin: "gal-lectin". The family of galectins has been known for about 30 years,[7] and although new members are still found and are relatively unexplored,[8] others have been studied extensively. These studies have even led to inhibitors of galectin-3 which reached clinical trials for the treatment of pulmonary fibrosis,[9,10] and fibrotic liver disease (cirrhosis) and cancer.[11,12] Despite all this research, the diverse nature and functions of galectins in physiological and pathophysiological (disease) contexts remain immensely complex.

> *"[Galectins] occur in a bewildering number of functional contexts, intracellular and extracellular, low and high concentrations, slow and fast, low or high specificity, and there is no clear statement like: 'this is the function of galectins, or even any particular galectin'".*[13]
>
> *– Prof. Hakon Leffler*

1.3.1 The galectin family

The protein family of galectins contains 16 members, which have similar functions and a largely similar structure. The proteins in this family are the target of our study and we will investigate their many aspects in depth. But first, a few galectins are excluded from this work: galectin-5 and -6 are only found in rodents, and galectin-11 and -15 only in caprinae (sheep and goats). These will not be mentioned in this dissertation again, since we are primarily interested in galectins in humans. Among those, galectin-13, -14, and -16 are found in humans but mainly in the placenta,[14] and are also not directly considered in our research.

The galectins bind to galactose saccharides with their carbohydrate recognition domain (CRD, **Figure 3**). This domain is highly conserved amongst the galectins and often has a low ligand specificity, allowing them to bind a variety of saccharides. Some galectin CRDs even have overlapping recognition and can bind the same ligands with comparable affinities.

1.3.2 Carbohydrate Recognition Domains

Galectins are divided into three categories based on the presentation of their CRDs (**Figure 3B**). The prototypical galectin-1, -2, -7, and -10, have one CRD, which are usually monomeric at physiological concentrations.[15] At higher local concentrations, such as when binding on surfaces, homo- and hetero-dimerisation can occur,[16] which allows these galectins to crosslink different binding partners. Galectin-3 is alone in its category; it has only one CRD, just like the prototypical galectins, but it also carries an N-terminal non-lectin region rich in glycine, proline, and tyrosine or tryptophan, which allows it to self-aggregate at higher local concentrations.[17] The oligomeric galectin-3 is found in complex interactions with cell surface glycoproteins and extracellular components.[18] The third category describes tandem-repeat type galectin-4, -8, -9, -12. The galectins in this category contain two CRDs in their singular protein chain. These two CRDs are not the same, and therefore they are distinguished as the C-terminal and N-terminal domain according to their occurrence in the protein sequence.

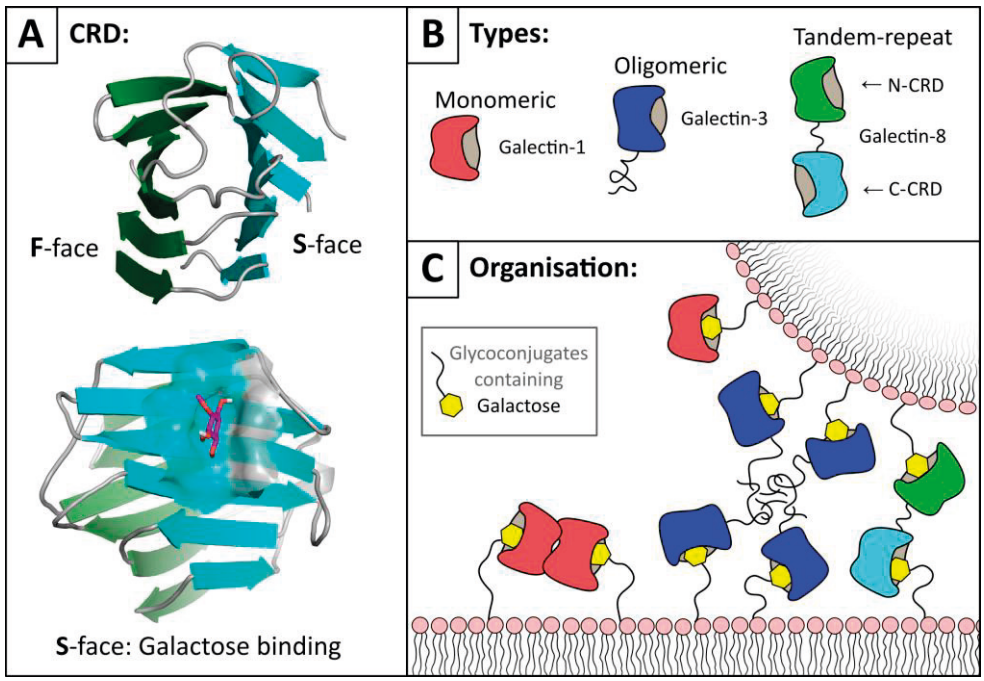


Figure 3. Galectin CRDs. Depicting (A) the F-face and the galactose-binding S-face of the CRD, (B) the types of galectins, and (C) the different possible organisations of CRDs in the galectin family and examples of their binding behaviour.

Galectin CRDs are arranged in two stacked β-sheets of 5 or 6 strands, the external surfaces of these are the F- and S-face (**Figure 3A**). The S-face contains the groove in which carbohydrate recognition occurs. The F-face is capable of binding peptides and proteins, of which galectin-1 can bind over 20.[19] Protein–protein interactions are also observed for galectin-8.[20,21] A galectin-8–NDP52 crystal structure was obtained as proof that protein–protein interactions occur at a site separate from the CRD and independent of glycans.[22]

As the carbohydrate recognition on the S-face and the protein-protein interactions on the F-face occur at opposite sides of the galectin CRDs, it means these two phenomena might not be exclusive or competitive. The two binding events might even have complementary functional results. Studying such aspects might be possible in the future with the development of novel research tools, which would allow dissecting the intricate and sometimes overlapping roles of galectins in numerous pathologies.

1.3.3 Localisation and Cellular Functions

The glycoconjugates that galectins bind to can be attached to many proteins in and on the cell (glycoproteins)[23] and to the lipids present in the cell wall (glycolipids).[24] Galectins, similarly, are found throughout almost all cell types and tissues,[25] although localisation and concentration differ per galectin.[13] Galectins can be found not only in the cytosol of the cell but are also exported to the extracellular matrix through an unknown, non-classical, secretion pathway. Here, galectins are not directly attached to the cell surface but can interact with different binding partners to cross-link surface proteins, components in the extracellular matrix, and other ligands on cells and pathogens.[26] Through binding and cross-linking galectins induce or facilitate different signals,[27] influence the organisation and longevity of membrane structures and, by increasing the local concentrations of glycosylated matrix components on the cell surface,[28] can enhance signals not directly attributed to galectins.[29,30]

1.3.4 Associated Pathologies

Different bacteria, viruses, and parasites interact with galectins during their infection.[31] Most of these interactions lead to activation of the immune system by galectins,[32] but some pathogens evolved to have galectin-recognition facilitate one or more stages of their infection.[33] Inside the cell, damaged vesicles containing bacterial, viral, or chemical disruptors are rapidly recognised by galectins,[34] which direct various autophagy cascades to destroy the ruptured vesicle and its contents.[35,36] Another example of galectins in pathological conditions is that galectin-1 is tightly associated with obesity, type 2 diabetes, and related

complications.[37] Several galectins have been shown to regulate cytokine activity,[38,39] one way in which galectin-8 alters the immune response,[40] and is involved in tumour progression.[41] Galectin-8 binds to glycosylated integrin receptors to modulate the function of white blood cells,[42] and also hinders cell adhesion and induces apoptosis.[43] Formation of blood and lymph vessels is affected by galectins,[44,45] which means they are important for the healing of damaged tissues and connected to fibrosis,[46] tumour progression,[47] and metastasis.[48] Galectin-8 is also shown to be involved in inflammatory disorders such as rheumatoid arthritis,[49] and neurodegenerative diseases such as Alzheimer's.[50]

The wide and varied functions of galectins may seem bewildering, which exactly underscores the premise of developing galectin inhibitors. Sometimes their functions appear to be contradictory or overlap, making it difficult to investigate these proteins individually. Gene knock-out animals can be studied, as was done for galectin-3 and galectin-8 deficient mice to analyse the effect of gene deletion on the overall biochemistry of the animal.[51] In such an approach, however, it is difficult to say if these are the result of the missing galectin or rather from downstream and secondary effects. On the contrary, selective high-affinity inhibitors of galectins might enable us to delineate with more precision the functions of individual galectins related to carbohydrate recognition and to further investigate the potential of galectin ligands as pharmaceutical agents. But first, we will explore the binding pocket of galectins and point out what makes them similar, and what differences may be exploited by ligands targeting galectins.

1.3.5 The Galectin Structure

The first human galectin X-ray structure was published in 1998 and hundreds more have been published since.[52] The existence of (numerous) X-ray crystal structures gives medicinal chemists searching for galectin ligands a great advantage; structures can be used in structure-based design of novel ligands, docking studies and *in silico* screening, and molecular dynamics studies. The compact carbohydrate recognition domains of galectins are quite stable, which means even non-optimised ligands may be crystallised to both confirm a binding hypothesis and reveal further potential optimisation. For example, the X-ray crystal structure of a previously discovered ligand,[53] the quinoline–galactose (**Figure 5c**) in galectin-8, will be presented in Chapter 4.1 This structure revealed to us a unique sub-pocket, which became the focus of the research covered in Chapter 4.3.

Comparing the Carbohydrate Recognition Domains

The many structures of galectins have allowed researchers to identify what makes galectins recognise galactose, and what structural features bring about the binding selectivity within the family. To help facilitate the comparison, the binding pocket

on the S-face of galectins is described using an ABCDE-subsite nomenclature, in which the C-subsite binds the D-galactose (**Figure 4**).[54] Out of eight highly-conserved glycan-binding amino acids in all galectin binding pockets, five surround the galactose in the C-subsite.[55] In subsite A,B and subsite D,E more variety among amino acids is observed between galectins, thus offering more options for specific interactions for different galectins and yield selectivity for galectin ligands, both natural and designed. Comparing the binding pockets of galectin-1, -3, and -8 (**Figure 4** and **Figure 5**), three galectins that are found throughout the human body,[56] it becomes clear that the differences in the binding sites are really small.

