Biotechnology Advances 58 (2022) 107928

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

# Methods of *in vitro* study of galectin-glycomaterial interaction

Viktoria Heine [a,b,1], Carina Dey [b,1], Pavla Bojarová [a,*], Vladimír Křen [a], Lothar Elling [b,*]

[a] Laboratory of Biotransformation, Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, CZ-14220 Prague 4, Czech Republic
[b] Laboratory for Biomaterials, Institute for Biotechnology and Helmholtz-Institute for Biomedical Engineering, RWTH Aachen University, Pauwelsstraße 20, D-52074 Aachen, Germany

ARTICLE INFO

*Keywords:*
Biosensor
Carbohydrate
Cancer
Galectin
Glycoconjugate
Inflammation

ABSTRACT

Galectins are a family of carbohydrate-binding lectins modulating cell events such as cell proliferation, apoptosis, adhesion or migration by cross-linking the glycan structures of cell membranes and/or extracellular matrix components. In a diseased organism, galectins are upregulated and trigger the progression of diseases such as inflammation, cancerogenesis, fibrosis, cardiovascular and metabolic disorders. Targeting galectins with glycomaterials for the aims of diagnostics or therapy is, therefore, a focus of biotechnological and biomedicinal research, and already led to candidates for clinical trials. Testing and evaluation of galectin-glycomaterial interactions require informative and versatile analytical methods at several levels of knowledge, from basic intermolecular interaction to complex cell-based assays. This review aims to classify and characterize a selection of the most promising methods to identify the prospective glycomaterials for translating galectin targeting from the molecular level to the level of tailored *in vivo* assays.

## 1. Introduction

The eminent role of complex carbohydrates in cell recognition and functions is becoming increasingly important for the development of targeted strategies to combat serious diseases such as cancer, chronic inflammation, and infection. Complex glycosylation pathways encode a wide variety of glycan structures on glycoproteins, glycolipids, and proteoglycans. Exposure on cell surfaces and in the extracellular matrix facilitates the specific binding of glycan-binding proteins (GBPs). Multivalency of the underlying glycan epitopes and the oligomeric composition of GBPs result in high binding avidity with functional effects on cellular responses such as signal transduction, cell migration, cell proliferation, and apoptosis. Galectins are a family of GBPs modulating such cell events (Elola et al., 2015; Johannes et al., 2018; Kamili et al., 2016). Their name derives from the early observation that all galectins bind to galactose moieties, which are mostly located on the outer (non-reducing) side of glycans. The galectin members are grouped into three sub-families - the prototype, chimeric, and tandem-repeat family – with distinct structural features (Fig. 1). In the human genome, galectins are annotated in the gene group LGALS (HUGO Gene Nomenclature Committee, https://www.genenames.org/). Based on their concentration in the micro-environment, the prototype galectins (nine members in

*Abbreviations:* AEB, 2-amino-*N*-(2-aminoethyl)-benzamide; AD, Alzheimer's disease; ASF, asialofetuin; BLI, biolayer interferometry; BSA, bovine serum albumin; CAM, chicken embryo chorioallantoic membrane; CRD, carbohydrate recognition domain; cryo-EM, cryogenic electron microscopy; DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DVP, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; ELISA, enzyme-linked immunosorbent assay; ELLA, enzyme-linked lectin assay; ESI, electrospray ionization; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; 2'-FL, 2'-fucosyllactose; FP, fluorescence polarization; Gal-1, galectin-1; Gal-3, galectin-3; Gal, galactose; GBPs, glycan binding proteins; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HEK, human embryonic kidney; HMOs, human milk oligosaccharides; HPLC, high-pressure liquid chromatography; HMPA, *N*-(hydroxypropyl)methacrylamide; HUVEC, human umbilical vein cell; $IC_{50}$, half-maximal inhibitory concentration; LacdiNAc, *N*,*N*-diacetyllactosamine; LacNAc type 1, *N*-acetyllactosamine type 1; LacNAc type 2, *N*-acetyllactosamine type 2; Lac-3'-sulfate, lactose-3'-sulfate; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; NMR, nuclear magnetic resonance; LOD, limit of detection; MMP, matrix metalloprotease; MS, mass spectrometry; NGPs, neo-glycoproteins; NHS, *N*-hydroxysuccinimide; PAA, polyacrylamide; SAM, self-assembling monolayer; SPR, surface plasmon resonance; TDG, thiodigalactoside; TFS, tryptophan fluorescence spectroscopy (TFS); VEGF, vascular endothelial growth factor..

* Corresponding authors.
*E-mail addresses:* bojarova@biomed.cas.cz (P. Bojarová), l.elling@biotec.rwth-aachen.de (L. Elling).
[1] these authors contributed equally

https://doi.org/10.1016/j.biotechadv.2022.107928
Received 27 September 2021; Received in revised form 7 February 2022; Accepted 14 February 2022
Available online 18 February 2022

humans, *e.g.,* galectin-1) form electrostatically bound homodimers, each carrying a carbohydrate recognition domain (CRD). Galectin-3 (Gal-3), the only member of the chimera galectin family and the only monomeric lectin *per se*, forms homo-oligomers, preferably pentamers, upon interaction with its oligosaccharide ligands. It is composed of a C-terminal CRD and a collagen-like N-terminal domain. The tandem repeat galectins (5 members in humans, *e.g.*, galectin-8) are covalent heterodimers with two different CRDs interconnected with a short peptide linker. Although β-D-galactopyranosyl is the common recognition motif for the galectin families, individual galectins exhibit a distinct selectivity for particular glycan epitopes (Kamili et al., 2016; Nielsen et al., 2018; Song et al., 2009). Since glycosylation is a highly dynamic process controlled by the health or disease state of cells, human galectins can decode the pathological glycosylation status of cells and trigger appropriate cellular responses (Rodrigues et al., 2018). It is therefore not surprising that a plethora of galectin-mediated pathological cellular responses has been reported, some of them being summarized in recent reviews (Elola et al., 2018; Girotti et al., 2020; Johannes et al., 2018; Laaf et al., 2019; Robinson et al., 2019; Wang et al., 2020).

Due to their role in vital pathological processes such as cancerogenesis, cardiovascular diseases (Suthahar et al., 2018), fibrosis (Martínez-Martínez et al., 2016), or metabolic disorders (Pugliese et al., 2015), galectins are the focus of biomedical research to develop glycomaterials and glycomimetics as novel diagnostic tools and therapeutics (Restuccia et al., 2016; Richards and Gibson, 2021). The first candidates in clinical trials (Girard and Magnani, 2018) illustrate the dynamic development of biomedical galectin research. Targeting individual galectins requires a profound knowledge of the molecular interactions of the respective glycan in the CRD. The protein crystal structures of human galectins have been solved; however, many involve only lactose as a surrogate glycan ligand. In addition, molecular modeling and molecular dynamics simulations support the rationalization of the specific interactions of glycans and glycomimetics. Apart from a few human galectins (Gal-1, Gal-3, Gal-4, Gal-7, and Gal-8) (Campo et al., 2016; Öberg et al., 2011), the development of potent inhibitors lacks knowledge of the glycan specificity of other galectins as well as feasible synthetic routes to these glycans. A careful screening and pre-selection of glycan candidates for targeting galectins that start from the assessment of the basic inter-molecular interaction may avoid controversial debates about the nature of the interaction, as seen recently in the binding assessment of modified citrus pectin or galactomanan preparations with galectin-3 (Balakrishnan et al., 2020; Eliaz, 2019; Eliaz and Raz, 2019; Fan et al., 2018a, 2018b; Stegmayr et al., 2016).

In this regard, methods of studying galectin-glycan interactions *in vitro* are crucial for refining the interactive interface in the CRD. Moreover, these methods can also reliably assess avidity, the gain in binding strength, through the multivalent presentation of potent glyco-epitopes and glycomimetics as well as their selectivity for particular galectins (Bertuzzi et al., 2020; Tavares et al., 2020; Zhang et al., 2018).

In the present review, we provide an overview of methods for analyzing galectin-glycomaterial interaction from different points of view. Since galectin-related *in vivo* processes are extremely complex, great emphasis should be placed on the analysis of corresponding interactions at the molecular level that mimic galectin-glycan interactions in body fluids or on cell surfaces. Here, several solid-phase and in-solution methods have been developed for *in vitro* determination of the binding potency and selectivity of glycomaterials. Solid-phase methods include enzyme-linked lectin assays (ELISA, ELLA) (Laaf et al., 2017), fluorescence detection on glycan microarrays (Campanero-Rhodes et al., 2020; Noll et al., 2016; Song et al., 2009; Stowell et al., 2014), surface plasmon resonance (Bumba et al., 2018; Zhou et al., 2019), and biolayer interferometry (Chan et al., 2018; Zhang et al., 2016). In-solution assays comprise isothermal titration calorimetry (ITC), fluorescence polarization (Noresson et al., 2018; Sörme et al., 2004), tryptophan fluorescence spectroscopy (Bernhard et al., 2020; Sindrewicz et al., 2019), and mass spectrometry (El-Hawiet et al., 2017; Kitova et al., 2020; Shams-Ud-Doha et al., 2017). Rational approaches to refine and resolve the selectivity of galectin-glycan interactions are also discussed in length, especially advanced NMR spectroscopy methods (Ippel et al., 2016), X-ray analysis of crystallized galectin-ligand complexes (Hsieh et al., 2016), engineering and mutagenesis of galectin variants (Ludwig et al., 2019) as well as computational methods such like *in silico* modeling (Zetterberg et al., 2018) and molecular dynamics simulation (Dahlqvist et al., 2019; Gómez-Redondo et al., 2021).

The next stage of validation of galectin inhibitors encompasses prospective designs of *in vitro* cell culture assays. Fluorescence-activated cell sorting (FACS) (Vašíček et al., 2020), histochemistry (Roy et al., 2017), and cell aggregation assays (Zhang et al., 2015; Zhang et al., 2016) monitor galectin binding and their inhibition by glycomaterials. Moreover, functional cell assays are being established to detect inhibition of galectin-induced cell responses (Girotti et al., 2020), such as apoptosis (Heine et al., 2021), cell adhesion, and migration (Filipová et al., 2020) as well as angiogenesis (Guha et al., 2013).

The final part of this review covers current advances in the field of galectin-detecting biosensors based on selective high-affinity carbohydrate-based ligands. Biosensors for glycan-lectin interaction have already been developed (Belický et al., 2016; Pacholski et al., 2020; Rosencrantz et al., 2016; Rosencrantz et al., 2019). However, only a few examples of galectin-specific biosensors have been published. We report on galectin-specific gold nanorods and their unique surface plasmon resonance properties (Zhao et al., 2016) and a label-free electrochemical biosensor based on gold nanoparticles (Long et al., 2019; Martos-Maldonado et al., 2020). An electrochemical biosensor based on molecularly imprinted polymers as well as an amperometric immunosensor have been developed for point-of-care diagnostics of the cancer biomarker galectin-3 (Cerqueira et al., 2021; Yola and Atar, 2020).

In summary, over the past twenty years, galectin-glycan research has shifted from galectin inhibition by natural disaccharides or simple

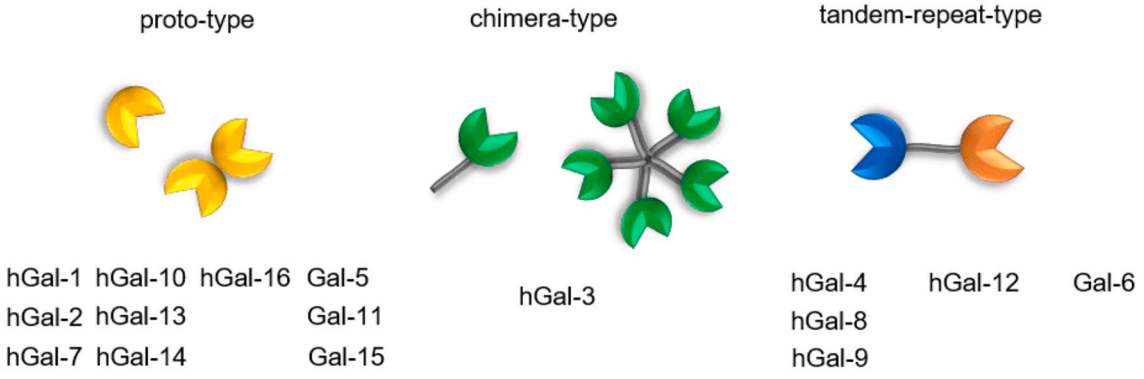


**Fig. 1.** Structural features of galectin families ('h' indicates human galectins) (Liu and Rabinovich, 2010; Than et al., 2009).

**Table 1**
Methods for analyzing galectin-glycomaterial interactions at the inter-molecular level. In most studies, different galectins were compared.
ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; BLI, biolayer interferometry; Gal-, galectin

| Assay | Method | Galectin | Reference |
|---|---|---|---|
| Solid-phase | ELISA | Gal-1, -3 | (Bertuzzi et al., 2021; Böcker and Elling, 2017b; Böcker et al., 2015; Laaf et al., 2017; Tavares et al., 2020; Vašíček et al., 2020; Vrbata et al., 2022; Zhang et al., 2018) |
| | | Gal-3 | (Filipová et al., 2020; Heine et al., 2021; Hoffmann et al., 2020) |
| | Microarray | Gal-1, -3 | (Shams-Ud-Doha et al., 2017; Stowell et al., 2014) |
| | | Gal-1, -3, -4, -7, -8, -9 | (Song et al., 2009) |
| | | Gal-1, -3, -7, -9 | (Noll et al., 2016) |
| | | Gal-1, -3, -7 | (Gao et al., 2019) |
| | | Gal-3 | (Dion et al., 2017) |
| | SPR | Gal-1, -3 | (Bertuzzi et al., 2021; Marchiori et al., 2015) |
| | | Gal-3 | (Bumba et al., 2018; Rosencrantz et al., 2019; Zhou et al., 2019) |
| | BLI | Gal-1, -3, -7 | (Hsieh et al., 2015; Vrbata et al., 2022) |
| In-solution | ITC | Gal-1, -3, -7 | (Hsieh et al., 2016) |
| | | Gal-3 | (Atmanene et al., 2017; Sindrewicz et al., 2020; Talaga et al., 2016) |
| | | Gal-8N | (Patel et al., 2020) |
| | Intrinsic Fluorescence spectroscopy | Gal-2, -3, -4 | (Sindrewicz et al., 2019) |
| | | Gal-3 | (Bernhard et al., 2020; Restuccia et al., 2018) |
| | Fluorescence polarization | Gal-1, -2, -3, -4N, -4C, -7, -8N, -9C, -9N | (Stegmayr et al., 2016) |
| | | Gal-1, -3, -7 | (Hsieh et al., 2015; Peterson et al., 2018) |
| | | Gal-4C | (Bum-Erdene et al., 2015) |
| | | Gal-3 | (Boza-Serrano et al., 2019; Noresson et al., 2018) |
| Structure-based | MS | Gal-1, -3, -7 | (Shams-Ud-Doha et al., 2017) |
| | | Gal-3 | (Atmanene et al., 2017; Kitova et al., 2020) |
| | NMR | Gal-1, -3, -7 | (Hsieh et al., 2016) |
| | | Gal-3 | (Ippel et al., 2016; Rauthu et al., 2015; Zhang et al., 2019) |
| | | Gal-1, -3 | (Bertuzzi et al., 2021) |
| | X-ray crystallography | Gal-8N | (Bohari et al., 2018; Patel et al., 2020) |
| | | Gal-1, -3, -7 | (Hsieh et al., 2015; Hsieh et al., 2016) |
| | | Gal-4 | (Bum-Erdene et al., 2015; Rustiguel et al., 2016b) |
| | | Gal-3 | (Noresson et al., 2018; Peterson et al., 2018; Zetterberg et al., 2018) |
| | Cryo-EM | Gal-1, -3 | (Bertuzzi et al., 2021) |
| | | Gal-1 | (Qi et al., 2018) |
| | *In silico* | Gal-1, -3 | (Vašíček et al., 2020) |
| | | Gal-8N | (Bohari et al., 2018; Patel et al., 2020) |
| | | Gal-3 | (Boza-Serrano et al., 2019; Bumba et al., 2018; Dahlqvist et al., 2019; Zetterberg et al., 2018) |
| | | Gal-9 | (Mahanti et al., 2020) |
| | | Gal-4 | (Bum-Erdene et al., 2015; Rustiguel et al., 2016b) |

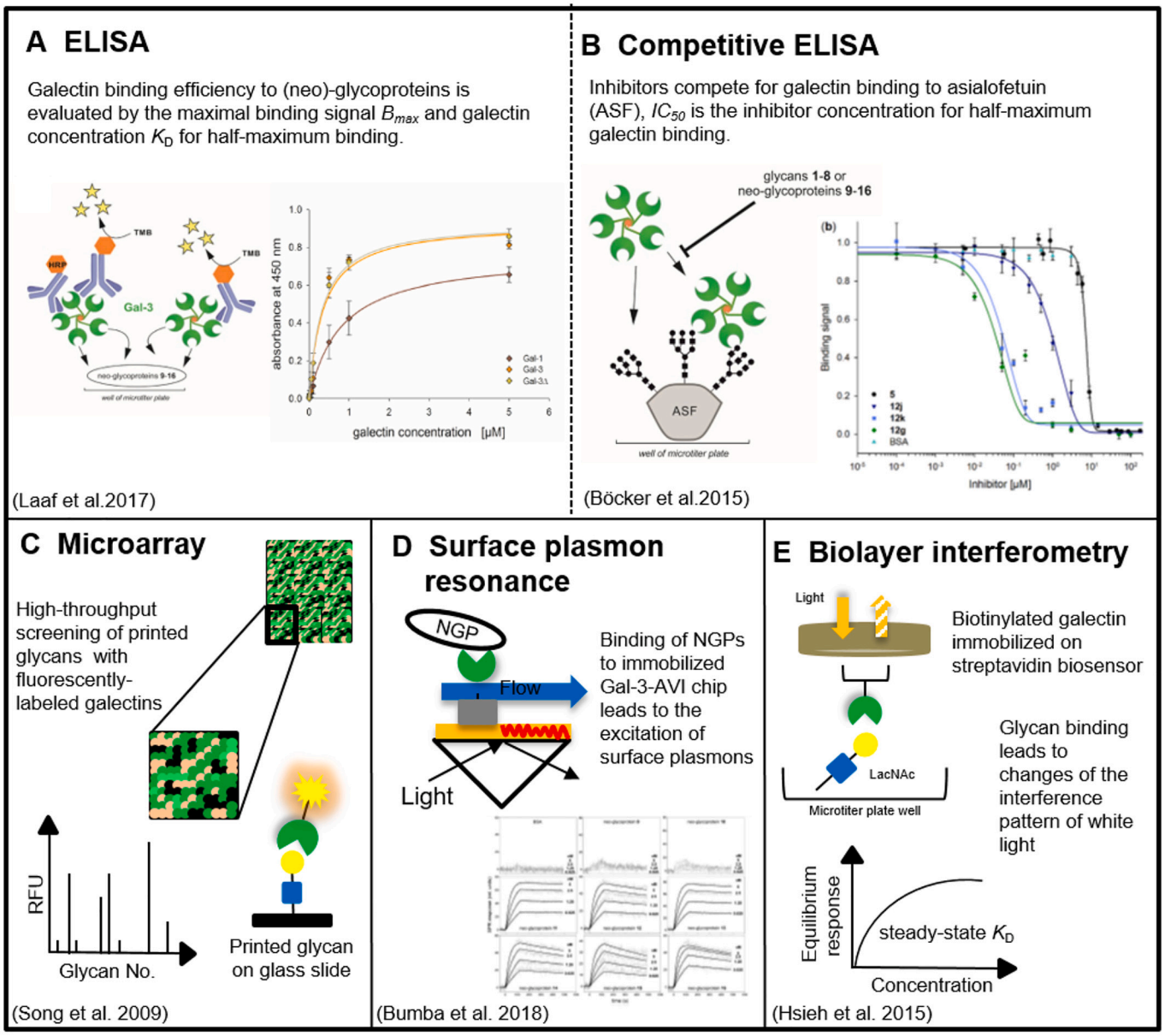


**Fig. 2.** Solid-phase assays for the detection of galectin-glycomaterial interactions. (A), Direct ELISA and (B), competitive ELISA; (C), Microarrays for high-throughput screening of lectin-glycan interactions; (D) surface plasmon resonance (SPR); (E), biolayer interferometry (BLI).

glycodendrimers to sophisticated multivalent systems and tailored glycomimetics that result from collaborative interdisciplinary efforts among modeling specialists, organic and material chemists, and molecular engineers. The current review aims to classify and characterize the most useful approaches to identify the promising candidates for translating galectin-targeting carbohydrate-based tools from the bench to the bedside.

## 2. Galectin-glycomaterial interactions at the inter-molecular level

Galectin interactions with glycomaterials are most often characterized by solid-phase-based analysis and in-solution assays. In this section, we report on different types of assays and their ability to evaluate galectin binding at the inter-molecular level (Table 1). Notably, the binding constants for carbohydrates and glycomimetics can hardly be compared across various assays. However, different assay formats may help to evaluate and rank the compounds for further use in biomedical applications, especially if a general, ideally a commercially available standard like lactose is always employed as a benchmark for comparison.

### 2.1. Solid-phase based assays of galectin-glycomaterial interaction

#### 2.1.1. ELISA and ELLA-type assays

Enzyme-linked-immunosorbent (ELISA) and enzyme-linked lectin (ELLA) assays are well-established solid-phase-based analytical tools for investigating the properties of galectin-mediated carbohydrate interactions or for efficient screening of novel highly specific ligands targeting galectins for biomedical applications (Laaf et al., 2019). A typical experimental setup is based on the immobilization of asialofetuin or another galectin binder of interest on adsorptive microwell plates (Fig. 2A). Asialofetuin (ASF) is the de-sialylated form of fetuin and is considered a standard glycoprotein ligand for galectins. ASF is composed of three N-glycans terminated with *N*-acetyllactosamine (β-D-Gal-(1→4)-D-GlcNAc; LacNAc) moieties (Thaysen-Andersen et al.,

2009). Subsequently, unoccupied binding sites of the plate are typically blocked with bovine serum albumin (BSA) to avoid nonspecific protein binding. This step is followed by the incubation with a series of highly diluted galectins of interest. In ELISA assays, galectin-carbohydrate interactions are detected, *e.g.*, by incubation with anti-galectin antibodies or anti-(histidine)$_6$-antibodies targeting the (poly)-histidine-tag of recombinant galectins (Fig. 2A). In ELLA, enzyme-conjugated galectins (De Melo et al., 2007) or fluorescently labeled galectins (Böcker and Elling, 2017a; Kupper et al., 2013; Nieminen et al., 2007) are employed in the incubation step. The latter gives a direct spectrophotometric readout, omitting further enzymatic conversion steps. For the enzyme conjugates (mostly conjugated to peroxidase or alkaline phosphatase) (Azevedo et al., 2003; De Melo et al., 2007), further incubation steps with suitable substrates create colored products, which are measured at their respective absorption maxima by a UV-Vis spectrophotometer. Important to note is that in ELISA and ELLA, intermediate washing steps are obligatory to reduce background signals. The binding properties of analyzed galectins are then visualized in concentration-dependent binding curves by plotting the absolute or relative background-subtracted absorbance against the applied galectin concentration (Fig. 2A). Graphical analysis software tools enable the non-linear fitting of obtained data to determine binding parameters such as the apparent equilibrium binding constant (apparent $K_D$) which is defined as the galectin-concentration at which half-maximal inhibition is reached. The ratio of the maximal binding signal and the apparent $K_D$ value gives the binding efficiency ($\mu M^{-1}$) of galectin (Laaf et al., 2017), which provides information on the binding affinity of galectin toward the assayed glycan target (*i.e.*, the binding partner immobilized on the plate).

Furthermore, competitive ELISA/ ELLA assays provide information on the inhibitory ability of potential galectin inhibitors (Fig. 2B). Here, a constant concentration of galectin is incubated with varying concentrations of an inhibitor of interest (such as benchmark lactose, glycomimetic, mono- or multivalent glycoconjugates), which competes for galectin binding with the immobilized standard binder (*e.g.*, ASF). The galectin bound to the immobilized binder is measured spectrophotometrically as described above. The half-maximal inhibitory concentration ($IC_{50}$) is determined by the non-linear fitting of the obtained inhibition data (Hunter and Cochran, 2016). It is very useful to compare the found $IC_{50}$ values with a standard ligand (lactose is most commonly used) for the sake of comparison across different set-ups in different laboratories. In recent years, a lot of data on galectin-carbohydrate interactions based on ELISA/ ELLA-type assays have been obtained to gain insight into the mechanisms of these biomolecular recognition events at the molecular range. In particular, the design and synthesis of galectin-inhibitors targeting the cancer-related Gal-1 and Gal-3 have attracted the attention of the glycoscience community.

We proposed novel multivalent neo-glycoproteins (NGPs) as high-affinity ligands for Gal-3 (Böcker et al., 2015; Hoffmann et al., 2020; Laaf et al., 2017). These neo-glycoproteins are based on serum albumin as a protein scaffold that can be decorated with glycans, modified glycans, or glycomimetic structures (Böcker and Elling, 2017b; Böcker et al., 2015; Heine et al., 2021; Laaf et al., 2017; Zhang et al., 2018) in a tunable manner for their multivalent presentation. A whole range of NGPs presenting glycan epitopes were synthesized, immobilized in microwell plates, and evaluated in ELISA-type assays for their selective binding of Gal-3 and their potential to inhibit galectin binding to immobilized ASF. Neo-glycoproteins bearing terminal *N*,*N*′-diacetyllactosamine (β-D-GalNAc-(1→4)-D-GlcNAc; LacdiNAc) showed high selectivity for Gal-3 compared to Gal-1 with binding constants in the nanomolar range (Böcker et al., 2015; Bumba et al., 2018; Laaf et al., 2017).

A new ELISA-like assay was developed for the selective capture of Gal-3 from serum samples using immobilized multivalent NGPs presenting terminal LacdiNAc motif in a hexasaccharide glycan (Laaf et al., 2017). The assay shows high sensitivity for the detection of functional intact Gal-3 in serum samples (> 90 ng/mL), which is in the range of elevated levels of circulating Gal-3 in the serum of patients suffering from gastrointestinal carcinoma (Iurisci et al., 2000). Furthermore, an antibody-free ELLA was developed by capturing Gal-3 between a sandwich of immobilized LacdiNAc-decorated NGPs and biotin-labeled LacdiNAc-decorated NGPs (Laaf et al., 2017).

The most potent ligands detected by ELISA were C3-modified thiodigalactoside (TGD)-derivatives multivalently presented on BSA as an aglyconic scaffold (Zhang et al., 2018). The binding and inhibition abilities of these NGPs for Gal-1 and Gal-3 were investigated by ELISA-type analysis. Results revealed apparent dissociation constants ($K_d$) in the nanomolar range, indicating that these TGD-derived substrates are high-affinity ligands for both galectins. Analysis by the competitive ELISA assay (Fig. 2B) revealed that multivalent presentation of TDG-derivatives on a protein carrier leads to a significantly increased inhibition potential towards Gal-3 with $IC_{50}$ values in the low nanomolar range. Interestingly, the strength of the multivalent effect is apparently strongly influenced by the type of carrier and the mode of presentation of carbohydrate ligands as exemplified in glycomimetic-decorated polymers (Tavares et al., 2020; Vrbata et al., 2022).

In contrast to colorimetry, fluorescent detection provides sensitivity enhanced by up to several orders of magnitude. Carbohydrate-functionalized poly(amidoamine) (PAMAM) dendrimers were immobilized to evaluate their multivalent binding potential for Gal-1 and Gal-3 in a competitive ELISA type assay (Wolfenden et al., 2015). In a fluorescence-linked immunosorbent assay (FLISA), Gal-3 was immobilized in microwell plates and binding of a fluorescently labeled glycan or glycoprotein was observed (Zhang et al., 2016). Competition with a non-fluorescent glycan probe resulted in a lower fluorescent signal. The competitive ELISA was also used to assess pectin binding to Gal-3. In conclusion, ELISA and ELLA-type assays provide a versatile and reproducible method for evaluating galectin inhibitors. The utilization of immobilized multivalent NGPs in galectin ELISA-type assays allows for the assessment of the multivalency effect of various glycans. Moreover, on the basis of NGPs, novel assay formats were created, such as an antibody-free ELLA (Laaf et al., 2017). Competitive ELISA assays are the first method of choice to classify carbohydrates and glycomimetics as potential biomedical and diagnostic agents that can be further tested in cell-based assays (Section 3).

### 2.1.2. Fluorescence-based microarray analysis

Microarrays are solid-phase high-throughput screening devices for analyzing the binding behavior of lectins or antibodies against a variety of targets. In recent decades, various microarrays have been developed, such as DNA-, lectin- or glycan microarrays (Mende et al., 2019; Oinam et al., 2020; Wöhrle et al., 2020). Glycan microarrays are based on solid-phase materials modified with functional groups for the covalent attachment of libraries of free or conjugated target glycans (Fig. 2C). In most cases, glass slides are used as surface materials due to their easy and versatile modification and compatibility with optical detection systems. Target glycans, free or modified, are covalently attached (*i.e.*, printed) to a chemically activated glass slide (Oyelaran and Gildersleeve, 2009; Rillahan and Paulson, 2011; Song et al., 2009). Fluorescence detection is used for analyzing carbohydrate-protein interactions and requires labeled lectins or other coupled binding proteins such as streptavidin. Fluorescence intensity is typically measured with a microarray scanner and the data obtained can be plotted as relative fluorescence units (RFUs) against the spot number, which identifies the target glycan on the glass slide (Hyun et al., 2017).

The Cummings group explored the glycan specificity of Gal-1 and Gal-3 by screening glycans derived from natural glycan sources (glycoproteins, glycolipids, glycosaminoglycans, and free glycans) in a microarray system (Song et al., 2009). A novel fluorescent-derivatization method was established for rapid extended analysis of glycan-carbohydrate interactions. Here, 2-amino-*N*-(2-aminoethyl)-benzamide (AEAB) was applied as a bifunctional fluorescent linker to conjugate free reducing glycans by reductive amination *via* its aryl

amine groups. The AEAB-conjugated glycans were purified by HPLC and then printed in the open-ring form on NHS-activated glass slides *via* the free alkylamine group. Fluorophore-conjugated streptavidin was used for detecting bound biotinylated galectins. The glycan arrays revealed concentration-dependent selective binding of galectins. At physiological concentrations (0.4 – 1.5 μM), Gal-1 showed selective binding toward a range of complex N-glycans with terminal LacNAc moieties whereas Gal-3 was preferentially bound to poly-LacNAc glycans. The same working group applied the glycan microarray technique for analyzing galectin - human milk oligosaccharide (HMO) interactions to evaluate the role of galectins in infant immunity (Noll et al., 2016). Here, a shotgun-microarray derived from human milk and a microarray with defined HMOs was generated to compare the binding of Gal-1, -3, -4, -7, -8, and -9. Each galectin showed specificities for particular glycan motifs. Surprisingly, Gal-2 did not bind to the tested HMOs although it is known from the literature to bind simple HMO structures. Furthermore, the preference of Gal-7 for β-D-Gal-(1→3)-D-GlcNAc (LacNAc type 1) carbohydrates was revealed.

Klassen and coworkers studied the binding properties of four human galectins (mutant Gal-1, C-terminal fragment of Gal-3, Gal-7, and N-terminal fragment of Gal-9) to HMOs, by comparing results from electrospray ionization mass spectrometry (ESI-MS), shot-gun glycan microarray analysis, and ITC measurements (Shams-Ud-Doha et al., 2017). There was only a moderate correlation between the relative binding affinities obtained from glycan array analysis and ESI-MS and ITC as in-solution assays. A possible explanation is the structural difference of the reducing glucose residue (open ring form) after printing HMOs on the microarray glass slide. This is one example of how the setup of glycan immobilization in a solid-phase assay can influence the assessment of galectin binding. Galectins were also assayed using a library of 32 complex-type N-glycans derived from egg yolk (Gao et al., 2019). Fluorescence-based microarray revealed preferential binding of Gal-1 to LacNAc type 2 terminated N-glycans and the tolerance to α2,3-linked sialic acids. Gal-3 also bound LacNAc type 2 terminated glycans with a preference for α2,3-linked sialic acids; however, recognition of bi-antennary glycan structures was not observed.

Glycan-based arrays have become emerging tools for analyzing bacterial surface glycans for the evaluation of potential vaccine candidates, identification of lectin ligands, or diagnosis of bacterial infections (Campanero-Rhodes et al., 2020). A compelling microarray setup for the detection of immune factor-microbe interactions was proposed to investigate the protective influence of galectins as a part of innate immunity (Stowell et al., 2014). A microbial glycan microarray (MGM) was generated based on purified glycans derived from a variety of microbes. The binding properties of biotin-conjugated Gal-3, Gal-4, and Gal-8 were measured *via* fluorescence intensity using a fluorophore-labeled streptavidin. It was concluded that galectins bind the same antigenic determinants presented by microbes and mammalian cells with the potential to eliminate microbes, demonstrating galectins as a distinct part of innate immunity.

Another microarray based on fluorescence detection was proposed for the examination of potential galectin inhibitors (Dion et al., 2017). Here, polyacrylamide (PAA)-conjugated glycosides and glycoproteins were printed on epoxy-coated-glass slides to investigate the inhibitory potency of various arylated LacNAc-type 2-derived inhibitors toward Gal-1, Gal-3C (Gal-3 truncated at the N-terminus, comprising only the CRD), and Gal-7. Inhibition of galectin binding was detected by measuring the fluorescence intensities of bound cyanine-3-conjugated galectins. The amide-based conjugation of aromatic moieties at the C-2 position of the glucosamine residue was found to be the structural feature efficiently recognized by Gal-3; diaromatic oxazoline was also found to be a specific inhibitor of Gal-3.

In sum, fluorescence-based microarray analysis of galectin binding represents an efficient and compelling approach for high-throughput screening of complex glycan targets derived from naturally occurring sources such as human milk or microbes. This solid-phase method requires small sample volumes and offers user-friendly and simple detection possibilities. However, there are still challenges to overcome in the modification of target glycans. The structural addition of linkers and the linker-dependent presence of the acyclic form of the reducing sugar may influence the binding behavior of some galectins, and therefore do not reflect the physiological binding conditions of these galectins. Furthermore, direct fluorophore-based conjugation of galectins could affect the binding affinities to glycan targets.

### 2.1.3. Surface-Plasmon Resonance

Surface plasmon resonance (SPR) spectroscopy is an optical analysis technique for the sensitive examination of binding interactions at a metal/ liquid interface. This method allows label-free real-time monitoring of biomolecular interactions and the determination of ligand specificities, affinities, and kinetic parameters by fitting to a range of kinetic models. SPR is based on the excitation of surface plasmons - evanescent waves induced by the collective excitation of free electrons in a metallic material that is excited parallel to the metal surface (Fig. 2D). Excitation of surface plasmons can be achieved with light using either *the* Otto configuration or the Kretschmann configuration (Nguyen et al., 2015). The latter is the most commonly used configuration, which consists of a highly refractive glass prism covered with a thin metal film (gold, silver). The incidence of a parallel polarized light beam through the glass prism is reflected at the prism-facing side and leads to the excitation of plasmons at the opposite side of the metal film. The resonance occurs at a specific SPR angle depending on the material used (light source, type of metal film, refractive material). The sensor is generated by functionalizing the metal side facing the liquid interface with biological recognition elements such as antibodies, enzymes, DNA, or carbohydrates. The binding of analytes to sensor surface causes changes in the refractive index of the surface layer and leads to an altered SPR angle and an absorption wavelength shift. The response intensity is mostly measured in response units (RU) and correlates with the layer thickness of the sensor surface, which provides information about the binding of the analyte of interest (Guo, 2012). SPR allows the determination of kinetic parameters such as association rate constant ($k_a = k_{on}$), dissociation rate constant ($k_d = k_{off}$), and equilibrium rate constant ($K_D$) as a result of the kinetical analysis (Helmerhorst et al., 2012). In recent years, SPR has been used to analyze specific galectin-carbohydrate interactions. Two modes for analysis are possible: immobilization of glycan targets or galectins on the sensor chip.