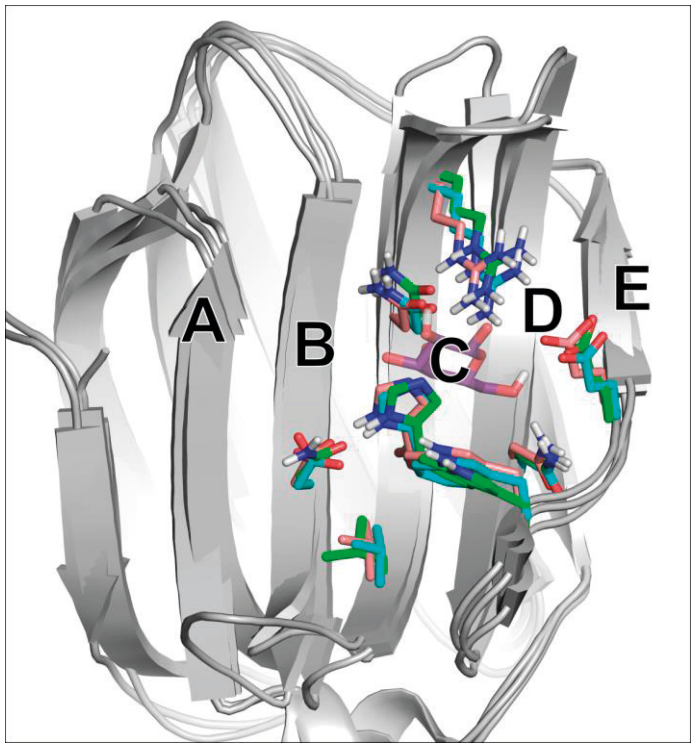


Figure 4. An alignment showing conserved residues of galectin-1 (green sticks, PDB ID: 5MWX), -3 (cyan sticks, PDB ID: 6QLN), and -8N (salmon sticks, PDB ID: 7AEN). Galectin binding pockets are often described and compared using the ABCDE-subsite nomenclature, in which galactose (transparent purple sticks) binds subsite C.

Galectin-1 is of the monomeric type. On the CRD of galectin-1 (**Figure 5a**), distinct features are the histidine-52 in subsite D and two aspartic acids in subsite A, which contribute to its unique binding specificity for certain carbohydrate structures and ligands.[57,58] Galectin-3 is a unique member of the galectin family that can form homo-oligomeric structures. In the binding pocket of galectin-3 (**Figure 5b**), the

specific amino acid arginine-144 can establish induced-fit interactions with ligands. By stacking of a phenyl ring underneath this accommodating arginine, inhibitors of galectin-3 have reached affinities in the nanomolar range[9] and high selectivity.[59]

Galectin-8 is a tandem-repeat type consisting of two differing carbohydrate recognition domains (CRDs) connected with a linker of variable length (most commonly 32 amino acids).[44] Although some galectin-8 functions are performed by each CRD independently,[60] other biological roles require both CRDs, especially for interactions on the cell surface.[61] The activity of truncated galectin-8 is altered in hemagglutination and apoptosis, indicating a cooperative interaction between the two CRDs and the linker itself.[62] Of the two galectin-8 CRDs, the N-terminal CRD has a higher affinity for its natural ligands.[61] For this reason, galectin-8N has been the primary target for novel glycomimetic ligands.

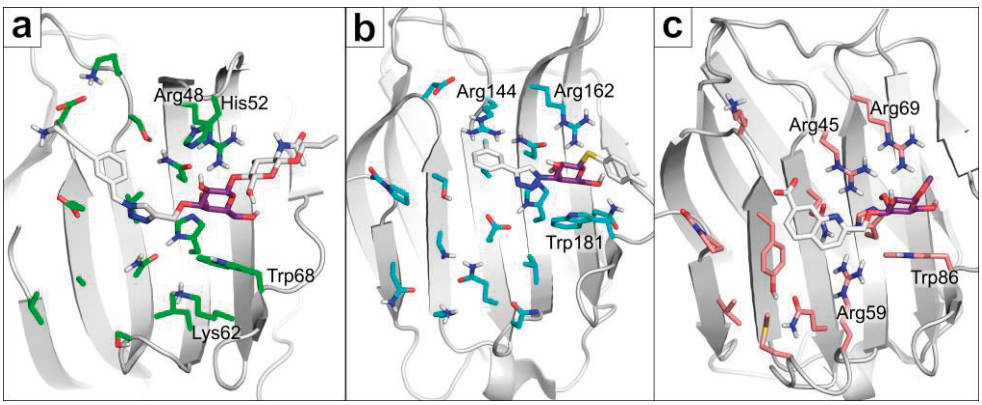


Figure 5. Comparison of binding pockets of X-ray crystal structures, containing ligands, of galectin-1 (green, PDB ID: 5MWX), galectin-3 (cyan, PDB ID: 6QLN), and galectin-8N (salmon, PDB ID: 7AEN).

In the CRD of galectin-8N (**Figure 5c**), three amino acids are of special interest. Arginine-45 is positioned in the same location as the arginine-144 in galectin-3. But, likely due to stronger interactions with the surrounding amino acids, arginine-45 does not appear to drift like its galectin-3 counterpart and an induced-fit has never been observed. Arginine-59 and tyrosine-141 are of interest because their surfaces form a shallow sub-pocket adjacent to subsite B, which is unique for galectin-8N. The importance of this sub-pocket for the recognition of a natural ligand fragment (lacto-*N*-neotetraose) was demonstrated by the strongly reduced affinity of this tetrasaccharide when tyrosine-141 was mutated to a serine.[61] This sub-pocket was also instrumental in the successful development of selective, high-affinity ligands for galectin-8N described in this dissertation.

1.4 Galectin ligands

All ligands designed to bind—and thereby block—the carbohydrate recognition domain of galectins occupy the groove on the S-face of galectins. A nomenclature was devised to describe and compare the binding pockets of galectins by dividing them into subsites (**Figure 6**). The conserved binding of D-galactose occurs in the C-subsite, with the A- and B-subsites located in extension of the galactose O3, and the D- and E-subsites extending from the anomeric carbon of the galactose.[54]

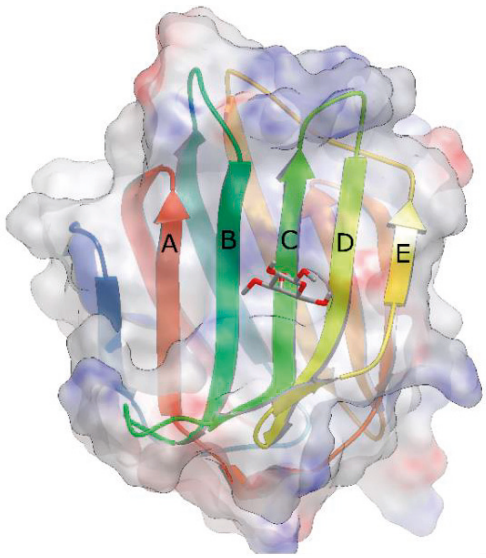


Figure 6. Galactose binds in subsite C of galectin-8N and other galectins.

1.4.1 Known galectin inhibitors

Research into galectin inhibitors has been ongoing for around 20 years, and several ligands of galectin-1, -3, and -8 have already been developed. Especially the development of galectin-3 inhibitors has received a lot of attention and has led to great results. At this time, two galectin-3 inhibitors have reached clinical trials. Thiodigalactoside GB0139 (**Figure 7**) has, in 2023,[10] concluded a phase 2b clinical trial investigating its efficacy.[63] GB0139 binds galectin-3 with a K_d of 2 nM and is administered to the lungs by a dry-powder inhaler to treat idiopathic pulmonary fibrosis. GB1211 is an orally available analogue of GB0139 that has completed two phase-1/2a clinical trials in 2023.[11,12] In this section, we will use the development of these two drug candidates as a guideline to describe a large part of galectin inhibitor development as it naturally introduces some discoveries that this thesis builds on.

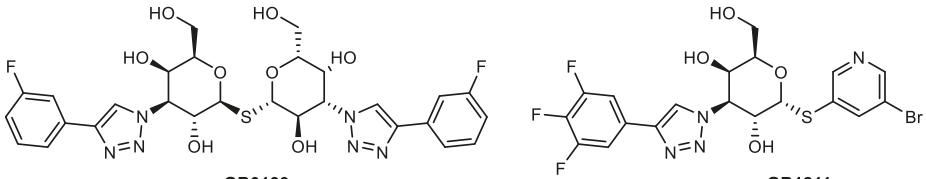


Figure 7. Two galectin-3 ligands that are currently in clinical trials.

A publication in 2002 described how *N*-acetyllactosamine disaccharide **1**,[64] a natural disaccharide binding subsite C and D of galectin-3 (K_d = 59 μM),[65] was optimised to occupy subsite B with a 3-methoxybenzamido-group (**2**, K_d = 2.5 μM)[66] or a [4-methoxy-2,3,5,6-tetrafluorobenzamido]-group (**3**, K_d = 0.88 μM)[67] on the galactose 3-position (**Figure 8**). The reported K_d's were determined using a fluorescence polarisation assay developed in the lab of Hakon Leffler and published a year later. This assay has since then been used to determine the affinities of all following ligands,[68,69] including those presented in this thesis. Further investigation of the disaccharide compounds led to the discovery of carboxynaphthalene lactoside **4** with a 0.32 μM affinity for galectin-3.