To immobilize glycans on the SPR biosensor, Marchiori and coworkers proposed amino acid-derived 1,2,4-triazole-galactosides and 1,2,3-triazole-divalent-lactose-derived glycoconjugates as high-affinity ligands for Gal-3 (Marchiori et al., 2015). An SPR sensor was fabricated based on a self-assembling monolayer (SAM) of cysteamine adsorbed on a gold surface (SAM/Au) that allowed immobilization of NHS-activated glycoconjugates. Another sensor was prepared by amino-group activation of the SAM/ Au itself for binding a divalent lactoside. SPR measurements showed the binding of human Gal-3 to amino-acid-derived glycoconjugates due to ionic interactions with arginine residues of Gal-3. The highest affinity was detected for the divalent lactoside with the ability to cross-link two different Gal-3 CRDs through an arginine residue. SPR was also used to study the binding of Gal-3 to linear lactose-based homoglycopolymers (Rosencrantz et al., 2019). The synthesis of homoglycopolymers based on reversible addition-fragmentation chain-transfer (RAFT)-polymerization of lactose-glycomonomers enabled direct immobilization of lactose on a gold-coated chip. Surprisingly, the equilibrium rate constant ($K_D$) for Gal-3 binding could not be determined due to an exponential binding behavior of Gal-3 to the glycopolymers with the absence of saturation effects. This phenomenon was attributed to the oligomerization of Gal-3 in the multivalent environment on the surface of the sensor chip. However, by immobilizing lactose-based linear homoglycopolymers on a gold sensor chip to enhance the layer thickness, this setup could be transferred to a 3D sensor chip, which prevented unspecific protein

adhesion effects due to the specific grafting process of the glycopolymers. The binding behavior of Gal-3 toward sequence-defined heteromultivalent macromolecules was investigated to find synthetic Gal-3 targeting ligands for use in therapeutic applications (Freichel et al., 2020). Here, glycomacromolecules were synthesized based on building blocks of different lengths bearing both glycosidic (lactose or glucose) and non-glycosidic moieties (neutral, amine, sulfated/ sulfonated). Preferential binding of Gal-3 to sulfated/sulfonated structures was further investigated by *in vitro* cell assays (Section 3), demonstrating the potential of these synthetic glycomacromolecules for the application as therapeutic agents.

Putnam and coworkers (Zhou et al., 2019) exploited the immobilization of galectins on the SPR biosensor for the analysis of selectively tunable glycopolymers. The glycopolymers were synthesized by grafting glycosylamines onto polyacrylic acid (PAA) *via* DMTMM coupling, which differed in carbohydrate identity (lactose, galactose, glucose, maltose, maltotriose) and the degree of substitution. The binding affinities of Gal-1 and Gal-3 were determined by immobilizing galectins on carboxymethylated dextran-coated sensor chips *via* amine coupling and by flowing the glycopolymers over the sensor chip. The binding affinities of Gal-1 and Gal-3 increased with the level of lactose conjugated in the glycopolymers. This was attributed to cluster valency effects. $K_D$ values were in the sub-nanomolar range ($10^{-11}$ M) for both galectins and rationalized by the strong interaction of the multivalent glycopolymers with oligomeric Gal-1 and Gal-3. Another method of immobilizing galectins consists in the use of an N-terminal fusion construct of Avi-Tag peptide to Gal-3, which enabled selective mono-biotinylation of Gal-3, and thus facilitated oriented immobilization on a neutravidin-coated biosensor surface (Bumba et al., 2018). SPR measurements with this Gal-3-AVI construct revealed strong binding of poly-LacNAc-modified NGPs to Gal-3 with equilibrium rate constants in the subnanomolar range. Especially, LacdiNAc-terminated tetra- and hexasaccharides turned out to be the strongest ligands for Gal-3, featuring the lowest $K_D$ values that were ever measured with an SPR setup.

In summary, SPR is a highly versatile label-free technique that even enables the determination of kinetic parameters. However, there are still challenges in the experimental set-ups. SPR can only detect macromolecule-type compounds as analytes (generally over 1,000 Da), and hence it is unsuitable for small-molecule inhibitors in the galectin-immobilization setup. Both the galectin and the carbohydrate ligand immobilization on the sensor chip may need to be evaluated to choose the appropriate design. In the case of galectin immobilization, the type of immobilization and the orientation of the galectin CRD has to be considered to prevent loss of binding activity.

### 2.1.4. *Biolayer interferometry*

The use of biolayer interferometry (BLI) for a specific analysis of galectin-carbohydrate interactions has recently gained attention. It is a solid-phase label-free optical technique for measuring biomolecular interactions (Fig. 2E*)*. The principle of biolayer interferometry is based on

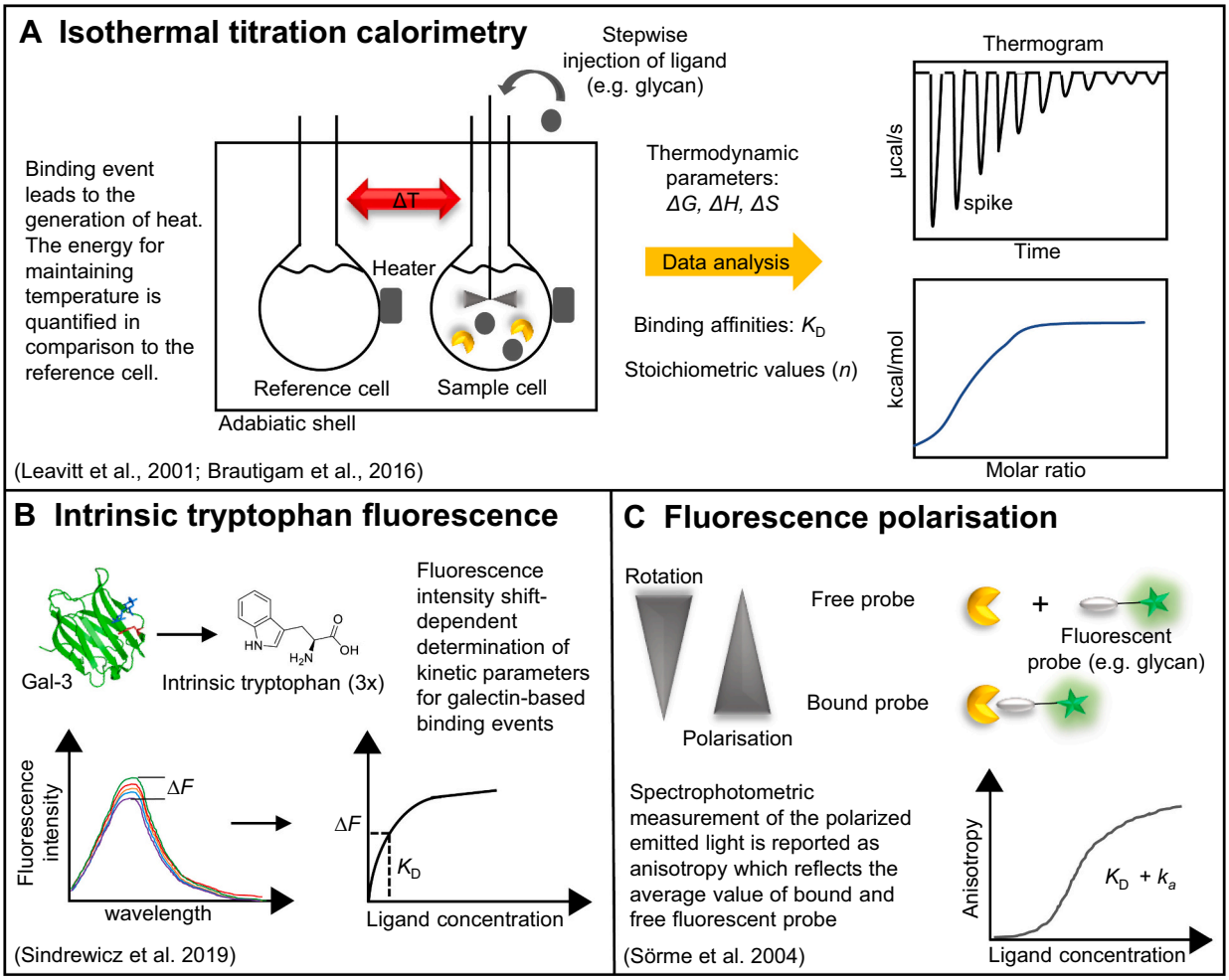


**Fig. 3.** In-solution assays for the detection of galectin-glycomaterial interactions. (A), Isothermal titration calorimetry (ITC); (B) intrinsic tryptophan fluorescence; (C), fluorescence polarization assay.

changes in the interference pattern/ refractive index of streamed white light induced by the binding event on a biosensor chip. The biosensor consists of a reference layer and a layer on the biosensor tip immobilized with a recognition element of interest. The reflection of white light streamed through the biosensor results in an interference pattern. If the analyte of interest binds to the recognition element on the biosensor tip, the thickness of the optical layer increases, and a wavelength shift is observed. This optical method can be applied for measuring real-time kinetics, affinity, or assessment of binder concentrations (Concepcion et al., 2009; Rich and Myszka, 2007; Wartchow et al., 2011). Furthermore, only the association and dissociation of the analyte causes changes in the interference pattern allowing the analysis of crude sample solutions without the need for prior purification steps (Fang, 2006). Hsieh and coworkers examined the affinity of recombinant Gal-1, Gal-3, and Gal-7 toward LacNAc type 1 and type 2 disaccharides (Hsieh et al., 2015). Biosensors were prepared by coating a streptavidin biosensor with biotinylated galectins. Changes in biosensor thickness due to binding of LacNAc type 1 and LacNAc type 2 were measured in a microtiter plate format (Fig. 2E). A higher affinity for LacNAc 2 was observed for Gal-1 and Gal-3 with $K_D$ constants in the μM ranges. BLI measurements with human Gal-7 revealed affinity toward LacNAc 1. The preference of the recombinant galectins was simultaneously determined by fluorescence polarization measurements, which revealed similar preference trends, demonstrating the reliability of this analytical tool for the analysis of galectin-carbohydrate binding events.

Advantageously, the concept of AVI-tagged galectins (section 2.1.3) can also be exploited in biolayer interferometry. The Gal-3-AVI construct immobilized on a streptavidin-coated biosensor was used to determine the binding kinetics of glycomimetic-decorated HPMA copolymers, which could not be assessed by SPR due to a poor response (Vrbata et al., 2022).

### 2.2. *In-solution assays of galectin-glycomaterial interaction*

#### 2.2.1. *Isothermal titration calorimetry*

Isothermal titration calorimetry (ITC) is a biophysical solution-based technique that is considered the „gold standard“ for quantitative analysis of biomolecular binding processes. ITC records the evolution of heat during the binding of a macromolecule with its binding partner. It allows the determination of thermodynamic parameters ($\Delta G$, $\Delta S$, $\Delta H$), affinity constants ($K_D$), and binding-related stoichiometric values ($n$). ITC provides information on thermodynamic parameters driving the biological binding events, such as the enthalpy of binding ($\Delta H$). The free enthalpy ($\Delta G$) can be calculated using the Gibbs-Helmholtz equation (Brautigam et al., 2016). A titration calorimeter consists of a thermally conductive material (such as gold), which is encased in an adiabatic shell (Fig. 3A). Within this shell, two identical fillable measuring cells are coupled with a thermal element to monitor the temperature of the liquids inside the cells, a reference cell and a probe cell (Ladbury, 2004). The experimental procedure typically involves stepwise injections of defined concentrations of ligand into the probe cell containing the macromolecule (*e.g.*, galectin), which results in the release of heat and the temperature rise in the surrounding liquid. The energy required for maintaining the temperature of the probing cell is quantified in comparison to the reference cell. Thermograms are then generated by plotting the energy input required to maintain the temperature [μcal/s] against time [s]. The peaks (spikes) that appear represent the energy change in dependence on the ligand concentration, and the heat generated during the reaction can be calculated from the area of each peak (Leavitt and Freire, 2001).

In recent years, ITC has been used for several studies on galectin-glycan interactions. Hsieh and coworkers investigated the structural relationship between TDG or its derivates and the galectin binding sites of Gal-1, -3, and -7 (Hsieh et al., 2016). ITC experiments revealed an enhanced binding affinity of Gal-7 toward the *bis*(fluorophenyl) TDG derivative TD139 with a $K_D$ value in the μM range. In addition, nanomolar binding constants were observed for Gal-1 and Gal-3 binding with a 1:1 ratio of galectin/inhibitor. Additional analysis gained detailed insights into the structural arrangement of the tested ligands within the binding sites of the galectins and identified interaction of an arginine residue located in the E-sub-site with arene groups of TDG derivatives. The found dual binding mode of galectins demonstrated the potential of substituted galactoside-based materials to increase galectin binding affinities and inhibition properties.

ITC experiments served as a biophysical tool to discuss whether aromatic substitution of LacNAc type 2 disaccharides contributed to higher binding affinities for Gal-3 (Atmanene et al., 2017). Here, unsubstituted LacNAc, as well as mono- or diarylated LacNAc, were tested and ITC data revealed a 50-fold increase in affinity for the diaromatic LacNAc with a $K_D$ value in the sub-micromolar range. ITC thermodynamic data showed a favorable enthalpic contribution of the aromatic substitutions and were attributed to the formation of cation-π interactions between the arginine residues of Gal-3 and the aromatic carbohydrate substituents.

Sindrewicz and coworkers employed a ligand displacement ITC competitive assay to characterize the binding properties of Gal-3 to four heparin-derived inhibitors (Sindrewicz et al., 2020). Here, LacNAc, used as a competitive ligand, was titrated against the heparin derivative/ Gal-3 mixture. The results showed weak binding of Gal-3 to all polysaccharide inhibitors in the low mM range, which was attributed to the absence of galactose residues in heparin-derived inhibitors. In an ITC binding experiment, Talaga *et al.* demonstrated micromolar multivalent binding of Gal-3 to chondroitin sulfate glycosaminoglycans (Talaga et al., 2016). Hill plot analysis revealed progressively increasing negative cooperativity, attributed to the interference of Gal-3 binding with the formation of a crosslinked network of multivalent chondroitin sulfate and Gal-3.

Recently, the tailored design of Gal-8 specific inhibitors and their ITC-based binding affinities was published (Patel et al., 2020). Based on X-ray crystallographic structures, novel monosaccharide-based galactomalonyl phenyl esters were chemically synthesized to selectively interact with amino acids of the Gal-8 N-terminal domain. Direct ITC binding analyses confirmed the binding of these compounds with $K_D$ values of 5-33 μM. In particular, 4-fluorophenyl-substituted galactomalonic acid displayed the strongest binding to Gal-8N demonstrating the antagonistic potential of these engineered Gal-8 ligands.

Altogether, ITC-based analysis of galectin-glycomaterial interactions offers a label-free in-solution determination of binding affinities. The additional generation of thermodynamic parameters provides a more detailed insight into the molecular mechanisms underlying the calculated binding affinities. This quantitative method is non-destructive for the tested samples and benefits from fast experimental procedures. However, there are still limitations to be considered, such as the sensitivity of this method to air bubbles and small fluctuations in the buffer composition during titration. Thus, samples and buffers need to be degassed to avoid incorrect readings and both binding partners must be dissolved in a perfectly identical buffer, which may pose challenges in the preparation of samples. Detection of extreme low- and high-affinity interactions is quite challenging but can be overcome with ITC-based competitive assays. Finally, ITC is more of a single experiment technique, and thus a low throughput method for the examination of galectin-mediated glycan binding. Nevertheless, it has become an indispensable part of the methodological collection of methods available

**Table 2**
Overview of galectin-specific intrinsic tryptophan residues (Sindrewicz et al., 2019).

| Number of Trp residues | Galectin family: Galectin |
|---|---|
| 1 | Prototype: Gal-1, Gal-2, Gal-7, Gal-13, Gal-14 |
| 2 | Prototype: Gal-10, Gal 16; Tandem-repeat: Gal-4, Gal-8 |
| 3 | Chimera-type: Gal-3; Tandem-repeat: Gal-9 |
| 4 | Tandem-repeat: Gal-12 |

to the glycoscience community.

### 2.2.2. Intrinsic tryptophan fluorescence

Within the group of proteinogenic amino acids, the amino acids tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp) are fluorescent due to their aromatic structure (Numata, 2020; Vivian and Callis, 2001). The Trp-based intrinsic fluorescence properties of proteins ($\lambda_{ex}$ = 280 nm; $\lambda_{em}$ = 348 nm; fluorescence lifetime 3.1 ns in water at neutral pH) are used for structural analysis of proteins due to their high sensitivity to changes in the surrounding medium. Ligand binding to a Trp-containing protein leads to changes in the local environment of the Trp moiety, which can result in shifts in the maximum fluorescence peak, changes in the emission intensity or changes in anisotropy (Ghisaidoobe and Chung, 2014) (Fig. 3B). This analytical technique, unlike other fluorescence-based methods such as fluorescence polarization and anisotropy measurements, does not require additional conjugation procedures of the analytes of interest. In recent years, intrinsic fluorescence spectroscopy has been successfully established as a reliable and label-free method for the quantitative analysis of galectin-glycomaterial interactions. Sindrewicz and coworkers investigated the presence of tryptophan residues within twelve human galectins (Table 2) and proposed fluorescence intensity-dependent determination of kinetic parameters for galectin-based binding events (Sindrewicz et al., 2019).

The binding properties of representative members of each galectin family, namely prototype (Gal-2), chimera-type (Gal-3), and tandem-repeat-type (Gal-4) galectins were analyzed with galactose, lactose, LacNAc type 2, and a semi-synthetic heparin ligand. Glycan ligand solutions were titrated into a solution of galectins at fixed concentrations and ligand binding-derived shifts of the emission wavelengths of Trp (at 285 nm excitation) were measured with a fluorescence spectrophotometer. Remarkably, using tryptophan fluorescence spectroscopy (TFS), measurements gave $K_D$ values in the μM range for lactose and LacNAc type 2. Additional ITC measurements confirmed the reliability of the obtained binding constants. Weak binding of galactose to Gal-4 in the mM range demonstrated the high sensitivity and ease of use of this fluorescence-based analytical method.

The binding properties of Gal-3 were investigated with lactoside-derived glycan-ligands, to highlight the potential of this technique in the analysis of low-affinity binding interactions (Bernhard et al., 2020). By applying direct waveform recording technology, fluorescence decay curves were obtained that revealed binding constants in the μM range. The authors demonstrated the adverse effects of fluorescence-intensity-based measurements and underlined the attractiveness of lifetime fluorescence measurements in contrast to other common methods for the examination of galectin-glycan binding. Specifically, low concentrations of analytes are required when compared with ITC or NMR. While ELISA or SPR measurements require the immobilization of analytes, here the galectin binding is measured under in-solution conditions. Concerning the fluorescence intensity, measuring real-time fluorescence is independent of experimental conditions such as pipetting errors or inconsistency of measuring devices (laser power, inner filter effects).

### 2.2.3. Fluorescence polarization

Fluorescence polarization (FP) is defined as the emission of polarized light from a fluorophore previously excited with linearly polarized light. Here, the fluorescence polarization of the fluorophore depends on its rotational speed in solution. The measurement of fluorescence polarization is based on the change in rotational speed of probes containing fluorophores. The binding of an analyte to fluorescent probe results in a decreased rotation speed, and thus more emitted light remains polarized. In contrast, the rotation of free fluorescent probes is very fast and leads to depolarization of the emitted light (Kakehi et al., 2001) (Fig. 3C). The Leffler group first introduced an FP-based analysis of galectin-carbohydrate interactions in the aqueous medium. Here, a constant concentration of a fluorophore-conjugated probe (carbohydrate or glycomimetic ligand) is titrated with varying concentrations of analytes (galectins) to be analyzed. Once the equilibrium is reached, the emitted light is measured using a fluorescence spectrophotometer in a microtiter plate format without separating the unbound fraction of the fluorescent probe. The received polarized emitted light is detected and reported as polarization value ($P$) or anisotropy ($A$); the latter reflects the averaged value of the bound and free fluorescent probes. Binding curves are obtained showing the increase in anisotropy of the unbound probe in solution ($A_0$) to a value where all the probe is bound by galectins ($A_{max}$). Kinetic parameters such as the equilibrium dissociation constant $K_D$ and $K_a$ can be obtained with corresponding analysis software (Sörme et al., 2004).

Hsieh and coworkers investigated the binding affinities of Gal-1, -3, and -7 for fluorescein isothiocyanate (FITC)-conjugated LacNAc type 2 and type 2 disaccharides using FP-based binding and competitive assays (Hsieh et al., 2015). Dissociation constants ($K_D$) in the μM range were obtained demonstrating the moderate affinities of Gal-1 and Gal-3 for LacNAc type 2 disaccharides whereas Gal-7 preferred the LacNAc type 1 motif. Moreover, additional crystallization experiments revealed the orientation of the LacNAc moiety as a critical factor for the galectin binding preference. An FP-based competitive assay was applied to elucidate the inhibitory potential of HMOs and blood group antigens. The blood group A tetrasaccharide was revealed as an inhibitor of Gal-4C binding of lactose with $K_D$ values in the low μM range (Bum-Erdene et al., 2015).

Recently, the enhancement of Gal-3 binding affinities by ligand-driven protein conformational changes (Noresson et al., 2018) has been demonstrated. Here, the affinities of Gal-3 for galactoside-derived 2-*O*-sulfated and non-sulfated aromatic substituents at C-3 were explored using competitive fluorescence polarization experiments. $K_D$ values in the μM range were obtained that demonstrated the preferred binding of Gal-3 to 2-*O*-sulfated galactosides bearing a 4-methoxy-2,3,5,6-tetrafluorobenzamido substitution at the C-3-position. Structure-based analysis contributed this effect to a conformational change of the Arg144 side chain induced by the fluorinated aromatic moiety that enabled favorable electrostatic interactions between the 2-*O*-sulfate group and Arg144. Based on these findings, Peterson et al. (Peterson et al., 2018) further demonstrated the effects of systematic structural tuning of glycan-based ligands for enhanced galectin binding. Symmetric and asymmetric TDG-derivatives were optimized to increase Gal-1 and Gal-3 binding affinities. The FP-based analysis revealed symmetrically substituted TDGs as exceptional Gal-1 ad Gal-3 inhibitors with $K_D$ values in the subnanomolar range. Furthermore, asymmetric TDGs bearing trifluorophenyltriazole and coumaryl substitutions induced selective binding of Gal-3 in comparison to Gal-1 by a factor of 46. The nM affinity binding of Gal-3 to symmetrically C-3-substituted TDGs was also confirmed in the study by Stegmayr and colleagues who examined the extra- and intracellular inhibitory potentials of the small Gal-3-inhibiting compounds (Stegmayr et al., 2019).

Among the huge variety of galectin-targeting inhibitors proposed for medical applications, plant-derived polysaccharides are the subject of discussion. In 2016, fluorescence anisotropy-based binding and inhibition studies provided detailed information on the binding and inhibition of a series of pectins and galactomannans toward a set of galectins (Gal-1,-2,-3,-4C,-4N,-7,-8N,-9C,-9N) (Stegmayr et al., 2016). Low or no galectin-CRD-specific inhibition of the tested polysaccharides was observed, and it was suggested that other binding mechanisms may be involved to confer beneficial medicinal properties of plant-derived polysaccharides.

Recently, the role of Gal-3 in the pathology of Alzheimer's disease (AD) was revealed by identifying Gal-3 as a ligand for the triggering receptor expressed on myeloid cells 2 (TREM-2) which is involved in the signaling pathway for diminishing AD-derived plaques in the brain (Boza-Serrano et al., 2019). Here, upregulation of Gal-3 was observed in AD patients and binding to TREM-2 in the nM range was achieved by fluorescence-anisotropy-based assays suggesting the inhibition of Gal-3 as a therapeutic approach to slow the progression of AD.

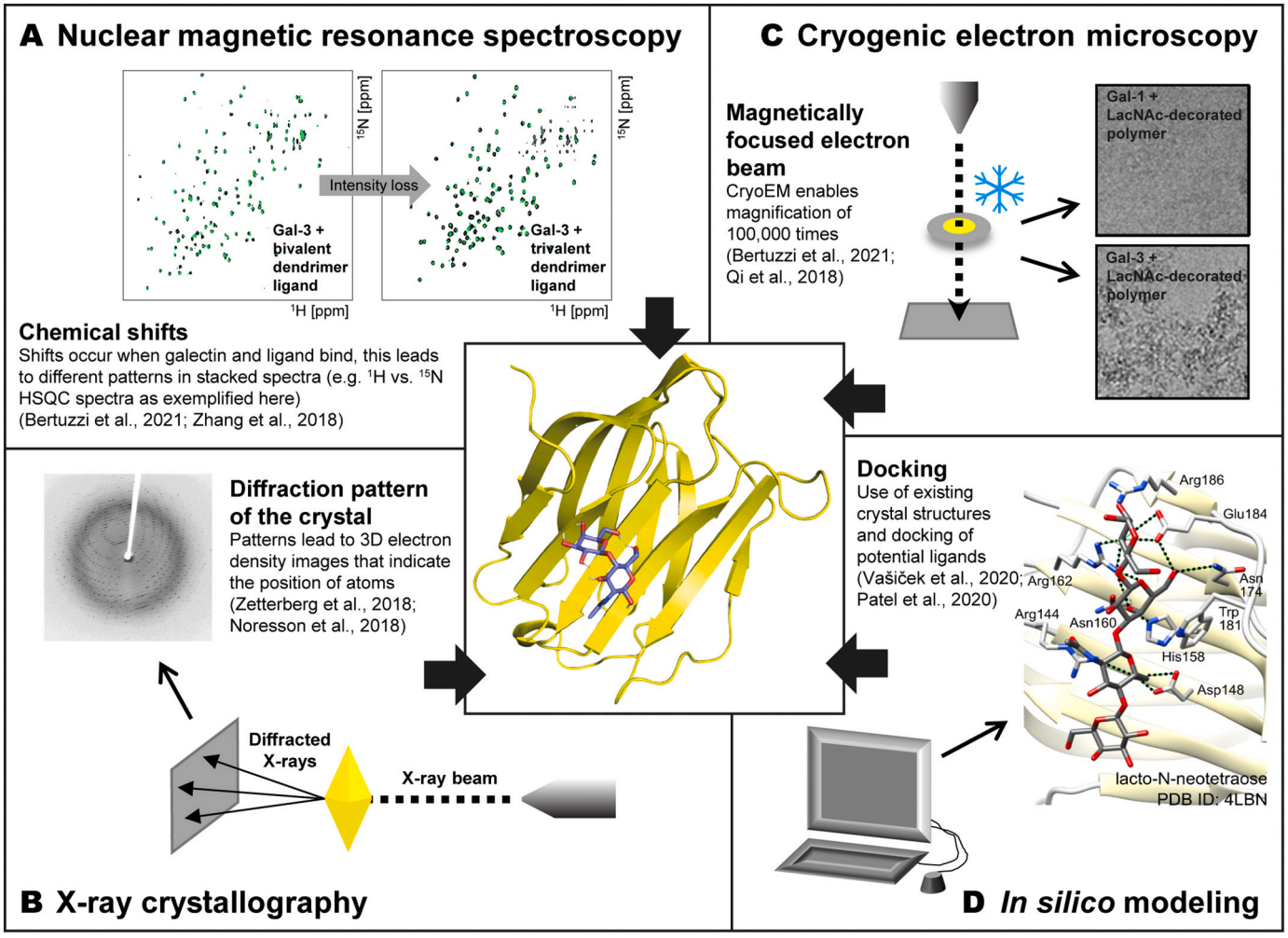


**Fig. 4.** Structure-based assays for evaluating the interaction between galectin and ligand. (A), NMR spectroscopy; (B), X-ray crystallography; (C), cryoEM and *in silico* modeling (D), all provide a prediction of the structure of the galectin-ligand complex.

Taken together, fluorescence polarization provides a simple and easy in-solution equilibrium method to study specific inter-molecular galectin-glycan interactions. This method offers affinity determination at the monovalent site and enables high-throughput analysis of small-sample volumes in the microwell-plate format. However, covalent labeling of samples with fluorescent protein tags such as fluorescein or FITC may lead to structural changes of the glycan moieties and may not reflect the binding behavior of galectins to native glycans.

### 2.3. Structure-based assays

For the aim of this review, the assays that exploit the structure of galectins are considered. Whereas solid-phase and in-solution assays primarily monitor the strength of interaction between galectin and its carbohydrate ligand, mass spectrometric methods take advantage of the molecular weight of the proteins and can detect galectin-ligand complexes. Crystallization and *in silico* studies of the proteins provide insights into the structure-function relationships concerning ligand binding (Fig. 4).

#### 2.3.1. Detection of galectin-ligand complexes by mass spectrometry

Mass spectrometry (MS) methods are used to detect complexes of lectins and their ligands in solution *via* mass determination, particularly for noncovalent interactions (Daniel et al., 2002). The method enables the determination of the affinities between two binding partners without the influence of immobilization linkers or labels. An example is the direct electrospray ionization assay (ESI-MS) or, in particular, the ESI-MS titration method, which measures the equilibrium of the protein-ligand interaction. The gas-phase ions of the free protein and the protein engaged in complexes with its ligand are detected; the abundance of the protein in both states can be determined (El-Hawiet et al., 2012). ESI is a gentle technique that facilitates the transfer of intact complexes from liquid to the gaseous phase and, hence, is suitable for the investigation of affinities between macromolecular binding partners with noncovalent interaction. The method provides values for the association constant $K_a$ that are calculated from the ratio of protein-ligand complexes to the free protein and ligand (Kitova et al., 2012). The values obtained represent the ratio of the complexes present; the higher the value, the stronger the interaction between galectin and ligand. Shams-Ud-Doha and coworkers determined the interaction between a library of human milk oligosaccharides (HMOs) and human galectins Gal-1, Gal-3C (C-terminal fragment), Gal-7, and Gal-9N (N-terminal fragment) by direct ESI-MS measurements (Shams-Ud-Doha et al., 2017). Free glycans were tested with the galectin constructs; the results were compared with those obtained from microarray screenings, where the HMOs were printed on the microarray (Noll et al., 2016). This comparison showed that the microarray provided several false negative and false positive results (Shams-Ud-Doha et al., 2017). In another approach, Atmanene et al. (Atmanene et al., 2017) used MS with Gal-3 and mono- and di-arylated LacNAc derivatives as ligands to obtain values for the equilibrium dissociation constant $K_D$. This value is reciprocal to $K_a$; the lower the $K_D$ value, the stronger the affinity of the ligand for galectin. Gal-3 was

incubated with different concentrations of *N*-acetyllactosamine and measured by MS. The measurements provided data for the construction of a titration curve to determine $K_D$ values (Atmanene et al., 2017). Kitova and coworkers investigated the influence of protein labeling on the affinities for ligands by direct ESI-MS measurements. The C-terminal domain of Gal-3 was covalently attached to different labels (biotin, Cy5, Bolton-Hunter reagent); the higher the degree of labeling, the lower the interaction of Gal-3 with the compound measured by ESI-MS (Kitova et al., 2020).

MS-based methods bring a range of advantages. In contrast to solid-phase assays, no immobilization or labeling is necessary that could interfere with the binding properties between galectin and ligand. In addition, the methods are very sensitive and allow the detection of low affinities. It is a good alternative to methods with large sample volumes; for example, only one fmol of sample is applied when nanoflow ESI is used (Kitova et al., 2012).

#### 2.3.2. Analysis of galectin-ligand interaction by nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy detects the interaction of atoms that are close to each other or share one bond; in this way, the interaction between lectins and ligands can be determined (Ardá and Jiménez-Barbero, 2018; Gimeno et al., 2020) (Fig. 4A). This method can reveal specific interacting atoms, as well as information about secondary structure (α-helices, β-strands, and random coils. In addition, NMR spectroscopy can elucidate whether lectins occur in a monomeric or oligomeric form or in which concentration oligomerization takes place. For investigation of galectin-ligand complexes and the influence of binding on protein structure, recombinant [$^{13}$C, $^{15}$N]-labeled galectins need to be produced in an isotopically enriched medium (Ippel et al., 2015; Ippel et al., 2016). When radiation pulses are applied to the measured molecule, every atom has a unique chemical shift, depending on its atomic surroundings (Howard, 1998). Nuclear relaxation effects give an indication of the three-dimensional structure of proteins based on the “through-space” NMR. These relaxation data combined with the chemical shifts of the protein with and without ligand give insights into the interaction between the ligand and the respective lectin. For example, if amino acids of the galectin CRD interact with ligands, changes in the NMR data are observable. Ippel et al. (Ippel et al., 2016) explored the interaction of di-LacNAc with Gal-3 by NMR spectroscopy and confirmed that the glycan binds to the canonical site, which is responsible for β-galactoside-binding. Hsieh et al. (Hsieh et al., 2016) titrated fluorinated TDG derivatives against Gal-1, Gal-3 (CRD), and Gal-7 in NMR titration experiments. The recorded $^{19}$F-NMR spectra revealed that the fluorophenyl-triazole moiety of the TDG derivatives binds into the subsites B and E in the CRD of Gal-1 and Gal-3 and into the subsite E of Gal-7 N-terminal CRD. In other studies, these galectins were examined regarding the interaction of ligands with their particular amino acid residues (Rauthu et al., 2015; Zhang et al., 2019). The strength of chemical shifts of the residues provided an indication of which amino acids are involved in the binding of the glycan ligand. A specific NMR method is saturation transfer difference NMR that can identify the ligand and assign stronger signals to the protons of the ligand involved in binding (Haselhorst et al., 2009). Bertuzzi and coworkers detected, which parts of the investigated multi-LacNAc HPMA copolymers were primarily involved in binding to Gal-1 and Gal-3, and facilitated a rating of interaction strength for the individual atoms within the ligands (Bertuzzi et al., 2021).

The advantage of NMR spectroscopy is that it provides accurate information on the structure of galectins and the changes induced by ligand binding. With this knowledge, more effective ligands can be tailored to find promising inhibitors for, *e.g.*, cancer therapy.

#### 2.3.3. Analysis of galectin-ligand interaction by X-ray crystallography

In crystallization experiments, proteins can be crystallized in complexes with their ligands; subsequently, the crystal structure of the complex is determined by X-ray crystallography (Fig. 4B). In most cases, the preparation of crystals requires cloning, expression, and purification of the particular lectin in high homogeneity and quantity. There are exceptions where the natural resources are rich in lectins, so that their purification for crystallization is possible (Pietrzyk-Brzezinska and Bujacz, 2020; Pietrzyk et al., 2015). It also requires sufficient long-term stability under crystallization conditions. It may often be extremely challenging to find the conditions under which the protein is most soluble and stable. In the next step, crystallization, a nucleus is first formed that is then grown into a crystal. This step is critical and can be tuned by using crystallization phase diagrams that show the dependence of protein crystallization on protein concentrations and adjustable parameters (*e.g.*, precipitant, pH, temperature, additives). Nucleation and crystal growth only occur under very specific conditions. There are various methods for crystallization that use different conditions and pathways for nucleation (Chayen and Saridakis, 2008). The crystals produced are then analyzed by X-ray crystallography (Zheng et al., 2015). The diffraction pattern of the X-rays is transferred into a 3D electron-density image that depicts the mutual position of the atoms and their bonds within the crystal (Rupp, 2009). This method provides the ultimate evidence, which particular amino acids bind the ligand. In recent studies, galectins have been crystallized by vapor diffusion (Hsieh et al., 2015; Patel et al., 2020). To acquire a crystal of the galectin-ligand complex, two approaches are possible: (*i*) the formation of apo crystals (from proteins without ligand) and subsequent soaking in a solution of the ligand or (*ii*) co-crystallization of both binding partners. Thus, Gal-4 and Gal-4C were co-crystallized with lactose (Lac) (Rustiguel et al., 2016a; Rustiguel et al., 2016b), Lac-3’-sulfate, 2’-FL, LNT, and LNnT (Bum-Erdene et al., 2015) as ligands. In contrast, the Gal-8N apo crystal was soaked in the ligand solution before X-ray (Bohari et al., 2018; Patel et al., 2020). For other galectins – Gal-3 CRD, Gal-1, Gal-7 – soaking and co-crystallization are varied (Hsieh et al., 2015; Hsieh et al., 2016; Noresson et al., 2018; Peterson et al., 2018; Zetterberg et al., 2018). The comparison of the apo-form crystals with complexes of protein and different potential ligands indicates how compounds alter the arrangement of atoms in the binding site of galectin, and thus the strength of interaction. Since crystallization experiments provide a detailed insight into the molecular nature of the interaction between galectin CRDs and potential ligands, the method is highly promising for targeted drug development. Nevertheless, it has some drawbacks. Optimization of crystallization conditions is time- and material-consuming and often very demanding. It is usually impossible to crystallize protein parts that are too flexible, which may require the preparation of adopted constructs. In addition, for some proteins, it is not possible to produce crystals of sufficient quality for X-ray measurements.