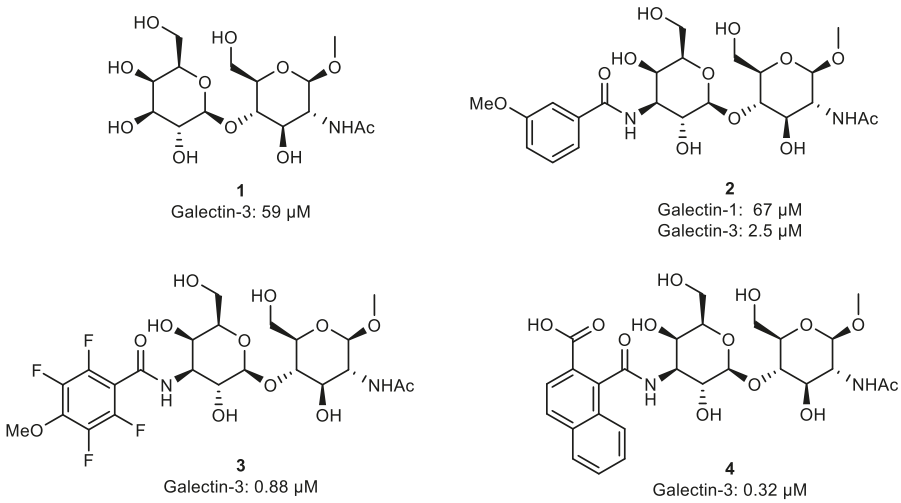
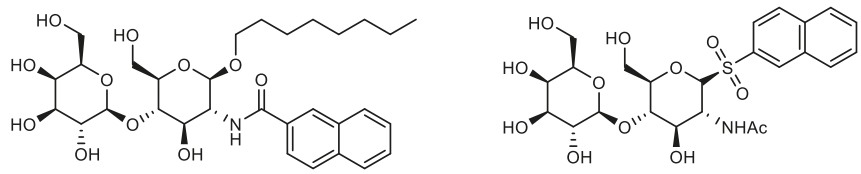


Figure 8. *N*-Acetyllactosamine disaccharides with increasing galectin-3 affinity.

The naphthalene, as found attached at the 3-position of the galactose in the *N*-acetyllactosamine of compound **4**, is also present in two other galectin inhibitors (**Figure 9**). Linked instead to the 1- or the 2- position of the glucosamine, these compounds also show a respectable affinity for their targets. Both published back

in 2006, one is the naphthamide-analogue **5** of *N*-acetyllactosamine **1** with a 6-fold increase in affinity for galectin-3.[70] The second is a 1-naphthalenesulfonyl **6** from the group of Roy with selectivity for galectin-1.[71] A potential problem with oligosaccharide-derived ligands, such as those presented above, is the hydrolytically labile glycosidic bond. This was addressed in the following discoveries involving monosaccharide ligands and thiodigalactosides connected by a sulfur atom at the anomeric carbon.



5
Galectin-3: 10.6 μM

6
Galectin-1: 40 μM
Galectin-3: 310 μM

Figure 9. Galectin-binding lactosides containing naphthalene.

The symmetrical thiodigalactoside (TDG) scaffold was introduced in 2005 (**Figure 10**) and would see the affinity of 3-methoxybenzamide **2** increase up to 50-fold for TDG analogue **7**, well into the nanomolar range.[72] The first group of monosaccharides targeting galectin-3 was reported in the same year and consisted of 3-triazolylgalactosides.[73] These were designed with a range of chemical moieties, including the benzylamide **8** with a 107 μM affinity for galectin-3. In 2006 the group of Roy also reported a monosaccharide O3-methyltriazole **9**, which showed 1.25 mM affinity for galectin-1 with selectivity over galectin-3, discovered en route to a trivalent lactoside with a 20 μM galectin-1 affinity.[74] The TDG ligands were analysed in detail,[75] and eventually combined with the 3-triazolyl functionality to give conjugate **10** with a 0.39 μM affinity for galectin-3, a 250-fold increase from the monosaccharide.[76] It is worth noting that these TDGs have high affinities for most other galectins, including galectin-1, but this trend does not appear as strong for galectin-8N affinity. Further research into the TDG class of compounds ultimately led to the discovery of **GB0139** (**Figure 7**),[9] with low nanomolar affinities for galectin-1 and -3 and a high pharmaceutical potential,[10] and dithiophene TDG **11** which is moderately selective for galectin-1.[77]

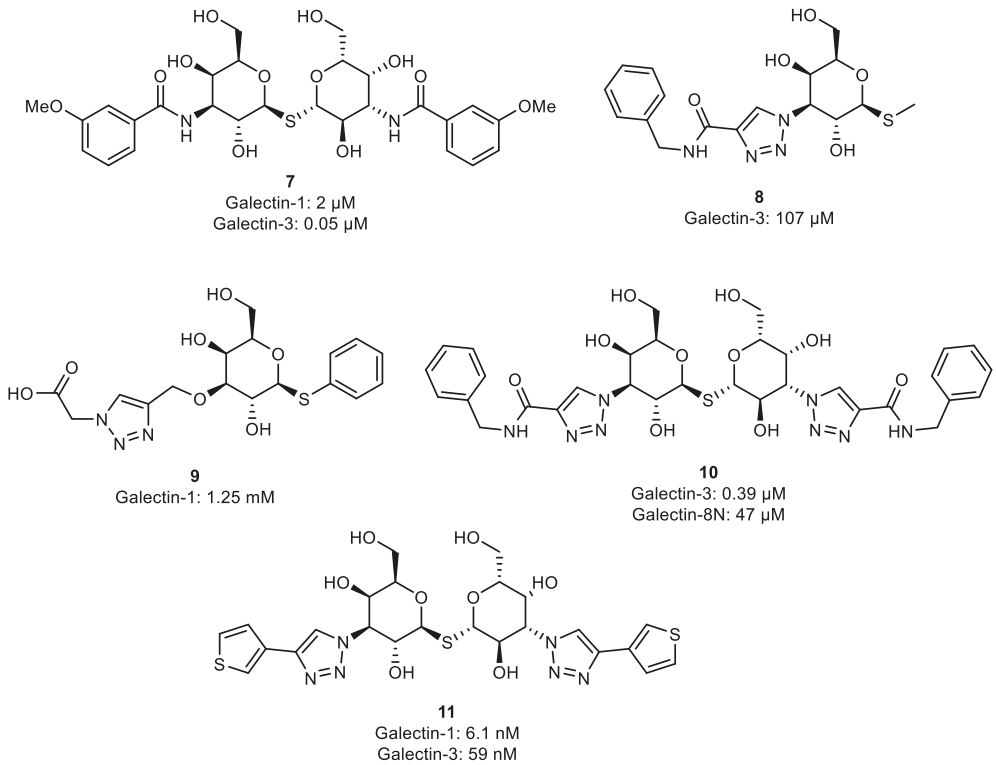


Figure 10. The first example of joining monosaccharides into digalactoside ligands, which eventually leads to very high-affinity ligands of galectin-1 and -3.

In the same years, the highest affinity TDG inhibitors were further investigated to attempt to create a drug with better solubility and bioavailability. For this purpose, the designed molecules were smaller and less polar monosaccharide ligands. β-Thiotoluene galactosides such as trifluorophenyl **12** were successfully used as screening ligands,[59] and showed promising passive permeability due to their lower polar surface area. A seemingly small adaptation provided great results with the inversion of the chirality of the anomeric position: compared to the β-analogue **12**, the α-thiotoluene analogue **13** had a much higher affinity for galectin-3 (**Figure 11**).[78] The α-dichlorothiophenyl **14** presented in the same paper even managed to reach a nanomolar affinity. Further optimisation of the aglycon moiety resulted in the discovery of **GB1211** in 2022,[12] which had a better hepatic stability compared to the chlorinated analogues and a more favourable *in vitro* safety profile. **GB1211** is the second small-molecule ligand in clinical trials targeting galectins.[79,80]

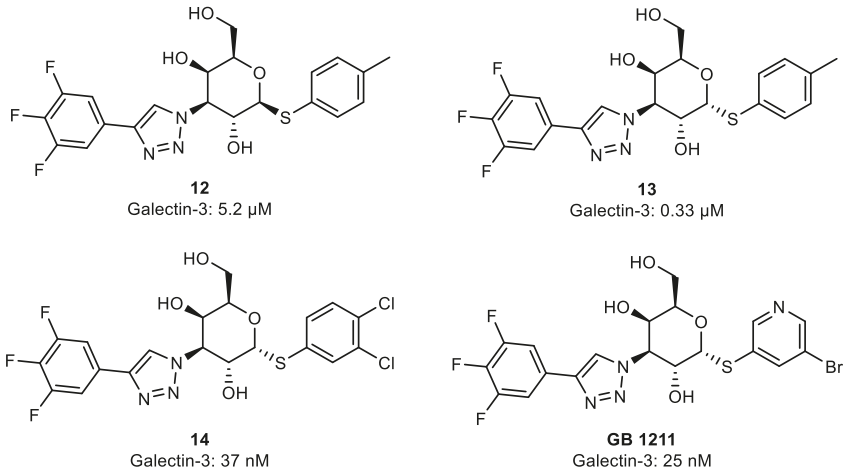


Figure 11. The optimisation of the aglycon part on trifluorophenyltriazole galactosides.