#### 2.3.4. High-resolution images of galectin-ligand complexes from cryogenic electron microscopy

If NMR spectroscopy and X-ray crystallography fail to determine the structure of galectin-ligand complexes, the relatively novel method of cryogenic electron microscopy (cryo-EM) might solve the problem (Yip et al., 2020; Zhang et al., 2020). In electron microscopy, a beam of electrons focused by magnets is applied to the measured sample (Fig. 4C). A magnification of 100,000 times is possible. Cryo-EM is a form of transmission electron microscopy (Ryu, 2017) where a specimen is frozen at cryogenic temperatures; this keeps the molecules in their intact and native state. Treatments of the samples that could alter their constitution are not necessary and the acquired data can be converted into high-resolution images. The best results are obtained with symmetric particles and homogeneous samples. Bertuzzi and coworkers (Bertuzzi et al., 2021) presented one example of the use of cryo-EM with galectins and their ligands. They observed the aggregation of Gal-1 and Gal-3 with LacNAc-HPMA copolymers and were able to examine different degrees of aggregation. Cryo-EM also allowed the detection of self-assembled micro-ribbons of Gal-1 with lactose- and galactoside-carrying ligands (Qi et al., 2018). The glycans were coupled to

Rhodamine B dye, which dimerized spontaneously. The glycan-Rhodamine B dimers bound one CRD of the dimeric Gal-1 per sugar unit; the other Gal-1 CRD could further interact with another glycan-Rhodamine B dimer. Thus, micro-ribbons were formed that were visualized by cryo-EM and prove the interaction of Gal-1 with the Rhodamine B-coupled glycan ligand. If no ribbons were formed, no interaction between galectin and ligand had occurred. Although cryo-EM produces high-resolution images, the method has its drawbacks. The analyzed molecules or complexes should preferably be homogeneous and symmetrical. If they are too small, it may be difficult to obtain the data; however, too large macromolecular complexes tend to increase linear magnification distortions, which limits the resolution of the method. For protein-ligand complexes, these conditions are sometimes not easy to fulfill.

### 2.3.5. *In silico modeling of galectin-ligand complexes*

Two different approaches are used to study the interaction between galectins and potential ligands *in silico*: molecular docking and molecular dynamics (Salmaso and Moro, 2018) (Fig. 4D). Molecular docking is used to predict the behavior of a protein with an interacting small

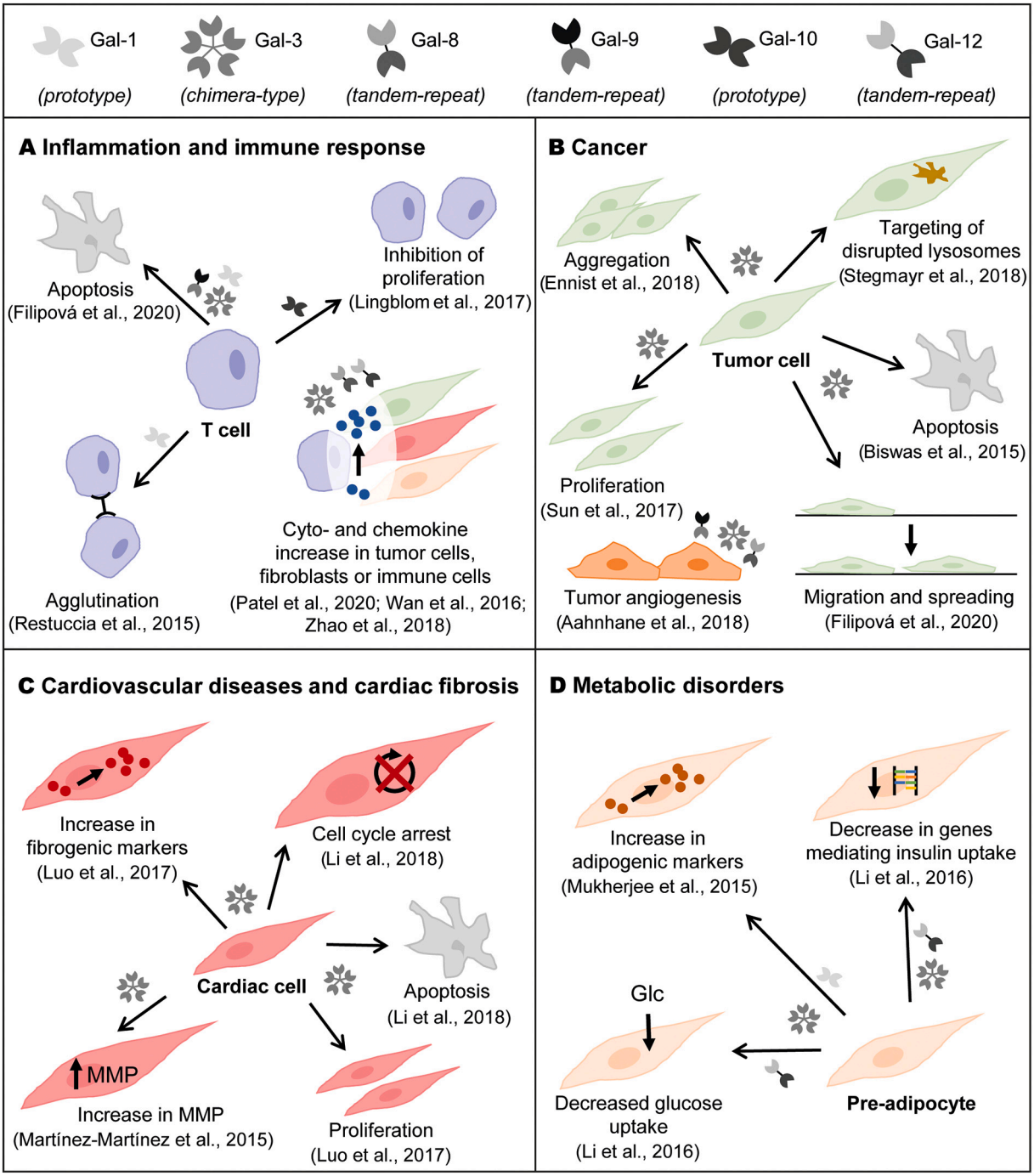


**Fig. 5.** Functional assays monitoring the effects of galectins on human cells. Assays related to (A), inflammation and immune response; (B), cancer; (C), cardiovascular diseases and cardiac fibrosis; (D), metabolic disorders. MMP, matrix metalloprotease; Glc, glucose.

molecule such as a ligand or inhibitor, or to perform a virtual screening for potential ligands (Meng et al., 2011; Morris and Lim-Wilby, 2008). It proposes the best pose of the ligand within the binding site of the protein and considers the degree of flexibility of both binding partners. For docking, the conformation of the ligand and its position within the binding site of the protein must first be predicted; then the binding affinity between protein and ligand is estimated. Corresponding computer programs use sampling algorithms and scoring functions to forecast the binding of the ligand to the protein. While docking investigates the interaction and is based on a relatively rigid system, molecular dynamics examines the conformational changes in the dynamic systems (Leach, 2007; Salmaso and Moro, 2018). It simulates that the ligand-lectin complex is in motion and calculates the surface energy as a function of time. Both approaches require knowledge about the structure of the complexes to be analyzed, *e.g.*, from X-ray crystallography or modeling of the protein. Vašíček and coworkers (Vašíček et al., 2020) used the crystal structures of Gal-3 (PDB ID: 5OAX) and Gal-1 (PDB ID: 3OYW) for docking and investigation of newly prepared ligands – 3,3'-*O*/*N*-disubstituted TDG derivatives. *In silico* docking provided results that allowed predictions for each ligand studied. A similar approach was used with Gal-8N (PDB ID: 3AP7) and methyl β-D-galactomalonyl phenyl ester ligands in docking experiments for structure-based drug design (Patel et al., 2020). Molecular docking often serves as a basis for molecular dynamics calculations. For example, molecular dynamics simulation of binding of selected 3,3'-*O*/*N*-disubstituted TDG derivatives to Gal-1 and Gal-3 revealed a dynamic equilibrium between binding, unbinding, flipping, and switching of binding modes within the galectin binding site (Vašíček et al., 2020). Molecular dynamics also allows us to find the preferred and most stable binding modes. Analyzes of, *e.g.*, Gal-3 (Boza-Serrano et al., 2019; Bumba et al., 2018; Zetterberg et al., 2018) and Gal-4 (Bohari et al., 2018; Bum-Erdene et al., 2015; Rustiguel et al., 2016a; Rustiguel et al., 2016b) with new ligands by molecular dynamics were exemplified in the literature, presenting some notable conclusions for future research.

Nilsson and coworkers performed pioneer work in the area of galectin-ligand interactions using molecular dynamics. They were able to explain the selectivity of 3*N*-aryl galactosides for the C-terminal and 3*N*-aryl gulosides for the N-terminal domain of Gal-9 (PDB ID: 3WLU) (Mahanti et al., 2020). Additionally, using molecular modeling techniques, they discovered (Dahlqvist et al., 2019) a unique subsite of Gal-3 (PDB ID: 3ZSL) that enabled the construction of highly selective and effective inhibitors.

The *in silico* analysis of galectin-ligand complexes is a helpful tool for the prediction of potential galectin ligands and drug design. However, a crystal structure of the particular galectin is required, at best in complex with a ligand of similar nature, which may not always be available. In larger screening sets, only simple docking studies are usually feasible due to the immense demand on time and computational capacity for molecular dynamics studies. Galectins are relatively difficult species for molecular modeling due to the shallow character of their binding site and often a relatively wide range of possible ligand positions. Still, *in silico* methods can pre-select promising compounds and provide direction for drug design, as in the case of a completely novel family of galectin inhibitors based on aminopyrimidine substitution (Dahlqvist et al., 2019).

## 3. *In vitro* assays in cell cultures

Galectins are multifunctional molecules and affect various disease-related processes in parallel (Sciacchitano et al., 2018). Galectin binding to cell surface glycans influences cellular signaling and cell-cell interaction processes; in particular, cancer-related events, development of fibrosis, immune response, and inflammation. Therefore, cell-based assays provide valuable information on the effect of particular galectins and their inhibitors. While binding/ inhibition studies described in Section 2 provide a deeper insight into the affinity of candidate compounds to the isolated galectin protein, functional cell assays reveal the efficiency of these compounds in the cellular environment including their biological consequences. In contrast to *in vivo* assays, cell-based *in vitro* assays are non-invasive and do not require animal testing. In this section, we specify cell-based functional assays that take advantage of *in vitro* or *ex vivo* cell lines (mainly mammalian or murine) to study the effect of galectins and their potential carbohydrate inhibitors on disease-related cell processes (Fig. 5).

### 3.1. Binding and inhibition assays in cell cultures

To elucidate the interaction between galectins and their glycan cell-surface binding partners – glycoproteins or glycolipids – flow cytometry or fluorescence-activated cell sorting (FACS) is the method of choice. This method allows cell counting and takes advantage of fluorescent labels (Bonner et al., 1972). Usually, labeled galectins or galectin-specific antibodies are used for this aim. Depending on the choice of the particular cell line, galectins bind specifically to cells depending on the presented glycans. Not only binding but also inhibition of galectin binding to the cells can be evaluated as demonstrated by applying carbohydrate inhibitors – *e.g.*, synthetic lactosides – that competed with the binding process (Roy et al., 2017). The stronger the inhibitor, the lower the amount of galectin that binds to the cells and the weaker the signal detected by flow cytometry. Vašíček and coworkers (Vašíček et al., 2020) performed binding inhibition experiments with galectin-overexpressing human embryonic kidney (HEK293) cells, Gal-3 and Gal-1, and a range of 3-*O*-substituted TDG inhibitors.

Visualization of galectin-cell complexes can be challenging because direct fluorescent labeling can inactivate the lectin activity of galectins. Another option, direct chemical biotinylation, often leads to a mixture of biotinylated products that are poorly defined and may also compromise lectin activity (Bumba et al., 2018). Therefore, the introduction of a so-called AVI-tag (GLNDIFEAQKIEWHE) for well-defined biotinylation of recombinantly produced galectins was established (Bumba et al., 2018). The AVI-tag contains a lysine that is specifically biotinylated by the biotin ligase (encoded by another plasmid) co-expressed during the production of the galectin construct in the recombinant *E. coli* strain. The distribution of the AVI-tagged galectin on the cell surface is detected by FACS using a fluorescently labeled streptavidin, which firmly binds the AVI-tagged biotinylated galectins. This method of fluorescent galectin labeling is reliable and does not influence the bioactivity of the proteins. Furthermore, since the streptavidin is bound to the biotinylated AVI-tag after galectins bind to the cells, the interaction between the galectin and the cell is not affected.

Besides monovalent inhibitors, scavenging of AVI-tagged Gal-3 by multivalent neo-glycoproteins carrying 3-*O*-substituted LacdiNAc mimetics was demonstrated with human adenocarcinoma DLD-1 cells using FACS (Heine et al., 2021). Filipová et al. (Filipová et al., 2020) detected the inhibition of bonding of AVI-tagged Gal-3 to the surface of HEK293 cells by various poly-LacNAc-decorated glycopolymers. Besford and coworkers (Besford et al., 2017) demonstrated an alternative approach, targeting PC3 prostate cancer cells with fluorescently labeled lactose-bearing nanoparticles. The binding of labeled nanoparticles was demonstrated by flow cytometry and the authors inferred that the nanoparticles target galectin presented on the cell surface.

Fluorescence microscopy is an alternative method to flow cytometry to closely follow the fate of labeled galectins and/ or their inhibitors within the cell or on its surface. Labeled ligands or galectins are incubated with specific cell lines; if they enter the cells, they are visible under the microscope; if they remain in the extracellular space, they are washed away unless they bind to the cell surface. The potential of the compounds to penetrate into the cells indicates their possible use as extracellular or intracellular galectin inhibitors. For example, human serum albumin-based neo-glycoproteins have been found not to specifically penetrate DLD-1 cells (Heine et al., 2021). In a study by Besford and coworkers (Besford et al., 2017), confocal microscopy showed the

attachment of lactose-decorated glycogen nanoparticles to the surface of PC3 cells, thus indicating the localization of Gal-3 on the cell surface.

The above-described methods provide the first insight into the binding of ligands to galectins and their effect on specific human cell lines. Nevertheless, galectin-related processes in diseases are much more complex than just interaction and transport. Hence, functional assays in cell cultures are crucial to complete the picture, considering the physiology and *modus operandi* of specific cell lines.

### 3.2. Functional assays

The involvement of galectins in cellular processes has been studied in a range of functional assays in cell cultures, monitoring, *e.g.*, apoptosis, angiogenesis, proliferation, migration, agglutination or adhesion. Functional cell-based assays provide clues as to how galectins may affect signaling and differentiation processes associated with a particular disease, helping to predict a suitable design of an *in vivo* assay or to preselect the most promising candidate compound for such assays. In particular, Gal-3 and Gal-1 are intensively studied in terms of their effects on tumor growth and development (Ruvolo, 2016; Thijssen et al., 2015), immune response, inflammation (Brinchmann et al., 2018; Parsonage et al., 2006), pathological fibrotic processes (Suthahar et al., 2018), and other disorders (Pugliese et al., 2015; Sciacchitano et al., 2018). Simple binding/inhibitory potency of compounds is combined with other prerequisites, such as the ability to penetrate the cell membrane, biocompatibility, bioavailability, and the general ability to trigger or inhibit the targeted processes in a highly complex environment of the cell culture. Typically, mammalian cell lines (*in vitro* – immortalized cell lines representing a specific type of cells; *ex vivo* – cell lines obtained directly from living organisms), are incubated with the galectin. Then either the interaction with the cells or the effect on the cellular mechanisms is evaluated by the assays described in this section. Using cell lines with knocked-down galectin genes, the pure effect of externally applied galectins can be investigated. Thus, the respective galectin-negative cell line can be obtained as a negative control or to selectively monitor the effect of one particular galectin; thus, endogenous galectins do not interact with exogenously applied recombinant galectins. While *in vitro* cell lines are more stable, affordable, and easier to obtain, *ex vivo* cell lines are physiological and more truly represent the conditions in the living organism. To test the influence of galectins, both types of cells are used.

The complexity of the biological pathways makes a reliable assessment of galectin inhibitors as potential therapeutics a complex task, often requiring a combination of several functional assays. It must be noted that the array of human disorders accompanied by the changes in galectin production is much broader than the one listed herein. In this review, we focus on the up-to-date available functional cell assays that investigate the effect of galectins on selected biological processes. It can be expected that the range of available assays will steadily increase along with the ever-expanding field of knowledge on the pathological functions of galectins and their potential pharmacological targeting.

#### 3.2.1. Inflammation and immune response

Responses of the immune system occur in practically all galectin-related diseases, such as cancer, heart failure, rheumatoid arthritis, and metabolic disorders (Elola et al., 2018; Girotti et al., 2020; Parsonage et al., 2006; Sundblad et al., 2017). Assays investigating the associated role of particular immune cells (T-cells and macrophages) are described in this section (Fig. 5A).

T-cells are critical for the cellular immune response and play a role in the protection against cancer. Galectins, such as Gal-3, bind to their cell-surface receptors, inducing apoptosis of T-cells (Ahmed and AlSadek, 2015; Xue et al., 2016), which weakens the immune response and allows cancer cells to proliferate and spread. Conversely, this means that scavenging of Gal-3 by glycomaterials protects immune cells and supports the elimination of cancer cells.

For studying the apoptosis and agglutination of T cells, the immortalized T cell line of Jurkat cells is often used. Apoptosis is followed by staining of the apoptotic marker Annexin V and FACS analysis. To test the anti-apoptotic effect of glycomaterials, Jurkat cells are incubated with the glycomaterial and externally added galectin, and the extent of the anti-apoptotic effect is observed (Filipová et al., 2020; Heine et al., 2021; Vrbata et al., 2022). HPMA-based glycopolymers and the neoglycoproteins were able to scavenge Gal-3, and thus protect Jurkat cells from apoptosis. Agglutination of Jurkat cells is considered an early sign of apoptosis. Restuccia and coworkers showed that LacNAc-decorated nanofibers effectively protected Jurkat cells from Gal-1-mediated agglutination (Restuccia et al., 2015). Multivalent nanofibers were more efficient than monovalent lactose and TDG. A preventive effect was also observed. Agglutinated and stained cells were examined microscopically.

Galectins are known to induce the expression of pro-inflammatory cytokines, which potentially promote cell cancer progression and metastasis (Ahmed and AlSadek, 2015). Therefore, upregulated expression of inflammatory markers can be used to assess the inhibitory effect of compounds on the interaction between galectins and mammalian cells. Expression levels are determined by reverse transcription of the cellular RNA into DNA. The amount of recovered DNA provides information about the up- or down-regulation of the respective genes, and thus about the corresponding processes. When breast cancer cells (SUM159) were incubated with Gal-8, they strongly overexpressed IL-8, IL-1β, and IL-6. In contrast, the expression of inflammatory markers was suppressed in cells incubated with Gal-8 and glycemic inhibitors (methyl β-D-galactomalonyl phenyl ester) (Patel et al., 2020).

#### 3.2.2. Cancer

Cancer-related processes are most studied in the field of pharmacological galectin inhibition, specifically targeting processes like metastasis, cancer cell migration, cell survival, tumor angiogenesis, and many more (Ahmed and AlSadek, 2015; Campo et al., 2016). As described in Section 3.2.1, cancer progression is closely related to the apoptosis of T cells and the production of pro-inflammatory markers. Here we give a structured overview of the methods used to investigate these processes and the effects of tested glycomaterials (Fig. 5B).

Gal-3 provides a protective microenvironment for cancer cells (Ruvolo, 2016), protecting cancer cells from the recognition by the immune system and, hence, preventing their death (Ahmed and AlSadek, 2015). Cancer cell apoptosis was observed when SU-DHL-6 lymphoma cells were deprived of the protective extracellular Gal-3 layer by incubation with TF-antigen-presenting gold nanoparticles. With an increasing concentration of the nanoparticles, apoptosis of the cancer cells increased (Biswas et al., 2015). Importantly, this effect was not observed in Gal-3-negative cells (RAJI cells) used as negative controls.

The proliferation of cancer cells is associated with tumor survival; inhibition of proliferation leads to stagnation of tumor development. Cancer cell proliferation is promoted by Gal-3 (Califice et al., 2004). The effect of glycomaterials on this process can be analyzed in cell viability assays such as cell counting (Filipová et al., 2020; Paz et al., 2018; Vrbata et al., 2022) or the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay (Besford et al., 2017; Sun et al., 2017). MTT is incubated with treated cells and imported by endocytosis. The compound is reduced to the purple insoluble formazan by NADPH-dependent cellular oxidoreductase enzymes in mitochondria, endosomes, and lysosomes (Kuete et al., 2017). As a result, dead cells show no response to MTT. To exclude the negative effect of MTT on living cells (Lü et al., 2012) and prove the positive effect of galectins on cancer cell proliferation, a galectin-negative control should always be included.

Migration of cancer cells is a step that follows proliferation and leads to metastasis and, ultimately, to the uncontrollable spread of the tumor. In scratch assays, tumor cells (*e.g.*, DLD-1, PC3) are first grown on multiwell plates to a confluent layer and then disrupted with a scratch;

migration of cells can be observed under the microscope when the scratch gap is closing in the cell layer in time. Thus, the positive effect of galectins on the migration of cancer cells can be detected. Moreover, the inhibition of migration by trapping galectins with glycomaterials indicates the efficacy of the anti-metastatic potential of the particular glycoconjugate (Filipová et al., 2020; Freichel et al., 2020; Vrbata et al., 2022). Cell migration can be detected not only in scratch assays but also in trans-well assays. Cells are grown in plates with pores to allow migration, using the upper chamber with the medium void of serum. The lower chamber is filled with medium containing serum to attract the cells. After a while, the cells from the upper chamber are removed and cells from the lower chamber are stained with crystal violet and solubilized. The cell extract collected from the bottom chamber is then analyzed spectrophotometrically; the higher the absorption, the more cells migrated. Although not yet performed to study the effect of galectin inhibitors on cell migration, this assay may be useful for such studies (Fan et al., 2018a, 2018b).

Angiogenesis is crucial for the metabolic supply of tumors by the formation of new blood vessels. This process is induced in tumors by eliciting growth factors such as the vascular endothelial growth factor (VEGF) and the release of galectins, which cluster the VEGF-receptor on endothelial cells. Besides the proliferation and migration of cells, tube formation is another indicator of angiogenesis that can be investigated *in vitro* (Aanhane et al., 2018; Stryker et al., 2019). In the chicken embryo chorioallantoic membrane (CAM) assay, fertilized chicken eggs were opened, and the chorioallantoic membrane was treated with galectins (Aanhane et al., 2018). After several days, the membrane was observed under the microscope to determine the effect of the treatment on vascularization. Although this assay was *in vivo*, it allowed comparison to *in vitro* experiments such as tube formation assays. In tube formation or sprouting assays, HUVEC cells are seeded in a matrix (Matrigel) and incubated with galectins and/ or potential inhibitors (Aanhane et al., 2018; Guha et al., 2013). Under the influence of Gal-3, HUVEC cells align and build tubular structures. By applying TF-antigen-containing glycopeptide inhibitor and lactose, tube formation was decreased since both carbohydrates served as Gal-3 inhibitors (Guha et al., 2013). A decrease in the extent of tube formation stimulated by intrinsic Gal-3 was also detected upon addition of HPMA-based glycopolymers carrying Gal-3-inhibiting glycomimetics (Vrbata et al., 2022). The outcome of the tube formation assay is examined microscopically. Optionally, the up- and downregulation of angiogenetic genes can be determined by PCR with the RNA from the treated HUVEC cells (Varinská et al., 2020). As also described in Section 3.2.1, RNA is isolated from the cells and reverse-transcriptase PCR is performed. The amount of so-produced DNA indicates the expression of specific angiogenetic markers. Gal-8, for example, had a positive effect on the formation of new capillaries in the presence of VEGF in the CAM assay. Furthermore, the Gal-8/ VEGF combination led to an increased expression of these genes compared to VEGF only, to Gal-8, or the control (Varinská et al., 2020). In the frame of these assays, Aanhane and coworkers found that the Gal-9 isoforms – C-terminal domain, N-terminal domain, and complete galectin – have different effects on angiogenesis (Aanhane et al., 2018). Notably, tube formation and CAM showed different results in this study; while all Gal-9 isoforms stimulated the formation of tubes in the tube formation assay, they inhibited this process in the CAM assay. This indicates that *in vitro* assays may not exactly mirror the conditions of *in vivo* studies. The tube formation assays can be modified by using not only galectins but also glycan ligands for co-incubation to test their inhibitory effect on the galectin-induced tube formation.

Homotypic aggregation of cancer cells is another process in cancer metastasis that is induced by Gal-3. It leads to the accumulation of malignant cells into aggregates and, later on, to metastatic formation (Glinsky et al., 2003). The effect of glycomaterials on the Gal-3-induced cell aggregation is tested and observed microscopically in homotypic aggregation assays. Glycomaterials are first incubated with Gal-3 and then with the cells; aggregates are monitored under the microscope. LacNAc-presenting poly(amidoamine) dendrimers were able to either enhance or inhibit cancer cell aggregation, depending on the generation and, thus, the number of terminally presented LacNAc units (Ennist et al., 2018). While dendrimers of a low generation inhibited Gal-3-induced aggregation of cancer cells, dendrimers of higher generations tended to promote aggregation in the presence of Gal-3. This is probably because the larger dendrimers more efficiently crosslinked the cells by binding higher amounts of Gal-3 and built Gal-3-dendrimer aggregates while the smaller dendrimers only bound low amounts of galectin, and hence did not promote crosslinking and aggregation. This shows that the results of individual assays must always be interpreted with great care to reveal the exact processes present.

The phenomenon of Gal-3 accumulation around disrupted lysosomes is a process that can be used to study carcinogenic processes. In cancer therapy, lysosomotropic agents (*e.g.*, glycyl-L-phenylalanine 2-naphthylamide) cause disruption of lysosomes, the release of hydrolases, and eventual apoptosis of affected cancer cells. In addition to enzymes, lysosomes or vesicles also release unique glycans that are not found in the cytoplasm and, hence, can be targeted by various galectins (Aits et al., 2015). When lysosomes rupture, Gal-3 accumulates around them and can be determined by immunocytochemical staining and confocal microscopy, quantifying the accumulation of Gal-3 as "*puncta per nucleus*" (Stegmayr et al., 2019). Inhibition of Gal-3 accumulation by carbohydrate inhibitors (TDG and galactose derivatives) was found to cause a decrease in Gal-3 puncta per nucleus. In contrast, pectins and galactomannans showed no direct effect on galectin accumulation in this type of assay (Stegmayr et al., 2016).

### 3.2.3. Cardiovascular diseases and cardiac fibrosis

Galectins are closely related to the pathological processes leading to or associated with heart disease and failures, such as pulmonary hypertension and cardiac fibrosis (He et al., 2017; Suthahar et al., 2018). Exogenous and endogenous galectins might have different effects on the different kinds of cells located in the walls of blood vessels and the heart muscle. In hypertension and fibrosis, galectins act in favor of fibrosis of cardiac cells (cardiomyocytes), vessels, and organs. Cardiomyocytes are smooth-muscle cells responsible for the contraction of the heart. If they are damaged by disease or injury, cardiac fibroblasts are activated and differentiate into myofibroblasts. These cells are responsible for wound healing but also for fibrosis formation (Hall et al., 2021). Both effects, death of cardiomyocytes and differentiation of cardiac fibroblasts, are supported or even induced by galectins and result first in wound healing, and then in fibrosis. Extensive fibrosis can lead to stiffening and hypertension of organs, in this case, the heart, and can finally cause heart failure. These processes are accompanied by increased production of α-smooth muscle actin and collagen (Yu et al., 2013).

Gal-3 was reported to diminish the survival of cardiomyocytes. Overexpression of Gal-3 led to the increase in apoptosis and to the induction of cell cycle arrest as detected by cell count and flow cytometry (Li et al., 2018) (Fig. 5C). Cell cycle arrest is generally detected with propidium iodide, which interacts with DNA, visualizing the amount of DNA present in cells during flow cytometry. Depending on the cell cycle phase, the cell contains a varying amount of DNA and can be distinguished from cells with an impaired cell cycle.

In fibroblasts, Gal-3 evokes a range of effects (He et al., 2017). Exogenous and endogenous Gal-3 enhanced the proliferation of fibroblasts. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium), an MTT analog, was used (Luo et al., 2017) to test the proliferation of rat primary adventitial fibroblasts induced by recombinant Gal-3 (Fig. 5C). LacNAc was able to inhibit the effect of the galectin and reduce proliferation to the level of non-treated cells. Gal-3 also induced the increase in the amounts of α-smooth-muscle actin and collagen III, which can be detected by specific fluorescently labeled antibodies using microscopy. These phenomena can be exploited to test glycomaterials as inhibitors of galectin-induced effects on cardiac cells. Extra- and intracellular glycan inhibitors can be applied to

investigate the interaction with galectins and their effect on cardiac fibroblasts (Luo et al., 2017; Yu et al., 2013). The effect of LacNAc on Gal-3-mediated cardiac remodeling was investigated with different kinds of fibroblasts (human dermal fibroblasts and primary adventitial fibroblasts). The fibroblast phenotype was confirmed by immunohistological staining of α-smooth-muscle actin, collagen, and vimentin (the fibroblast intermediate filament) (Luo et al., 2017; Robinson-Bennett and Han, 2005). Although treatment with Gal-3 did not lead to an increase of vimentin in the fibroblasts, and hence the application of LacNAc did not inhibit this effect, this method can be used to determine the profibrotic activity of other galectins. In contrast, the expression of α-smooth muscle actin and collagen was decreased, compared to the control level, by the application of LacNAc (Luo et al., 2017). Furthermore, the same studies revealed a Gal-3-mediated increase of expression of genes involved in the production of collagen and α-smooth-muscle actin. These genes were downregulated when LacNAc was applied as an inhibitor.

Gal-3 acts as a pro-fibrotic compound and it increased the secretion of the matrix metalloproteases (MMPs) in cardiac fibroblasts (Hall et al., 2021; Martínez-Martínez et al., 2015) (Fig. 5C). MMPs are expressed in various cardiac conditions and play a role in 'pathogenic remodeling', such as tissue disintegration and remodeling (Papazafiropoulou and Tentolouris, 2009). MMPs were secreted into the medium of human cell cultures and were detected by a fluorescently labeled peptide substrate (Carmona et al., 2009). MMPs activity was measured by an increase of fluorescence in the medium.

#### 3.2.4. *Metabolic disorders*

In recent years, the issue of galectin involvement in metabolic disorders, such as diabetes and obesity, attracted interest (Pugliese et al., 2015). Gal-1 and -3 were identified as the main players (Marin-Royo et al., 2018; Mukherjee et al., 2015). Related changes in cell physiology are commonly detected by PCR (upregulation of disease-related markers) and western blot (detection of disease-related proteins in cells, on cell surfaces, and in the medium) (Fig. 5D). A high-fat diet led to the increased expression of galectins in adipose tissues of rats. When adipocytes in cell cultures were treated with TDG, accumulation of fat and expression of adipogenic markers were reduced, which correlated with the inhibition of Gal-1 by TDG (Mukherjee et al., 2015). Gal-12 initiated the differentiation of 3TL-L1 cells to adipocytes (Lo et al., 2018), as reflected on the upregulated adipogenic markers. In contrast, the deficiency or inhibition of Gal-12 improved the sensitivity to insulin, which may be applied in the therapy of diabetes. Treatment of 3TL-L1 cells with recombinant Gal-3 in *in vitro* assays led to an increased expression of inflammation markers and an enhanced production of ECM components (collagen), as evaluated by western blot of the cell extracts with specific antibodies (Martínez-Martínez et al., 2016). The effect of Gal-3 on insulin sensitivity in obesity was detected by the uptake of glucose in cells. The 3TL-L1 cells were incubated with recombinant Gal-3, insulin, and 2-deoxy-D-glucose; the uptake of the glucose derivative was determined by HPLC analysis of the metabolic successor of 2-deoxy-glucose, 2-deoxy-glucose-6-phosphate, from the cell lysate (Li et al., 2016). Furthermore, insulin signaling is inhibited by Gal-3, which was determined in the study on the downregulation of the corresponding markers on the genetic level. A Gal-3 inhibitor, a 1*H*-1,2,3-triazol-1-yl thiodigalactoside derivative (Salameh et al., 2010), was able to improve uptake of glucose and, hence, to inhibit binding of Gal-3 to insulin receptors (Li et al., 2016). All these effects can be exploited to test the efficiency of putative galectin ligands on cell physiology.

**Table 3**
Disease-related serum concentrations of biomarkers Gal-1 and Gal-3.

| Disease | Biomarker | Serum concentration [ng/mL] | Reference |
|---|---|---|---|
| Pancreatic cancer | Gal-1 | 21.6-37.3 | (Martinez-Bosch et al., 2018) |
| Lung cancer | Gal-1 | 3.1 | (Kuo et al., 2011) |
| High-grade glioma | Gal-1 | 4-18 | (Verschuere et al., 2013) |
| Heart failure | Gal-3 | 17.8-25.0 | (Primo et al., 2018) |
| Breast cancer | Gal-3 | 23.5 | |
| Gastro-intestinal cancer | Gal-3 | 20.0 | |
| Lung cancer | Gal-3 | 21.7 | (Iurisci et al., 2000) |
| Melanoma | Gal-3 | 35.1 | |
| Ovarian cancer | Gal-3 | 36.0 | |

## 4. Galectin-specific biosensors

Recent years have seen huge progress in the development of assays for the analysis of galectin-carbohydrate interactions, such as ELISA-binding assays, fluorescence-based methods, or NMR (Section 2). Here, the focus is on the examination of binding potencies and kinetic characterization of galectins to large libraries of carbohydrate ligands. In the clinical environment, the methodical application of galectin-carbohydrate binding events is receiving increasing attention. The current methods of early-stage diagnosis and monitoring of various disorders like cancer or heart failure focused mostly on Gal-1 and Gal-3 as biomarkers due to their increased expression level in human blood and sera samples from patients (Table 3) (Felker et al., 2012; Grandin et al., 2012; Hara et al., 2020; Kuo et al., 2011; Martinez-Bosch et al., 2018; Verschuere et al., 2013).

In invasive tumor diagnostics, tumor tissue biopsies are linked to molecular diagnostic methods such as PCR or immunohistochemistry (Marshall et al., 2013). In addition, the need for trained and experienced personnel, time-consuming multistep analysis, and complex sample preparation usually hinder bedside and point-of-care applications. Therefore, advanced diagnostics of biomarkers from body fluids, whole blood or serum samples require robust, selective, and sensitive biosensors (Giljohann and Mirkin, 2009; Marshall et al., 2013). Early disease diagnosis and monitoring of clinically relevant tumor biomarkers that meet hospital-specific requirements are of particular interest.