A separate branch of investigation, no doubt inspired by the impressive increase in affinity achieved through the optimisation of the aglycon on the anomeric position shown in **Figure 11**, continued to explore different galactose-C1 functionalisations. Often involving intriguing chemical modifications, especially for the sub-field of carbohydrate chemistry, examples of galactoside C1-heterocycles are shown in **Figure 12**. From the group in Lund came another set of novel triazoles. Aiming for selective binding to galectin-1 over galectin-3, C1 triazole **15**[81] and methyltriazole **16**[82] showed an equal galectin-1 affinity, although snapshots from their respective molecular dynamics simulations indicate these triazoles do not interact with the same amino acid residues. Others have also investigated the potential of galactose-C1 functionalisation. The group of Roy presented a range of interesting aglycons in 2008, including a set of 2-aminothiazole galactosides such as **17**.[83] Unfortunately, these ligands did not reveal the true potential of C1 functionalisation and the affinities of these ligands were low. Nonetheless, these molecules, perhaps together with the triazoles in **Figure 12**, may have inspired the development of a new orally available galectin-3 inhibitor **18** with the highest galectin-3 potency of any monosaccharide reported to date.[84]

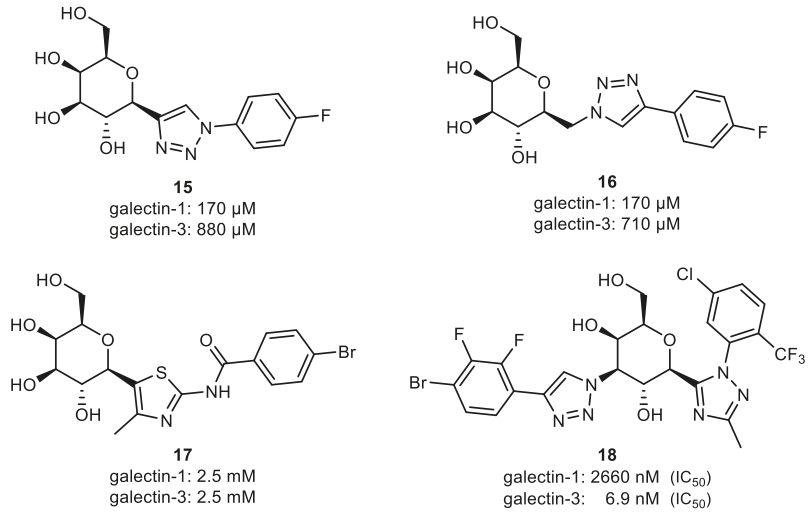


Figure 12. Development in anomeric heterocycles.

1.4.2 Ligands for Galectin-8N

In recent years, there has been a growing interest in inhibitors targeting galectin-8. Important and interesting structures that were found include the galactose–quinoline hybrid **19** (**Figure 13**), which showed a good affinity for galectin-8N already combined with a moderate selectivity over galectin-1 and -3.[53] The interesting galactose-O3-malonyl ester **20** was published in 2020 and showed promising galectin-8N antagonism in cell lines,[85] but no reported affinities for other galectins.

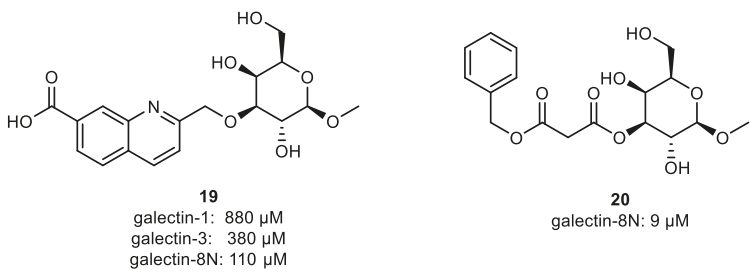


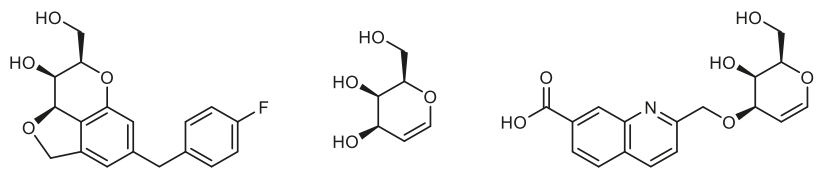
Figure 13. Galectin-8N inhibitors.

In a rough summary of all design strategies for galectin ligands discussed in this chapter, the optimisation depended on the functionalisation of the 1- and 3-position of galactose. Different galactose functionalisations appeared either as a group attached to the O3 of the galactose or by modification of this group into an amide

or a triazole to carry aromatic moieties further into the A,B-subsite of the galectin CRD. Investigations into the aglycon have led to a wide range of galactosides with interesting binding properties and some selectivity for different galectins. Broadly speaking, these C1 functionalisations include not only the precisely optimised aromatic groups such as those found in **GB1211** and galactoside **18**, but also the larger thiodigalactosides.

The last group of galectin ligands that require introduction have a more ingenious approach. These ligands have an altered monosaccharide core, hoping to improve even upon the highly conserved binding of the galactose scaffold in subsite C. Aiming to replace the galactose, these can also be called galactomimetics: chemical moieties that are not, strictly speaking, carbohydrates, but have some molecular properties that are similar enough to mimic their effective (binding) properties. Please note that the term "glycomimetic" generally includes all molecules that mimic carbohydrates,[86,87] including the decorated carbohydrates that have been discussed until now, but I like using the term galactomimetics for those molecules that have adaptations in the very hexose structure itself.

Published first, in 2020, an interesting tricyclic galactomimetic scaffold **21** (**Figure 14**) had relinquished a part of the galactose sugar stereochemistry with a benzene ring fused on C1 and C2.[88] With an additional cycle linked through the O3 and a peripheral 4-fluorobenzene, this ligand showed an admirable galectin-8N affinity and a slight selectivity over galectin-1. Discovered separately in our group and published in 2021 was the D-galactal **22**, which was expediently combined with the quinoline **19** to give the O3-functionalised D-galactal ligand **23**.[89] The quinoline–D-galactal showed a great affinity for galectin-8N, and also a promising selectivity over galectin-1 and -3.



21
galectin-1: 420 μM
galectin-3: 4100 μM
galectin-8N: 180 μM

22
galectin-1: 1600 μM
galectin-3: 3300 μM
galectin-8N: 1300 μM

23
galectin-1: 3600 μM
galectin-3: 590 μM
galectin-8N: 48 μM

Figure 14. Galactomimetic ligands targeting galectin-8N.

From the galectin ligands shown in this chapter, the research presented in this thesis has derived the most inspiration from the triazole compounds such as **12**, and quinoline–galactose **19** (which was published shortly before this project began) and the switch from galactose to D-galactal **22** (published during the early phases of this

project). Additionally, the previously successful strategies of incorporating the α-dichlorothiophenyl moiety (as observed for galactoside **14**) or transforming a monosaccharide ligand into its thiodigalactoside analogue (exemplified for *N*-acetyllactosamine **2** into TDG **7** and for monosaccharide **8** into TDG **10**) to boost the affinity of galectin ligands have been applied in this work.

The previous overview of the ongoing search for ever more potent galectin inhibitors, together with the following research manuscripts that build on all this history, hints at the true nature of medicinal chemistry; every step, however small, that improves ligand affinity, selectivity, or safety contributes to a more complete understanding of galectin inhibition.

2 Hypotheses

This thesis focuses on the discovery of galectin inhibitors and, in particular, on the discovery of selective ligands for galectin-8. At the onset of the project, the research has the character of a hit identification effort in the drug discovery pipeline. This means that, to some extent, we already have some structural information and some non-optimised ligands for galectin-8. These ligands have been the result of previous investigations into other galectins. At this stage, the main focus is to further study the galectin-8 target, to allow better ligands to be developed. This is a codependent stage of exploration and validation, where higher affinity ligands equally contribute to the understanding of what might make better ligands in future iterations.

Hypothesis 1

Benzoic acid and -ester derivatives of 3-triazolylgalactosides are a suitable scaffold to probe CRDs for increased affinity and selectivity for various galectins.

Based on the most contemporary galectin-8N targeting ligand **19** (see Chapter 1.4), a carboxylic acid group proved to be a favourable functionality on a heteroaromatic moiety placed on the O3 of galactose. To validate this discovery and to enhance its impact, we synthesised a focused library of benzoic acids with a variety of substitutions on a 3-(1,2,3-triazol-1-yl)galactoside scaffold. This scaffold was chosen to present the benzoic acids because of the proven applicability of the triazole heterocycle in galectin inhibitors. The intermediate benzoic esters were also purified for testing to better profile the contribution of the corresponding acids. The main goal was to confirm the added benefit of the carboxylic acid and to identify the optimal substitution on the heterocycle to create high-affinity ligands for various galectins.

Hypothesis 2

The investigation of phthalazinone structures on galactose C3 allows obtaining potent galectin-8N ligands with K_d values in the low micromolar range.

Galactoside structures have a preferred binding in subsite C. From there, any chemical moiety linked at the O3 is directed into the A,B-subsite of a galectin. As is described in detail in Chapter 1.4, the A,B-subsite of galectin-8N is a region with several amino acids—such as arginine-59—that are non-conserved among galectins. We conceptualised a ligand design that incorporates heterocycles bearing nitrogen and carbonyl groups. Placed directly adjacent to the linker to the galactoside, these electron-rich groups would be ideally placed to appease the electron-poor arginines that line the B-subsite. In our design, these heterocycles also served to direct an attached phenyl ring into a sub-pocket, adjacent to the A,B-subsite, which is unique for galectin-8N.

A phthalazinone scaffold was selected for this purpose and a set of related compounds were synthesised. Based on recent discoveries, D-galactal was chosen over β-D-galactoside structures for this compound library. Sub-structures of the phthalazinone–galactal conjugate were also synthesised for a dissection of the structure–affinity relationship of the phthalazinone scaffold. Ultimately, the phthalazinone scaffold was further decorated aiming to achieve ligands with K_d values in the micromolar range for galectin-8N and with an excellent selectivity over other human galectins.

Hypothesis 3

The screening of a 3-triazolylgalactoside-based compound library allows identification of potent galectin-1 or galectin-8N ligands.

Our further attempt was to screen the binding pockets of galectins for interactions that could induce a high affinity and, ideally, selectivity among these highly conserved binding pockets. For this purpose, a compound library with varied functional groups targeting the A,B-subsite of these galectins was constructed. The reliability and speed in the synthesis of the compound library were central. The synthetic approach revolved around the core structure, a tolyl 3-azido-1-thio-β-galactoside, and a late-stage diversification into the final compounds through ‘click’-chemistry and a facile deprotection. Click reactions were chosen for their renowned modular applicability, robust reaction conditions and high yields.

After synthesis of the library and determining the affinity for galectin-1, -3, and -8N, structure–activity relationships (SARs) were established to help identify beneficial substructures and functional groups. Additionally, one structure was further optimised to validate the capacity of the screening effort to identify substructures that can lead to potent ligands of these galectins. The optimised structures were also analysed in molecular dynamics simulations.

3 List of Methods

Organic synthesis

- Microwave-promoted synthesis
- Thin-layer chromatography (TLC)
- Reversed-phase chromatography
- High-performance liquid chromatography (HPLC)
- Mass spectrometry (MS)
- Nuclear magnetic resonance (NMR)

Computational methods

- Structure-based virtual screening (OpenEye Scientific Software)
- Molecular modelling, docking, ligand-protein complex analysis (OpenEye Scientific Software, Schrödinger, Inc., PyMOL Molecular Graphics System)
- Molecular dynamics simulations (Desmond, Schrödinger, Inc.)
- Molecular drawings (ChemDraw®; PerkinElmer Informatics, Inc.)
- X-ray crystal structure refinement (CCP4i, CCP4i2, Xia2, Aimless, Refmac5, Coot, Molprobity)
- Analysis of NMR spectra (MestReNova; Mestrelab Research S.L.)

In vitro biological and physicochemical evaluation

- Competitive fluorescence anisotropy assay
- Chemical stability (by LC-UV)
- $LogD_{octanol/water}$ (by LC-UV)
- Human blood-plasma stability (by LC-MS/MS)
- Human and mouse microsomal stability (by LC-MS/MS)
- Parallel Artificial Membrane Permeability Assay (by LC-UV)

4 Results

List of Papers

Paper I: **Benzimidazole–Galactosides Bind Selectively to the Galectin-8 *N*-Terminal Domain: Structure-Based Design and Optimisation.**

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. *Eur. J. Med. Chem.* **2021**, *223*, 113664.

Contributions The project was conceptualised together with the co-authors. M. Hassan, J. Dernovšek, F. Baussière and I performed the synthesis, purification, and characterisation of all compounds. I performed the docking, docking analysis, and created the images. The X-ray crystallisation was performed by M. Hassan, M. Håkansson, and R. Kovačič. Molecular dynamics simulations were performed by A. P. Sundin. Together, M. Hassan and I wrote the manuscript, and the co-authors edited it.

Paper II: **Design and Synthesis of Novel 3-Triazolyl-1-Thiogalactosides as Galectin-1, -3 and -8 Inhibitors.**

Van Klaveren, S.; Dernovšek, J.; Jakopin, Ž.; Anderluh, M.; Leffler, H.; Nilsson, U. J.; Tomašič, T. *RSC Adv.* **2022**, *12* (29), 18973–18984.

Contributions: The project was conceptualised together with the co-authors. Together, J. Dernovšek and I performed the synthesis, purification, and characterisation of all compounds. I performed the docking, the molecular dynamics simulations, and created the images. I wrote the manuscript, and the co-authors edited it.

Paper III: Selective Galectin-8N Ligands: The Design and Synthesis of Phthalazinone-D-Galactals.

Van Klaveren, S.; Sundin, A. P.; Jakopin, Ž.; Anderluh, M.; Leffler, H.; Nilsson, U. J.; Tomašič, T. *ChemMedChem* **2022**, *17* (6), e202100575.

Contributions: The project was conceptualised together with the co-authors. I performed the synthesis, purification, and characterisation of all compounds. Together, B. Kahl-Knutson and I performed the fluorescence polarisation assays. I performed the molecular dynamics simulations and the binding pose metadynamics was performed together with A. P. Sundin. I wrote the manuscript, and the co-authors edited it.

Paper IV: Halogenated Galactal–Phenylphthalazinone Hybrids as Highly Selective Galectin-8N Ligands

Van Klaveren, S.; Hassan, M.; Håkansson, M.; Johnsson, R. E.; Larsson, J.; Jakopin, Ž.; Anderluh, M.; Leffler, H.; Tomašič, T.; Nilsson, U. J. *Manuscript.*

Contributions: The project was conceptualised together with the co-authors. I performed the synthesis, purification, and characterisation of all compounds. I performed the molecular dynamics simulations. Together, M. Håkansson and I performed the crystallisation of the galectin-8N–ligand complex. I built, refined, and deposited the X-ray crystal structure (PDB ID: 8CM8) in the RSCB Protein Data Bank. I performed the physicochemical and *in vitro* ADME analyses. I wrote the manuscript, and the co-authors edited it.

4.1 Benzimidazole–Galactosides Bind Selectively to the Galectin-8 *N*-Terminal Domain: Structure-Based Design and Optimisation

Publication

This chapter has been published as a research article in the *European Journal of Medicinal Chemistry*. The article authors and identifiers are as follows:

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. *Eur. J. Med. Chem.* **2021**, *223*, 113664.

Content

The dual goal of the scientific paper that comprises this chapter was (1) a systematic investigation of a carboxylic acid placed in the A-subsite of galectin-8N, and (2) using this SAR together with our X-ray crystal structure data to design optimally functionalised galectin-8N ligands. The first of these goals aligns with the premise of Hypothesis 1: to probe the CRD of galectins using the 3-triazolylgalactoside scaffold, and the second goal expands upon the SAR thus obtained.

The research and results presented in this publication are presented in support of Hypothesis 1.

4.2 Design and Synthesis of Novel 3-Triazolyl-1-Thiogalactosides as Galectin-1, -3 and -8 Inhibitors

Publication

This chapter has been published as a research article in the *RSC Advances* journal. The article authors and identifiers are as follows:

Van Klaveren, S.; Dernovšek, J.; Jakopin, Ž.; Anderluh, M.; Leffler, H.; Nilsson, U. J.; Tomašič, T. *RSC Adv.* **2022**, *12* (29), 18973–18984.

Content

The goal of the scientific paper that comprises this chapter was to synthesise a library of 3-triazolylgalactosides and to screen for their binding affinities against galectin-1, -3, and -8N. This allowed for several SARs to be deduced, and one of the hits was further optimised to reach sub-micromolar affinities. The optimised ligands were further analysed using molecular dynamics simulations to identify and further analyse their binding poses.

The research and results presented in this publication are presented in support of Hypothesis 3.

4.3 Selective Galectin-8N Ligands: The Design and Synthesis of Phthalazinone-D-Galactals

Publication

This chapter has been published as a research article in the *ChemMedChem* journal. The article authors and identifiers are as follows:

Van Klaveren, S.; Sundin, A. P.; Jakopin, Ž.; Anderluh, M.; Leffler, H.; Nilsson, U. J.; Tomašič, T. *ChemMedChem* **2022**, *17* (6), e202100575.

Content

The goal of the scientific paper that comprises this chapter was to explore the potential of phthalazinone-based ligands with a D-galactal glycomimetic core. It led to the discovery of galectin-8N ligand with a 100 μM affinity and good selectivity over other galectins. This investigation was compounded by a detailed SAR of the components of the phthalazinone scaffold.

The research and results presented in this publication are presented in support of Hypothesis 2. Further investigation on the optimisation of the phthalazinone ligand class is described in Chapter 4.4.

4.4 Halogenated Galactal–Phenylphthalazinone Hybrids as Highly Selective Galectin-8N Ligands

Publication

This chapter has been prepared as a research article which has been submitted to the *ACS Medicinal Chemistry Letters* journal. The article authors are as follows:

Van Klaveren, S.; Hassan, M.; Håkansson, M.; Johnsson, R. E.; Larsson, J.; Jakopin, Ž.; Anderluh, M.; Leffler, H.; Tomašič, T.; Nilsson, U. J., *Manuscript.*

Content

The goal of the scientific manuscript that comprises this chapter was to investigate the functionalisation of the phthalazinone-D-galactals to increase their affinity and selectivity for galectin-8N. Halogenated analogues were made with the ability to occupy a sub-pocket that is specific for this galectin, the best of which had an affinity of 25 μM and at least 60-fold selectivity over galectin-4C. No affinity was found for any of the other tested galectins. The discovery was substantiated with an X-ray crystal structure and relevant ligands were tested for some physicochemical and *in vitro* ADME properties, to facilitate further development of related galectin-8N inhibitors.

The research and results presented in this publication are presented in support of Hypothesis 2.

5 Discussion

5.1 The first selective and μM-affinity ligands for galectin-8N

Ligand-based screening

The search for galectin-8 ligands with optimised affinities started with a ligand-based screening of potential interactions, as described in Chapter 4.1. The ligands in the screening library (**Figure 15**) were based on a triazolyl scaffold that is common in galectin inhibitors (see **Figure 10** in Chapter 1) but with slight variations. Ligands targeting the galectin-8N CRD have often benefitted from presenting a carboxylic acid moiety into the subsite A of the binding pocket, showing higher affinities than their non-functionalised analogues.[53] One goal was to identify the optimal location of the carboxylic acid on the scaffold. To this end, the library covered all possible positions (*ortho*-, *meta*-, *para*-) of carboxylic acids (Chapter 4.1, **10a–f**) and their corresponding methyl esters (Chapter 4.1, **9a**–**f**). Additionally, the combined interaction engaging also with subsite D was investigated by comparing the affinities of ligands with a small β-methoxy moiety on the anomeric carbon of the galactose (Chapter 4.1, **9a**–**c**, **10a–c**) with those bearing the larger β-thiotoluene group (Chapter 4.1, **9d**–**f**, **10d**–**f**). Using an established fluorescence polarisation assay,[68,69] the ligands were tested for their affinities for galectin-3 and -8N. Most ligands had a slight preference for galectin-3 with affinities between 200 μM and 1000 μM, compared to the K_d's between 650 μM to 1500 μM found for galectin-8N. To inspect the differences in the binding poses and interactions of the benzoic acids and esters, we applied a molecular docking approach to virtually place the ligands in the binding pocket of galectin-8N. The structural basis for these docking studies was the X-ray crystal structure of galectin-8N with the quinoline–galactose ligand (PDB ID: 7AEN), which was published as part of this paper. The SAR revealed that, overall, the benzoic acids had better affinities than the methyl benzoate compounds. Additionally, the *meta*-substituted benzoic acid analogues performed better than the *ortho*- and *para*-analogues, indicating that this position facilitates interactions in the subsite A. The β-anomeric functionalisation targeting subsite D did not reveal a clear preference for galectin-8N, but did show that the β-methyl galactosides had a slight preference—affinity wise—for galectin-3.