Biosensors are biotechnical tools consisting of four different modules for the quantitative determination of information about biological binding events (Fig. 6A). In this process, an analyte of interest binds to a biorecognition element that acts as a receptor for the selective binding of an analyte. The biorecognition element itself is connected to a transducing element (amperometric, electrochemical, optical). This element is capable of translating the biological binding event into a measurable signal that is directly proportional to the concentration of the analyte bound to the biosensor platform. The resulting signal is further processed by an electrical detector and gives a user-specific visualization output (Belický et al., 2016).

For the detection of lectin-glycan interactions, a wide variety of biosensors have been published (Belický et al., 2016). These biosensing tools are based on electrochemical, microcantilever, quartz crystal microbalance, and optical detection methods. They are supposed to be efficient recognition tools for a diversity of analytes such as viral/antiviral proteins, lectins, cancer-related tumor markers, or for the profiling of stem cells. An example, which can be transferred to galectins, is the development of a label-free localized surface plasmon resonance glycan biosensor revealed binding constants of the plant lectin GSII from *Griffonia simplicifolia* in nanomolar ranges to GlcNAc glycopolymer brushes in a flow-through configuration (Rosencrantz et al., 2016). Glycopolymer brushes were further modified by surface-located two-step enzymatic synthesis, affording the Galili-trisaccharide (α-D-Gal-(1→3)-β-D-Gal-(1→4)-β-D-GlcNAc-R), which enabled the binding of the *Clostridium difficile* toxin receptor-binding domain in concentrations suitable for clinical application (2.5 ng/mL). This disposable biosensor platform offers a tailor-made synthesis of glycan structures based on multiple glycosyltransferase reactions for the detection of clinical analytes of interest. In the galectin field, specific antibodies are applied as biorecognition elements in biosensors (Primo et al., 2018; Tang et al., 2018; Yola and Atar, 2020). Also, a molecularly imprinted polymer-

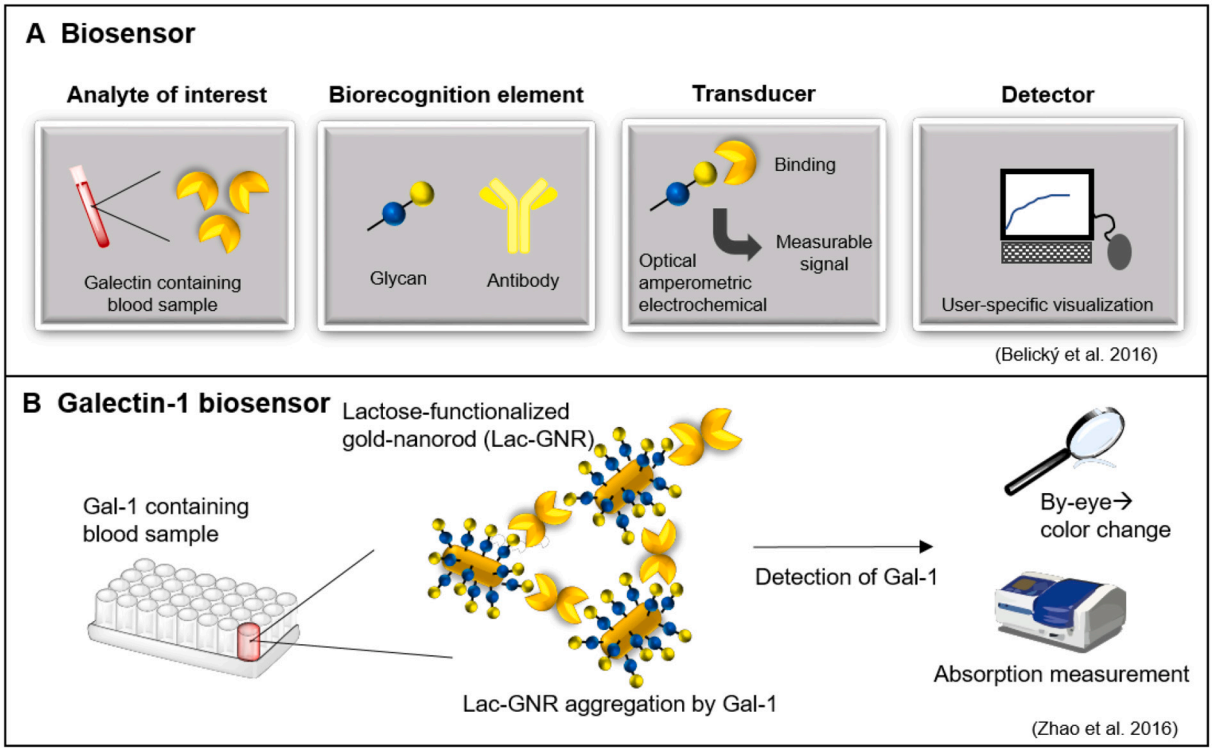


**Fig. 6.** Biosensor for galectin detection from blood samples. (A), The principle of analyzing lectin-carbohydrate interaction by a biosensor; (B), Gal-1 biosensor based on lactose-functionalized gold nanorods.

**Table 4**
Overview of galectin-specific biosensors LOD: limit of detection; the lowest concentration of galectin that can be reliably measured by the biosensor device (Armbruster and Pry, 2008).

| Reference | Analyte | Biorecognition element | Backbone | Transducer | LOD | Linear range | Response time | Serum tested |
|---|---|---|---|---|---|---|---|---|
| (Long et al., 2019) | Gal-1 | Lactose | $Cu_2O$@Au Nanocomposites | Amperometric | | 0.1 pg/mL -10 ng/mL | | no |
| (Zhao et al., 2016) | Gal-1 | Lactose | Gold nanorods | Optical | $10^{-13}$ M | | 60 min | yes |
| (Martos-Maldonado et al., 2020) | Gal-3 | Lactose-ferrocene | AuNP and PAMAM-dendrimers | Electrochemical | 160 nM | | 10 min | no |

based biosensor was developed for selective capturing Gal-3 from serum samples (Cerqueira et al., 2021).

Only a few examples of specific carbohydrate-based biosensors have been published focusing on targeting human Gal-1 and Gal-3 as cancer-related biomarkers (Table 4). The biosensors display lactose as a bio-recognition element. An interesting example of a label-free selective non-invasive screening of the tumor marker Gal-1 consisted in lactose-functionalized gold nanorods (GNRs) with unique SPR properties (Zhao et al., 2016). The lactose-gold nanorods (Lac-GNRs) were easily functionalized using sulfhydryl lactose ligands for the selective capture of Gal-1 (Fig. 6B). The resulting SPR signal relied on the aggregation of Lac-GNRs, which is induced by the binding of homodimeric Gal-1. Interestingly, the biosensor distinguished between sera of healthy and cancer patients by capturing trace amounts of Gal-1 from human serum samples of different cancer patients with a limit of detection (LOD) in low nanomolar ranges ($10^{-13}$ M). Furthermore, a simplified application method was presented using these Lac-GNRs with a microplate reader and a readout at 770 nm. The significant color differences of the serum samples of healthy and cancer patients enabled a visual detection of Gal-1, which is attractive for clinical serological cancer diagnosis. A label-free in-solution amperometric biosensor based on $Cu_2O$@Au nanocomposites was designed for the point-of-care detection of Gal-1 (Long et al., 2019). Lactose-functionalized cuprous oxide nanocrystals covered with gold nanoparticles ($Cu_2O$@Au) were produced and coated on a glass carbon electrode. The excellent signal amplification properties of this particular biosensor resulted in high sensitivity with a linear response of Gal-1 binding in the range between 0.1 pg/mL and 10 ng/mL as demonstrated by cyclic voltammetry and differential pulse voltammetry (DPV) measurements. However, it remains to be investigated whether this biosensor is selective for Gal-1 detection and covers clinical application demands, such as the compatibility with serological samples and re-usage, as well as the critical LOD. The electrochemical biosensor platform appears to be promising for the sensitive detection of cancer biomarkers due to its economic production and exceptional signal amplification features. A gold nanoparticle-based electrochemical biosensor was used for specific sensing of Gal-3 by coupling lactose-ferrocene building blocks (Fc-Lac) to disulfide-functionalized reactive groups on gold nanoparticles (AuNPs), to enable the production of a multivalent ligand-presenting biosensor platform (AuNP@Fc-Lac) (Martos-Maldonado et al., 2020). The response of Gal-3 binding was monitored by recording the signal variations in optical (UV-VIS) and DPV measurements induced by the formation of cross-linked complexes between the galectin and the lactosyl ferrocenylated-gold nanoparticles. The AuNP@Fc-Lac based biosensor displayed a high electrochemical sensing ability for the detection of Gal-3 at nanomolar concentrations (the LOD of 160 nM). However, specificity for Gal-3 over Gal-1 has yet to be demonstrated.

In summary, several promising highly sensitive biosensor devices have been published specifically for the early detection of Gal-1 and Gal-3 in various disease states. The majority of the proposed biosensors were

able to selectively bind and detect galectins from complex human serum or spiked serum samples, covering relevant serum concentrations. Glycan-based biorecognition of galectins was accomplished by multivalently presented glycan ligands such as lactose taking advantage of the cluster glycoside effect. In contrast to immunosensors, glycan-based biosensors monitor "active" galectins. This may be an advantage for serum diagnostics. However, for these glycan-based galectin biosensors, selectivity must be demonstrated by the proper choice of the glycan ligand. In immune-biosensors, galectins are detected by highly specific antibodies. The transduction of the detection signal was mainly achieved by using optical, amperometric, or electrochemical detection methods. Gold has emerged as a favorable material for the design of biosensing tools due to its outstanding optical and ligand functionalization properties. However, even though promising biosensors are available, there are still some challenges to focus on when it comes to application-based transfer from the laboratory to medical point-of-care and bedside use. The corresponding practical applicability of these biosensors could be improved by reducing the level and complexity of instrumentation for better accessibility of the measurement devices in hospitals. In addition, the development of simple handling protocols could obviate the need for specially trained personnel or costly training courses. In addition, there could be economic and environmental considerations, such as the commercialization of biosensors as disposable/ single-use or reusable devices. Considering all of the above, there are also regulatory issues in the approval of medical devices (for the country concerned), which have to be considered in the design and processing of biomedical biosensors targeting galectins.

## 5. Outstanding questions and perspectives

- Which route to clinically applicable galectin inhibitors is optimal in terms of price, speed, and reliability: direct *in vivo* assays or the gradual analytical sequence „inter-molecular interaction – cell culture assays – *in vivo* assays“?
- Are the results of a single affinity assay even relevant for the ranking or screening of compounds? Or should we always consider at least two different assays to draw trustworthy conclusions?
- What properties are vital for galectin inhibitors intended for therapeutic purposes? Is it high binding/ inhibition efficiency or their properties *in vivo*, such as bioavailability, compound metabolism, or biocompatibility?
- Is the clinical impact of rare tandem-repeat galectins at all relevant and worth the search for selective inhibitors? Or will their influence always be overshadowed by the most abundant Gal-1 and/ or Gal-3?
- What properties are required of a good ligand for diagnostic purposes, apart from high affinity?

## 6. Conclusions

Carbohydrate-based inhibitors of galectins have the significant and yet unappreciated potential for the diagnosis and therapy of a variety of diseases associated with galectin overexpression. Based on the functional mechanisms known to date, carbohydrate compounds primarily target the inhibition of extracellular galectins. Though current clinical research is mainly focused on Gal-3 and its application in anti-tumor therapy and pulmonary fibrosis (Laaf et al., 2019), basic research in this area is rapidly expanding to other galectins and disorders as evidenced by the steadily growing number of patents and solved crystal structures (https://www.rcsb.org/). Fighting the COVID-19 pandemic, Gal-3 inhibitors are discussed for the treatment of COVID-19 by reducing the release of cytokines in the inflammatory response (cytokine storm) and by blocking the cell attachment of the SARS-CoV2 virus *via* the spike protein (Caniglia et al., 2020; Sethi et al., 2020). However, despite intense interest from the scientific public and extensive research, very few compounds have reached clinical evaluation so far (http://clinicaltrials.gov/).

The route to a potential candidate worthy of reaching this final stage of evaluation is usually quite complicated. The goal of this review is to define and classify important milestones along this path, which ranges from a simple assessment of inter-molecular interaction with the target galectin and goes over the understanding of its effects at the cellular level to the results of *in vivo* assays in animal models. As well known from *in vivo* experiments, a careful selection of the appropriate model and conditions can determine the success or failure of the whole concept. The previous screening procedures summarized herein should help minimize this risk and present strong and reliable background data, on which to build. A careful pre-screening of galectin-targeting candidates alone, starting with the assessment of intermolecular interaction, may avoid *culs-de-sac* like in the case of modified citrus pectin (Fan et al., 2018a, 2018b; Stegmayr et al., 2016).

Over the past twenty years, galectin-glycan research has shifted from galectin inhibition by simple disaccharides or undefined polysaccharides to sophisticated multivalent systems and tailored glycomimetics that are the result of collaborative interdisciplinary efforts of modeling specialists, crystallographers, organic and material chemists, and molecular engineers. The development of informative and versatile methods to study galectin-carbohydrate interactions should keep pace with this process, as only a wide range of tailored methods to identify potential candidates can lead to rapid translation of galectin-targeting carbohydrate-based tools from the laboratory to the bedside.

## Author contributions

L.E., P.B., and V.K. conceived the project scope. C.D., V.H., and L.E. performed the literature search. C.D. and V.H. designed the figures. V. H., P.B., C.D., V.K., and L.E. drafted the manuscript, all authors contributed to the discussion, editing, and completion of the manuscript and all authors approved the final submitted version.

## Credit author statement

Viktoria Heine and Carina Dey contributed equally.

## Declaration of Competing Interest

The authors declare no conflicts of interest.

## Acknowledgments

V.H., P.B., and V.K were supported by the Czech Science Foundation (grant project 20-00215S) and by the Ministry of Education, Sports and Youth of the Czech Republic (mobility projects LTC20069, LTC18041, and LTC20072). L.E. and V.K. gratefully acknowledge the financial support from the German-Czech joint project by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG, project no. EL 135/12-1) and the DAAD-AVČR mobility project No. 20-04. The authors are thankful for mobility and networking support from the EU COST actions CA15135 MuTaLig, CA16225 EU-CARDIOPROTECTION, CA18132 GLYCONanoPROBES, CA18103 INNOGLY, and CA16122 BIONECA. C.D. and L.E acknowledge support by the Collaborative Research Center (CRC) grant SFB 985 project C3 from DFG (Deutsche Forschungsgemeinschaft).

## References

Aanhane, E., Schulkens, I.A., Heusschen, R., Castricum, K., Leffler, H., Griffioen, A.W., Thijssen, V.L., 2018. Different angioregulatory activity of monovalent galectin-9 isoforms. Angiogenesis 21, 545–555. https://doi.org/10.1007/s10456-018-9607-8.

Ahmed, H., AlSadek, D.M., 2015. Galectin-3 as a potential target to prevent cancer metastasis. Clin. Med. Insights Oncol. 9, 113–121. https://doi.org/10.4137/CMO.S29462.

Aits, S., Kricker, J., Liu, B., Ellegaard, A.M., Hamalisto, S., Tvingsholm, S., Corcelle-Termeau, E., Hogh, S., Farkas, T., Holm Jonassen, A., Gromova, I., Mortensen, M., Jaattela, M., 2015. Sensitive detection of lysosomal membrane permeabilization by