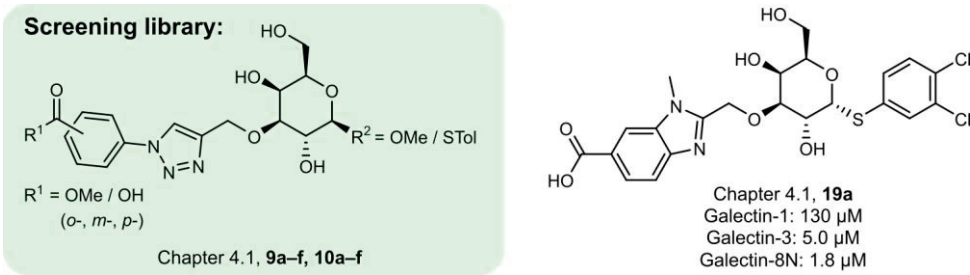


Figure 15. Overview of the synthesised screening library and the optimised ligand presented in Chapter 4.1.

Benzimidazole–galactosides

The further work described in Chapter 4.1 makes use of the SARs derived from the library of 3-triazolylgalactosides. A parallel ligand-based virtual screening identified benzimidazoles, amongst other privileged heterocyclic structures, as the most suitable scaffold to occupy subsite B of galectin-8N. This benzimidazole scaffold, joined with the optimal orientation of the carboxylic acid identified in the SAR, led to the discovery of the 6-carboxy-benzimidazole–galactoside (Chapter 4.1, **16a**) as a 7.4-fold selective galectin-8N ligand (190 μM affinity compared to 1400 μM for galectin-3). Further optimisation on the α-anomeric position with a known 3,4-dichloro aglycon (**Figure 15**) gave an analogue (Chapter 4.1, **19a**) with a high affinity for galectin-8N (1.8 μM), but only a 2.8-fold selectivity over galectin-3 (5 μM).

The discovery of selective and μM affinity ligands for galectin-8N in Chapter 4.1 is a direct result of the investigation of the binding pocket with benzoic acid and ester derivatives of 3-triazolylgalactosides. The suitability of these derivatives to probe CRDs of galectins is demonstrated by the increased affinity and selectivity of the benzimidazole–galactosides (Chapter 4.1, **16a** and **19a**).

5.2 A library for screening galectin-1, -3, and -8N

Structure-based virtual screening

While the research presented in Chapter 4.1 was ongoing, we also initiated a structure-based investigation of the binding pockets of three galectins presented in the paper in Chapter 4.2. From a targeted ligand library for galectin-1, galectin-3, and galectin-8N, we aimed to uncover those features in the CRDs that would allow us to create high-affinity ligands for any or all of these three galectins. Additionally, we hoped to achieve selectivity among these galectins. As presented in Chapter 4.2, a virtual library of ligands was created (**Figure 16**). The ligands were selected based

on their ease of synthesis from available starting materials. This library was then screened *in silico* by employing the X-ray crystal structures of galectin-1, -3, and -8N. From this screening, a subset of the ligands was selected for synthesis.

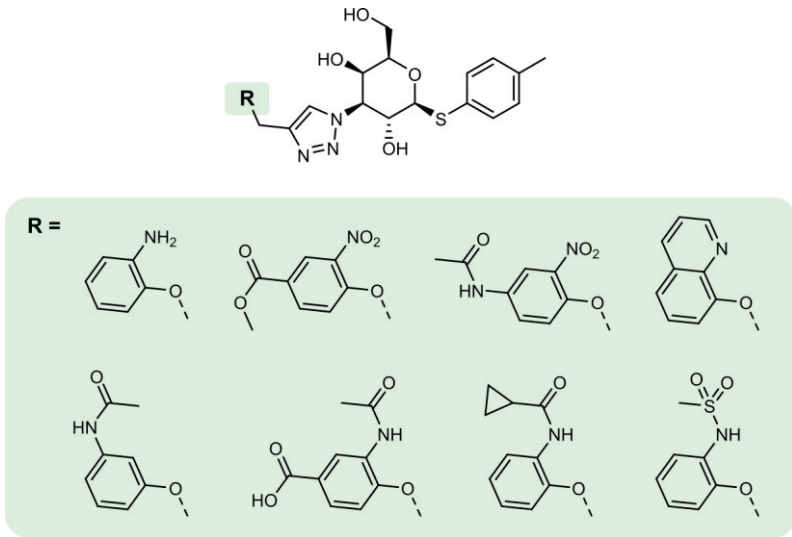


Figure 16. Examples of compounds in the ligand library in Chapter 4.2.

Placed at the onset of the research project presented in this dissertation, the ease of synthesis and ready availability of the starting materials were selected as the most important factors in this sub-project to allow greater speed in the synthesis of the selected library. In this way, quickly attained research results such as binding affinities and any discovered SARs could create an informed repository of galectin inhibition and potential features to be targeted and exploited. In total, 29 ligands were synthesised with relative ease and in a short time, and their binding affinities were obtained using the same fluorescence polarisation assays applied previously.[90] Several compounds showed binding affinities well below 1 mM, which served as good hit compounds for further analysis.

Beneficial structural motifs were identified on several ligands, for example, the *ortho*-amido and nitro group functionalisations proved generally favourable for each of the galectins. For galectin-3, introducing carboxylic acid increased the affinity and selectivity over the other two tested galectins, which was not seen for the corresponding methyl ester analogues. This observation is consistent with the findings presented in Chapter 4.2, where an increase in binding affinities for galectin-3 is observed after hydrolysis of benzyl esters ligands to the corresponding benzoic acids.

Ligand optimisation and binding analysis

To further validate the approach described in Chapter 4.2, an *ortho*-acetamide analogue (Chapter 4.2, **36**) was selected for further optimisation emulating two known galectin-targeting ligands presented previously in Chapter 1.4 (**Figure 10** and **Figure 11**). The two optimised ligands (**Figure 17**) showed an improved affinity for each of the three galectins compared to the parent galactoside. For galectin-8N, the affinity increased from virtually non-binding (K_d > 1500 μM) to an impressive 100 μM for the dichlorothiophenyl monosaccharide (Chapter 4.2, **59**). Compared to the parent compound (Chapter 7, **36**), the symmetrical thiodigalactoside analogue (Chapter 4.2, **62**) showed an over 100-fold and 1000-fold higher affinity for galectin-1 and galectin-3 respectively, with affinities in the (sub)-micromolar range.

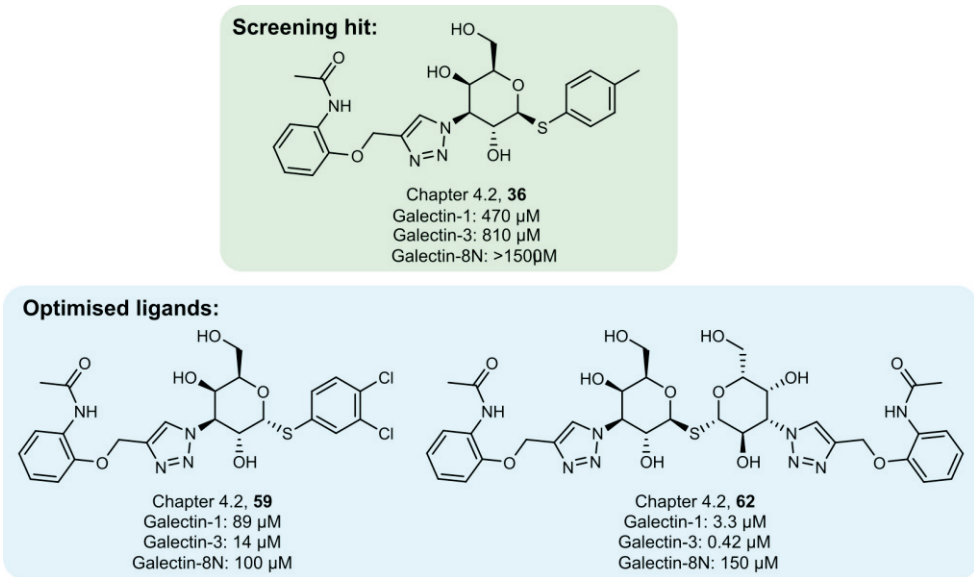


Figure 17. The ligands, with corresponding binding affinities, in the hit optimisation in Chapter 4.2.

These high-affinity ligands were analysed in molecular dynamics (MD) simulations to produce binding interaction analyses (Chapter 4.2, **Figure 3**) based on which representative MD snapshots were selected for display in the manuscript (Chapter 4.2, **Figure 4**). Amongst the most interesting findings were the different binding modes of the two optimised ligands in the CRD of galectin-3. The thiodigalactoside scaffold (Chapter 4.2, **62**) reveals a potential for the arginine-144 to engage in hydrogen bonding with the electron-rich triazole heterocycle, whereas the monosaccharide analogue (Chapter 4.2, **59**) shows that the same arginine can be displaced to accommodate the phenylacetamide in an induced-fit binding mode, also

observed for GB0139 (Chapter 1.4, **Figure 7**). Another important discovery, which has also impacted our following projects, was gathered from the binding modes observed in the MD simulations with galectin-8N. Both optimised ligands showed a strong interaction of the electron-rich triazole with arginine-59 (which is positioned opposite arginine-144 in galectin-3, respectively) in subsite B. The phenylacetamide moiety of the ligands is then found extending into a sub-pocket, adjacent to the A,B-subsites, which is not as well defined in the other two galectins. In this sub-pocket, the phenylacetamide is observed interacting, through π-stacking interactions, with both the arginine-59 and the tyrosine-141. These two amino acids, as the sub-pocket which they form, are specific for galectin-8N, and therefore a potential feature that can be used to impose selectivity among the three studied galectins. The successful development of selective galectin-8N ligands described below supports this approach and thereby supports the hypothesis that the 3-triazolylgalactoside-based compound library helped to identify potent ligands which are, additionally, selective for galectin-8N.

5.3 Phthalazinone-based ligands

Electron-rich scaffolds

The subproject of phthalazinone-based ligands, which spans Chapter 4.3 and Chapter 4.4, started from a structure-based perspective, with a visual inspection of the binding site of the galectin-8N CRD in the X-ray crystal structure with the quinoline–galactoside(PDB ID: 7AEN) presented in the paper in Chapter 4.1. Particularly the subsite B, directly adjacent to the conserved galactose binding subsite C, has a very interesting characteristic that it is made to bind a chemical group between two arginine residues. These residues are cationic, and therefore electron deficient. With this awareness, we suggested investigating ligands which would present electron-rich heteroaromatic scaffolds into subsite B, similar to the triazole binding to arginine-59 described above in Chapter 4.2. Structures with multiple carbonyl groups and/or ring-nitrogens were considered and, after a structure-based investigation, phthalazinone and uracil scaffolds were selected to be incorporated in ligands targeting the galectin-8N binding pocket.

Phthalazinone–galactals: Methylene linker synthesis

In the designed ligands, the traditional D-galactose was replaced with D-galactal. This smaller and less polar glycomimetic, which had only shortly before been discovered in our group,[89] has a 4-fold higher affinity for galectin-8N than the comparative methyl β-D-galactoside and an increased selectivity over galectin-3. The two proposed parts of the ligand, i.e., the heterocycle and the D-galactal, would ideally be joined with a methylene linker, -CH_2-, between an aromatic nitrogen and

the O3 of D-galactal. Longer linkers did not show a promising binding mode in prospective MD investigations. However, the synthesis of the methylene bridge was not very straightforward and became the subject of some experimentation.

Out of various synthetic approaches, two procedures were successful for fashioning the methylene linker. The first is a single-step approach, in which formaldehyde–sodium bisulfite-adduct reacts with the phthalazinone heterocycle to directly give the corresponding methylchloride. The reaction conditions are harshly acidic, refluxing the mixture in thionyl chloride.[91] This synthesis did proceed reliably but with rather low yields, ranging between 38–46%. An alternative two-step approach was deemed superior.[92] With, first, the instalment of an *N*-methyl pivalate using chloromethyl pivalate in standard basic conditions (sodium hydride in THF, or caesium carbonate in DMF), and second, substitution of the pivalate with a bromine, the *N*-methylbromide phthalazinone analogues were obtained with 63–83% overall yield. Besides the higher overall yield, the reaction conditions were more amenable and had simpler work-up procedures. Each of the halogenated intermediates would be attached to the D-galactal using a dibutyltin-mediated, O3-selective alkylation.[93] In this reaction, the dibutyltin forms an *in situ* complex bridging two specific alcohols of the hexopyranose; the geometric alignment of the tin coordinating simultaneously with the equatorial O3 and the axial O4 of galactal (or galactose) is favourable over alternative complexes and induces a higher reactivity specifically for the O3 to act as a nucleophile.[94] The nucleophilic attack is the actor in the nucleophilic substitution of bromide on the phthalazinone–methylbromide which produces the final compounds.

The investigation of the phthalazinone structure

The synthetic approaches described above were used to link a range of heterocycles to the galactal structure. Based on the binding affinities of the linked phthalazinones and related (sub)structures, we were able to identify those parts of the heterocycle which contribute to the binding affinity or selectivity of these ligands for galectin-8N. The SAR (**Figure 18**) reveals a continuous increase in galectin-8N affinity in linking the small pyridazinone heterocycle to D-galactal and the subsequently adjoined phenyl rings (Chapter 4.3, **Figure 4**). The addition of the 4-phenyl ring to phthalazinone (Chapter 4.3, **16**) to give 4-phenylphthalazinone (Chapter 8, **5**), has an almost negligible increase of affinity from 120 μM to 100 μM, for galectin-8N. However, this modification is tremendous for the selectivity; the affinity for galectin-1 drops from 210 μM to 3000 μM, and for galectin-3 from 290 μM to an affinity that is too low to be determined in our fluorescence polarisation assay. This display of selectivity is exceptional for galectins, and in this discovery, it can be attributed to the presence of a single phenyl group occupying a sub-pocket that is special for galectin-8N. The significance of the glycomimetic moiety in the phthalazinone–galactal ligands was confirmed by comparing it against the

phthalazinone–methyl β-galactoside (Chapter 4.3, **18**), as a reassuring three-fold higher affinity was observed for the D-galactal analogue (Chapter 4.3, **5**).

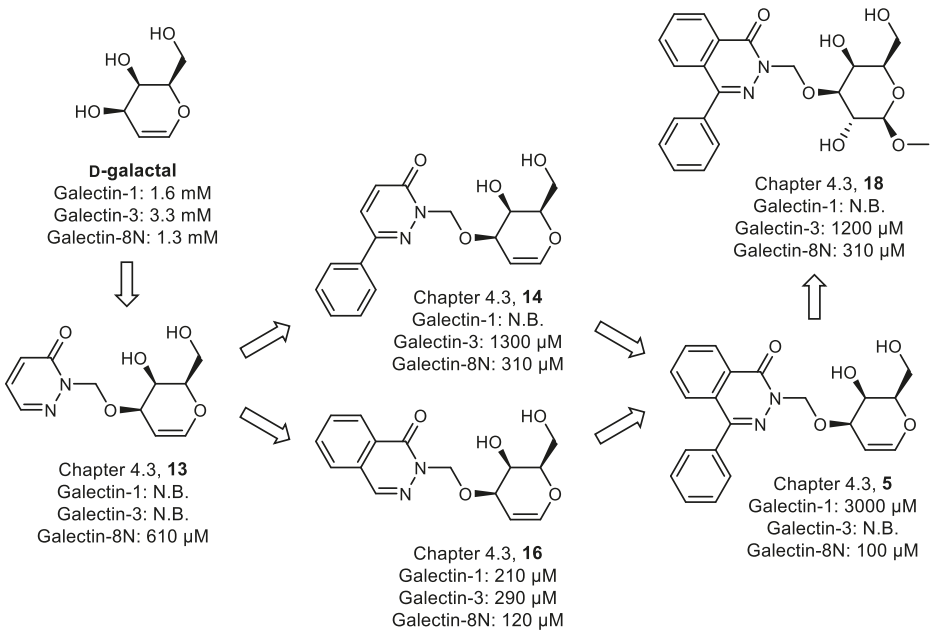


Figure 18. The structure–activity relationship of the 4-phenylphthalazinone (sub)structures.

The galectin-8N sub-pocket

Naturally, we wished to explore the galectin-8N CRD and the sub-pocket that allowed ligands to achieve such differences in affinity over other galectins. The sub-pocket, adjacent to the B-subsite, is formed by arginine-59 and tyrosine-141 and capped with the S3-S4 loop (**Figure 19A**). This loop connects strand 3 to strand 4 of the β-sheet on the S-face of the carbohydrate recognition domain. The S3-S4 loop is relatively flexible, as observed by the higher B-values in crystal structure data, and carries the arginine 59 as well as residue 56 which exists in two different point mutations (i.e., valine-56 or methionine-56) in different galectin-8 isoforms.[95] At the same location in other galectins, both the arginine-59 and tyrosine-141 are different amino acids and, for example in galectin-1 and -3, the S3-S4 loop consists of fewer residues, making the loop shorter and less flexible. This explains why the sub-pocket adjacent to the B-subsite is so specific for galectin-8N.

Molecular dynamics simulations and binding pose metadynamics analysis

To further investigate the galectin-8N ligands that achieved such remarkable selectivity, we turned to MD simulations. With the galectin-8N crystal structure

from Chapter 4.1 (PDB ID: 7AEN), the quinoline–galactose ligand was replaced with the 4-phenylphthalazinone–galactal (Chapter 4.3, **5**). The dynamics of the protein–ligand complex were simulated over a 1000 ns timespan. During the simulation, it was observed that the aromatic part of the ligand moved out of the predicted binding pose after around 200 ns to adopt a new binding pose in which the ligand–protein complex persisted for the remainder of the simulation (**Figure 19B**). In the alternative binding pose, the aromatic group would rotate out of the sub-pocket, allowing arginine-59 to reposition and engage a cation–π stacking on the 4-phenyl group from the opposite side as it was stacking originally (Chapter 4.3, **Figure 5**). This is, by the nature of the required motility of the protein binding pocket to accommodate the ligand, an induced-fit binding mode.

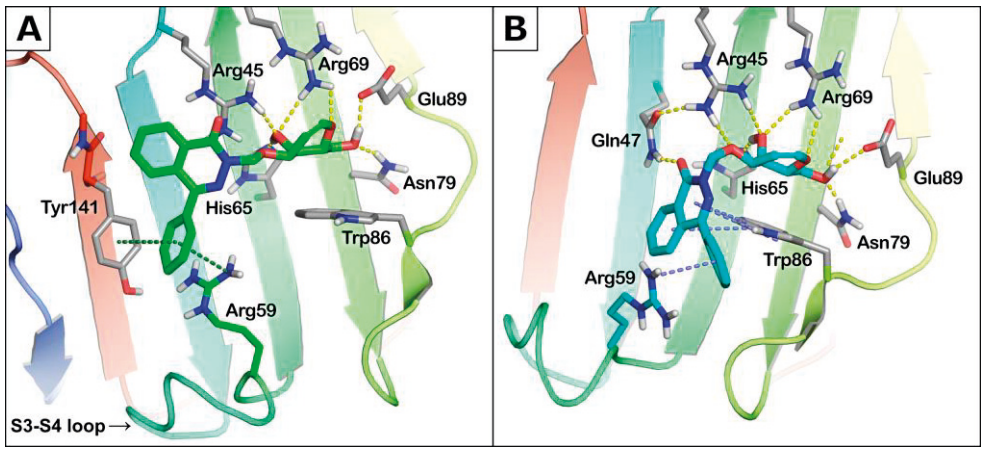


Figure 19. Molecular dynamics snapshots showing A: the predicted binding pose and B: the induced-fit binding pose of the 4-phenylphthalazinone–galactal ligand revealed during the simulations.

A relatively novel approach in computational chemistry was applied to be able to quantify and compare the two binding poses of the 4-phenylphthalazinone–galactal ligand in the galectin-8N binding pocket. Binding pose metadynamics analysis was initially developed to validate *in silico*-predicted ligand docking poses.[96] Instead, in the paper in Chapter 4.3, we applied this method to evaluate different binding poses of the same protein–ligand complex, as postulated by MD simulations, and to compare them. For the metadynamics, the ligand is placed in either of the two likely binding poses identified during the MD run. In reiterative simulations, ligand–protein complexes were then forced to increased motility by an included energy penalty for previously observed poses. A ligand (binding) pose that continues to persist under stressed conditions is expected to have a higher contribution to the overall ligand binding event. This persistence is expressed in two parameters which enumerate the movement of the ligand and the preserved hydrogen bonding

interactions. Based on these parameters and the longer residence time observed in the MD simulation, our metadynamics analysis concluded that the induced-fit binding mode is likely found to a greater extent than the originally expected binding mode in which the 4-phenyl group occupies the galectin-8N specific sub-pocket.

Halogenated 4-phenylphthalazinones

The induced-fit was not observed for the analogues of 4-phenylphthalazinone–galactal that were optimised (with halogens, mainly) on the *para*-position of the phenyl, presented in Chapter 4.4. These ligands were investigated because additional space was observed protruding past the 4-phenyl substituent in the galectin-8N-specific sub-pocket in the originally expected binding mode. This additional space could potentially be 'filled' with a space group on the *para*-position on the phenyl, according to *in silico* structural exploration. The synthesis of the decorated phenylphthalazinones (Chapter 4.4, **6**–**10**) was performed through the two-step methylbromide installation on the respective phthalazinones, followed by the protecting-group free, tin-mediated alkylation of the galactal. If not commercially available, the phthalazinone was first synthesised from the precursor 2-benzoylbenzoic acid by an efficient hydrazine cyclocondensation.[97]

From the affinities determined for the ligands of Chapter 4.4, a convincing SAR could be constructed by relating the size, expressed as the Van der Waals radius, of the atom or group attached at the *para*-position of the 4-phenyl group. From the SAR, we derived that the size of the ligand might be dictating the affinity of the ligands, as the methyl-substituted ligand (Chapter 4.4, **9**) showed a comparable affinity to the bromide analogue (Chapter 4.4, **10**) being about equal in size. These two ligands showed the highest affinity for galectin-8N of all the phthalazinone–galactal ligands presented in this work, with the 4-(*p*-bromophenyl)phthalazinone–galactal showing an impressive 25 μM affinity in addition to an almost complete selectivity profile over all other tested human galectins. The high affinity and selectivity profile of these compounds is a strong support for the second hypothesis in this dissertation.

X-ray structural analysis of a galectin-8N:phthalazinone complex

We continued our investigation of the obtained galectin ligands to facilitate any further potential research. For the newly obtained 4-(*p*-bromophenyl) phthalazinone–galactal (Chapter 4.4, **10**), a cocrystal structure with the ligand in the binding pocket of galectin-8N was obtained by soaking the ligand into a galectin-8N–lactose complex. The protocol was based on the same conditions that had produced the quinoline–galactoside structure published in Chapter 4.3.[90] The X-ray crystal structure of the protein–ligand complex was solved at a resolution of 1.22 Å (PDB ID: 8CM8, **Figure 20**) and gave us strong evidence to support the binding mode of the phthalazinone–galactal ligand with the *p*-bromophenyl group positioned in the specific sub-pocket of galectin-8N. The structural data of this

25 μM affinity ligand (presented in Chapter 4.4), underpinning the SAR that was established (in Chapter 4.3), is supportive of the rationale with which these phthalazinone-based structures targeting galectin-8N were designed. That is, to present heteroaromatic structures with carbonyl groups and ring-nitrogens into subsite B from the C3-position of a galactose (or, more opportunely, a galactal) leads to potent, high-affinity ligands of galectin-8N.

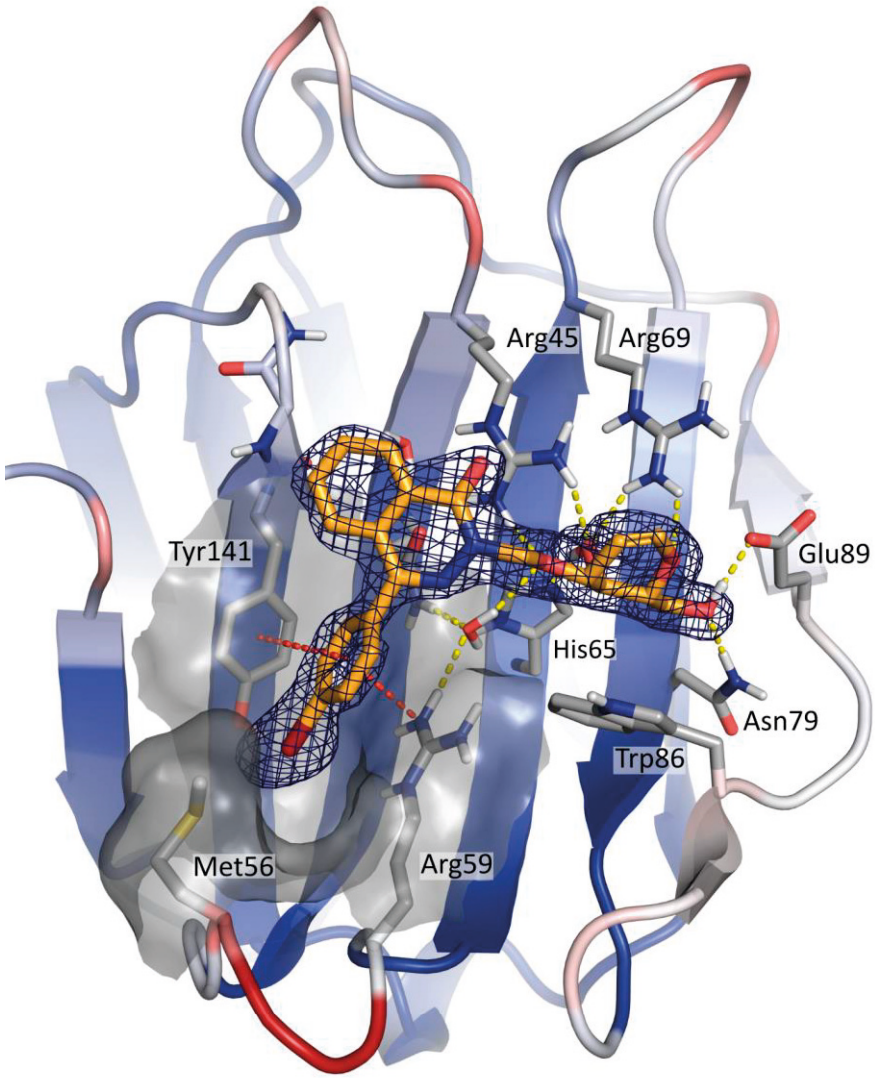


Figure 20. The crystal structure of galectin-8N in complex with the 4-(*p*-bromophenyl)phthalazinone–galactal **10** shown with its electron density map (PDB ID: 8CM8).

6 Conclusion

The different strategies and approaches that were selected and developed for the synthesis of a range of galactose-based and galactomimetic ligands and their detailed interaction analyses have been described in the previous chapters. A wide range of (rapid) synthesis strategies, virtual screening efforts, and molecular dynamics analyses, amongst many others (including *in vitro* ADME-Tox not included in this dissertation), have been reared and combined to further this project and its findings.

We have constructed two different libraries of galectin-targeting ligands following ligand-based and structure-based approaches, which has provided the informative background enabling the discovery of a third library of galactal–heterocycle ligands. Both uracil and phthalazinone representatives in this series showed a high selectivity for galectin-8N ligands, and the latter were further optimised to show a micromolar affinity with an almost complete selectivity profile over all other tested galectins. The hypotheses rely on creating a supporting information repository, through probing galectin-Carbohydrate Recognition Domains and the screening of compound libraries. These hypotheses are confirmed by the succeeding discovery of potent, selective phthalazinone inhibitors, which thereby also support the remaining hypothesis.

Hypothesis 1

Benzoic acid and -ester derivatives of 3-triazolylgalactosides are a suitable scaffold to probe CRDs for increased affinity and selectivity for various galectins.

This hypothesis is confirmed. We synthesised a library of benzoic acid and -ester derivatives of 3-triazolylgalactosides and used these ligands to probe the CRDs of galectin-3 and galectin-8. Based on the affinities and selectivities observed for the variations on this scaffold, selective and high-affinity ligands were developed. The results were reported in an original research article.

Hypothesis 2

The investigation of phthalazinone structures on galactose C3 allows obtaining potent galectin-8N ligands with K_d values in the low micromolar range.

Phthalazinone structures placed on the C3-position of galactose and galactal were investigated. Further optimisation of these ligands resulted in potent ligands, with high galectin-8N selectivity and micromolar affinity. This is in support of the hypothesis. The results were reported in two original research articles.

Hypothesis 3

The screening of a 3-triazolylgalactoside-based compound library allows identification of potent galectin-1 or galectin-8N ligands.

This hypothesis is confirmed. A 3-triazolylgalactoside-based compound library was designed and synthesised, which allowed the identification of important structural motifs. These findings directly led to the discovery of the potent and selective phthalazinone-based inhibitors of galectin-8N.

7 Concluding remarks

Our research of the past few years, which has culminated in the production of this doctoral dissertation, has focussed on the discovery and development of galectin ligands. Such ligands, acting as inhibitors, could block many or some specific functions of galectins and influence their natural biological behaviour in cell-cell and cell-matrix interactions, altering and enhancing the communication on the cell surface. Acting to influence galectins in pathophysiological settings, galectin inhibitors might see a potential future application in organ transplantation and wound healing, or high-impact fields such as immunotherapy or cancer treatments. More closely on the horizon, these ligands could be used, or inspire the development of research probes which allow us to further research the intricate roles of galectins. Ligands with high inter-galectin selectivity are especially promising as they might allow us to dissect the seemingly complex and overlapping function of this family of proteins.

The galectin ligands discovered as part of this research are built upon the results of giants and may, in the future, have their biggest impact be to inspire another generation of ever-more enhanced inhibitors of galectins with a valuable pharmaceutical application.

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