lysosomal galectin puncta assay. Autophagy 11, 1408–1424. https://doi.org/10.1080/15548627.2015.1063871.
Ardá, A., Jiménez-Barbero, J., 2018. The recognition of glycans by protein receptors. Insights from NMR spectroscopy. Chem. Commun. 54, 4761–4769. https://doi.org/10.1039/C8CC01444B.
Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. Clin. Biochem. Rev. 29 (Suppl. 1), S49–S52.
Atmanene, C., Ronin, C., Téletchéa, S., Gautier, F.M., Djedaïni-Pilard, F., Ciesielski, F., Vivat, V., Grandjean, C., 2017. Biophysical and structural characterization of mono/di-arylated lactosamine derivatives interaction with human galectin-3. Biochem. Biophys. Res. Commun. 489, 281–286. https://doi.org/10.1016/j.bbrc.2017.05.150.
Azevedo, A.M., Martins, V.C., Prazeres, D.M., Vojinović, V., Cabral, J.M., Fonseca, L.P., 2003. Horseradish peroxidase: a valuable tool in biotechnology. Biotechnol. Annu. Rev. 9, 199–247. https://doi.org/10.1016/s1387-2656(03)09003-3.
Balakrishnan, B., Subramanian, S., Mallia, M.B., Repaka, K., Kaur, S., Chandan, R., Bhardwaj, P., Dash, A., Banerjee, R., 2020. Multifunctional core–shell glyconanoparticles for Galectin-3-targeted, Trigger-responsive combination chemotherapy. Biomacromolecules 21, 2645–2660. https://doi.org/10.1021/acs.biomac.0c00358.
Belický, Š., Katrlík, J., Tkáč, J., 2016. Glycan and lectin biosensors. Essays Biochem. 60, 37–47. https://doi.org/10.1042/EBC20150005.
Bernhard, S.P., Goodman, C.K., Norton, E.G., Alme, D.G., Lawrence, C.M., Cloninger, M. J., 2020. Time-dependent fluorescence spectroscopy to quantify complex binding interactions. ACS Omega 5, 29017–29024. https://doi.org/10.1021/acsomega.0c03416.
Bertuzzi, S., Quintana, J.I., Ardá, A., Gimeno, A., Jiménez-Barbero, J., 2020. Targeting galectins with glycomimetics. Front. Chem. 8, 593. https://doi.org/10.3389/fchem.2020.00593.
Bertuzzi, S., Gimeno, A., Martinez-Castillo, A., Lete, M.G., Delgado, S., Airoldi, C., Tavares, M.R., Bláhová, M., Chytil, P., Křen, V., Abrescia, N.G.A., Ardá, A., Bojarová, P., Jiménez-Barbero, J., 2021. Cross-Linking Effects Dictate the Preference of Galectins to Bind LacNAc-Decorated HPMA Copolymers. Int. J. Mol. Sci. 22, 6000. https://doi.org/10.3390/ijms22116000.
Besford, Q.A., Wojnilowicz, M., Suma, T., Bertleff-Zieschang, N., Caruso, F., Cavalieri, F., 2017. Lactosylated Glycogen Nanoparticles for Targeting Prostate Cancer Cells. ACS Appl. Mater. Interfaces 9, 16869–16879. https://doi.org/10.1021/acsami.7b02676.
Biswas, S., Medina, S.H., Barchi Jr., J.J., 2015. Synthesis and cell-selective antitumor properties of amino acid conjugated tumor-associated carbohydrate antigen-coated gold nanoparticles. Carbohydr. Res. 405, 93–101. https://doi.org/10.1016/j.carres.2014.11.002.
Böcker, S., Elling, L., 2017a. Binding characteristics of galectin-3 fusion proteins. Glycobiology 27, 457–468. https://doi.org/10.1093/glycob/cwx007.
Böcker, S., Elling, L., 2017b. Biotinylated *N*-Acetyllactosamine- and *N*,*N*-Diacetyllactosamine-Based Oligosaccharides as Novel Ligands for Human Galectin-3. Bioengineering 4, 31. https://doi.org/10.3390/bioengineering4020031.
Böcker, S., Laaf, D., Elling, L., 2015. Galectin Binding to Neo-Glycoproteins: LacDiNAc Conjugated BSA as Ligand for Human Galectin-3. Biomolecules 5, 1671–1696. https://doi.org/10.3390/biom5031671.
Bohari, M.H., Yu, X., Kishor, C., Patel, B., Go, R.M., Eslampanah Seyedi, H.A., Vinik, Y., Grice, I.D., Zick, Y., Blanchard, H., 2018. Structure-Based Design of a Monosaccharide Ligand Targeting Galectin-8. ChemMedChem 13, 1664–1672. https://doi.org/10.1002/cmdc.201800224.
Bonner, W.A., Hulett, H.R., Sweet, R.G., Herzenberg, L.A., 1972. Fluorescence Activated Cell Sorting. Rev. Sci. Instrum. 43, 404–409. https://doi.org/10.1063/1.1685647.
Boza-Serrano, A., Ruiz, R., Sanchez-Varo, R., García-Revilla, J., Yang, Y., Jiménez-Ferrer, I., Paulus, A., Wennström, M., Vilalta, A., Allendorf, D., Davila, J.C., Stegmayr, J., Jimenez, S., Roca-Ceballos, M.A., Navarro-Garrido, V., Swanberg, M., Hsieh, C.L., Real, L.M., Englund, E., Linse, S., Leffler, H., Nilsson, U.J., Brown, G.C., Gutierrez, A., Vitorica, J., Venero, J.L., Deierborg, T., 2019. Galectin-3, a novel endogenous TREM2 ligand, detrimentally regulates inflammatory response in Alzheimer's disease. Acta Neuropathol. 138, 251–273. https://doi.org/10.1007/s00401-019-02013-z.
Brautigam, C.A., Zhao, H., Vargas, C., Keller, S., Schuck, P., 2016. Integration and global analysis of isothermal titration calorimetry data for studying macromolecular interactions. Nat. Protoc. 11, 882–894. https://doi.org/10.1038/nprot.2016.044.
Brinchmann, M.F., Patel, D.M., Iversen, M.H., 2018. The Role of Galectins as Modulators of Metabolism and Inflammation. Mediat. Inflamm. 2018, 9186940. https://doi.org/10.1155/2018/9186940.
Bumba, L., Laaf, D., Spiwok, V., Elling, L., Křen, V., Bojarová, P., 2018. Poly-*N*-Acetyllactosamine Neo-Glycoproteins as Nanomolar Ligands of Human Galectin-3: Binding Kinetics and Modeling. Int. J. Mol. Sci. 19, 372. https://doi.org/10.3390/ijms19020372.
Bum-Erdene, K., Leffler, H., Nilsson, U.J., Blanchard, H., 2015. Structural characterization of human galectin-4 C-terminal domain: elucidating the molecular basis for recognition of glycosphingolipids, sulfated saccharides and blood group antigens. FEBS J. 282, 3348–3367. https://doi.org/10.1111/febs.13348.
Califice, S., Castronovo, V., Van den Brûle, F., 2004. Galectin-3 and cancer. Int. J. Oncol. 25, 983–992. https://doi.org/10.3892/ijo.25.4.983.
Campanero-Rhodes, M.A., Palma, A.S., Menéndez, M., Solís, D., 2020. Microarray Strategies for Exploring Bacterial Surface Glycans and Their Interactions With Glycan-Binding Proteins. Front. Microbiol. 10 https://doi.org/10.3389/fmicb.2019.02909.
Campo, V.L., Marchiori, M.F., Rodrigues, L.C., Dias-Baruffi, M., 2016. Synthetic glycoconjugates inhibitors of tumor-related galectin-3: an update. Glycoconj. J. 33, 853–876. https://doi.org/10.1007/s10719-016-9721-z.
Caniglia, J.L., Guda, M.R., Asuthkar, S., Tsung, A.J., Velpula, K.K., 2020. A potential role for Galectin-3 inhibitors in the treatment of COVID-19. PeerJ 8, e9392. https://doi.org/10.7717/peerj.9392.
Carmona, A.K., Juliano, M.A., Juliano, L., 2009. The use of Fluorescence Resonance Energy Transfer (FRET) peptides for measurement of clinically important proteolytic enzymes. An. Acad. Bras. Cienc. 81, 381–392. https://doi.org/10.1590/s0001-37652009000300005.
Cerqueira, S.M.V., Fernandes, R., Moreira, F.T.C., Sales, M.G.F., 2021. Development of an electrochemical biosensor for Galectin-3 detection in point-of-care. Microchem. J. 164, 105992 https://doi.org/10.1016/j.microc.2021.105992.
Chan, Y.-C., Lin, H.-Y., Tu, Z., Kuo, Y.-H., Hsu, S.-T.D., Lin, C.-H., 2018. Dissecting the Structure–Activity Relationship of Galectin–Ligand Interactions. Int. J. Mol. Sci. 19, 392. https://doi.org/10.3390/ijms19020392.
Chayen, N.E., Saridakis, E., 2008. Protein crystallization: from purified protein to diffraction-quality crystal. Nat. Methods 5, 147–153. https://doi.org/10.1038/nmeth.f.203.
Concepcion, J., Witte, K., Wartchow, C., Choo, S., Yao, D., Persson, H., Wei, J., Li, P., Heidecker, B., Ma, W., Varma, R., Zhao, L.S., Perillat, D., Carricato, G., Recknor, M., Du, K., Ho, H., Ellis, T., Gamez, J., Howes, M., Phi-Wilson, J., Lockard, S., Zuk, R., Tan, H., 2009. Label-Free Detection of Biomolecular Interactions Using BioLayer Interferometry for Kinetic Characterization. Comb. Chem. High Throughput Screen. 12, 791–800. https://doi.org/10.2174/138620709789104915.
Dahlqvist, A., Zetterberg, F.R., Leffler, H., Nilsson, U.J., 2019. Aminopyrimidine–galactose hybrids are highly selective galectin-3 inhibitors. MedChemComm. 10, 913–925. https://doi.org/10.1039/C9MD00183B.
Daniel, J.M., Friess, S.D., Rajagopalan, S., Wendt, S., Zenobi, R., 2002. Quantitative determination of noncovalent binding interactions using soft ionization mass spectrometry. Int. J. Mass Spectrom. 216, 1–27. https://doi.org/10.1016/S1387-3806(02)00585-7.
De Melo, F.H.M., Butera, D., Medeiros, R.S., De, S., Andrade, L.N., Nonogaki, S., Soares, F.A., Alvarez, R.A., da Silva, A.M.M., Chammas, R., 2007. Biological Applications of a Chimeric Probe for the Assessment of Galectin-3 Ligands. J. Histochem. Cytochem. 55, 1015–1026. https://doi.org/10.1369/jhc.7A7174.2007.
Dion, J., Advedissian, T., Storozhylova, N., Dahbi, S., Lambert, A., Deshayes, F., Viguier, M., Tellier, C., Poirier, F., Téletchéa, S., Dussouy, C., Tateno, H., Hirabayashi, J., Grandjean, C., 2017. Development of a Sensitive Microarray Platform for the Ranking of Galectin Inhibitors: Identification of a Selective Galectin-3 Inhibitor. Chembiochem 18, 2428–2440. https://doi.org/10.1002/cbic.201700544.
El-Hawiet, A., Kitova, E.N., Klassen, J.S., 2012. Quantifying Carbohydrate-Protein Interactions by Electrospray Ionization Mass Spectrometry Analysis. Biochemistry 51, 4244–4253. https://doi.org/10.1021/bi300436x.
El-Hawiet, A., Chen, Y., Shams-Ud-Doha, K., Kitova, E.N., St-Pierre, Y., Klassen, J.S., 2017. High-Throughput Label- and Immobilization-Free Screening of Human Milk Oligosaccharides Against Lectins. Anal. Chem. 89, 8713–8722. https://doi.org/10.1021/acs.analchem.7b00542.
Eliaz, I., 2019. Letter to the Editor: Not all modified citrus pectins are the same: size does matter. Am. J. Physiol. Heart Circ. Physiol. 316, H1232–H1233. https://doi.org/10.1152/ajpheart.00118.2019.
Eliaz, I., Raz, A., 2019. Pleiotropic Effects of Modified Citrus Pectin. Nutrients 11, 2619. https://doi.org/10.3390/nu11112619.
Elola, M.T., Blidner, A.G., Ferragut, F., Bracalente, C., Rabinovich, Gabriel A., 2015. Assembly, organization and regulation of cell-surface receptors by lectin–glycan complexes. Biochem. J. 469, 1–16. https://doi.org/10.1042/bj20150461.
Elola, M.T., Ferragut, F., Méndez-Huergo, S.P., Croci, D.O., Bracalente, C., Rabinovich, G. A., 2018. Galectins: Multitask signaling molecules linking fibroblast, endothelial and immune cell programs in the tumor microenvironment. Cell. Immunol. 333, 34–45. https://doi.org/10.1016/j.cellimm.2018.03.008.
Ennist, J.H., Termuehlen, H.R., Bernhard, S.P., Fricke, M.S., Cloninger, M.J., 2018. Chemoenzymatic Synthesis of Galectin Binding Glycopolymers. Bioconjug. Chem. 29, 4030–4039. https://doi.org/10.1021/acs.bioconjchem.8b00599.
Fan, Y., Sun, L., Yang, S., He, C., Tai, G., Zhou, Y., 2018a. The roles and mechanisms of homogalacturonan and rhamnogalacturonan I pectins on the inhibition of cell migration. Int. J. Biol. Macromol. 106, 207–217. https://doi.org/10.1016/j.ijbiomac.2017.08.004.
Fan, Y., Sun, L., Yang, S., He, C., Tai, G., Zhou, Y., 2018b. The roles and mechanisms of homogalacturonan and rhamnogalacturonan I pectins on the inhibition of cell migration. Int. J. Biol. Macromol. 106, 207–217. https://doi.org/10.1016/j.ijbiomac.2017.08.004.
Fang, Y., 2006. Label-Free Cell-Based Assays with Optical Biosensors in Drug Discovery. Assay Drug Dev. Technol. 4, 583–595. https://doi.org/10.1089/adt.2006.4.583.
Felker, G.M., Fiuzat, M., Shaw, L.K., Clare, R., Whellan, D.J., Bettari, L., Shirolkar, S.C., Donahue, M., Kitzman, D.W., Zannad, F., Pina, I.L., O'Connor, C.M., 2012. Galectin-3 in ambulatory patients with heart failure: results from the HF-ACTION study. Circ. Heart Fail. 5, 72–78. https://doi.org/10.1161/CIRCHEARTFAILURE.111.963637.
Filipová, M., Bojarová, P., Rodrigues Tavares, M., Bumba, L., Elling, L., Chytil, P., Gunár, K., Křen, V., Etrych, T., Janoušková, O., 2020. Glycopolymers for efficient inhibition of Galectin-3: *In vitro* proof of efficacy using suppression of T lymphocyte apoptosis and tumor cell migration. Biomacromolecules 21, 3122–3133. https://doi.org/10.1021/acs.biomac.0c00515.
Freichel, T., Heine, V., Laaf, D., Mackintosh, E.E., Sarafova, S., Elling, L., Snyder, N.L., Hartmann, L., 2020. Sequence-Defined Heteromultivalent Precision Glycomacromolecules Bearing Sulfonated/Sulfated Nonglycosidic Moieties Preferentially Bind Galectin-3 and Delay Wound Healing of a Galectin-3 Positive

Tumor Cell Line in an In Vitro Wound Scratch Assay. Macromol. Biosci. 20, 2000163. https://doi.org/10.1002/mabi.202000163.
Gao, C., Hanes, M.S., Byrd-Leotis, L.A., Wei, M., Jia, N., Kardish, R.J., McKitrick, T.R., Steinhauer, D.A., Cummings, R.D., 2019. Unique Binding Specificities of Proteins toward Isomeric Asparagine-Linked Glycans. Cell Chem. Biol. 26 (535-547), e534 https://doi.org/10.1016/j.chembiol.2019.01.002.
Ghisaidoobe, A.B.T., Chung, S.J., 2014. Intrinsic Tryptophan Fluorescence in the Detection and Analysis of Proteins: A focus on Förster Resonance Energy Transfer Techniques. Int. J. Mol. Sci. 15, 22518–22538. https://doi.org/10.3390/ijms151222518.
Giljohann, D.A., Mirkin, C.A., 2009. Drivers of biodiagnostic development. Nature 462, 461–464. https://doi.org/10.1038/nature08605.
Gimeno, A., Valverde, P., Ardá, A., Jiménez-Barbero, J., 2020. Glycan structures and their interactions with proteins. A NMR view. Curr. Opin. Struct. Biol. 62, 22–30. https://doi.org/10.1016/j.sbi.2019.11.004.
Girard, A., Magnani, J.L., 2018. Clinical Trials and Applications of Galectin Antagonists. Trends Glycosci. Glycotechnol. 30 https://doi.org/10.4052/tigg.1744.1SE. SE211-SE220.
Girotti, M.R., Salatino, M., Dalotto-Moreno, T., Rabinovich, G.A., 2020. Sweetening the hallmarks of cancer: Galectins as multifunctional mediators of tumor progression. J. Exp. Med. 217 https://doi.org/10.1084/jem.20182041.
Glinsky, V.V., Glinksy, G.V., Glinskii, O.V., Huxley, V.H., Turk, J.R., Mossine, V.V., Deutscher, S.L., Pienta, K.J., Quinn, T.P., 2003. Intravascular metastatic cancer cell homotypic aggregation at the sites of primary attachment to the endothelium. Cancer Res. 63, 3805–3811. https://doi.org/10.1016/j.urolonc.2003.12.009.
Gómez-Redondo, M., Delgado, S., Núñez-Franco, R., Jiménez-Osés, G., Ardá, A., Jiménez-Barbero, J., Gimeno, A., 2021. The two domains of human galectin-8 bind sialyl- and fucose-containing oligosaccharides in an independent manner. A 3D view by using NMR. RSC Chem. Biol. 2, 932–941. https://doi.org/10.1039/D1CB00051A.
Grandin, E.W., Jarolim, P., Murphy, S.A., Ritterova, L., Cannon, C.P., Braunwald, E., Morrow, D.A., 2012. Galectin-3 and the development of heart failure after acute coronary syndrome: pilot experience from PROVE IT-TIMI 22. Clin. Chem. 58, 267–273. https://doi.org/10.1373/clinchem.2011.174359.
Guha, P., Kaptan, E., Bandyopadhyaya, G., Kaczanowska, S., Davila, E., Thompson, K., Martin, S.S., Kalvakolanu, D.V., Vasta, G.R., Ahmed, H., 2013. Cod glycopeptide with picomolar affinity to galectin-3 suppresses T-cell apoptosis and prostate cancer metastasis. Proc. Natl. Acad. Sci. U. S. A. 110, 5052–5057. https://doi.org/10.1073/pnas.1202653110.
Guo, X., 2012. Surface plasmon resonance based biosensor technique: a review. J. Biophotonics 5, 483–501. https://doi.org/10.1002/jbio.201200015.
Hall, C., Gehmlich, K., Denning, C., Pavlovic, D., 2021. Complex Relationship Between Cardiac Fibroblasts and Cardiomyocytes in Health and Disease. J. Am. Heart Assoc. 10, e019338 https://doi.org/10.1161/JAHA.120.019338.
Hara, A., Niwa, M., Noguchi, K., Kanayama, T., Niwa, A., Matsuo, M., Hatano, Y., Tomita, H., 2020. Galectin-3 as a Next-Generation Biomarker for Detecting Early Stage of Various Diseases. Biomolecules 10, 389. https://doi.org/10.3390/biom10030389.
Haselhorst, T., Lamerz, A.C., von Itzstein, M., 2009. Saturation Transfer Difference NMR Spectroscopy as a Technique to Investigate Protein-Carbohydrate Interactions in Solution. Methods Mol. Biol. 534, 375–386. https://doi.org/10.1007/978-1-59745-022-5_26.
He, J., Li, X., Luo, H., Li, T., Zhao, L., Qi, Q., Liu, Y., Yu, Z., 2017. Galectin-3 mediates the pulmonary arterial hypertension-induced right ventricular remodeling through interacting with NADPH oxidase 4. J. Am. Soc. Hypertens. 11, 275–289.e272. https://doi.org/10.1016/j.jash.2017.03.008.
Heine, V., Hovorková, M., Vlachová, M., Filipová, M., Bumba, L., Janoušková, O., Hubálek, M., Cvačka, J., Petrásková, L., Pelantová, H., Křen, V., Elling, L., Bojarová, P., 2021. Immunoprotective neo-glycoproteins: Chemoenzymatic synthesis of multivalent glycomimetics for inhibition of cancer-related galectin-3. Eur. J. Med. Chem. 220, 113500 https://doi.org/10.1016/j.ejmech.2021.113500.
Helmerhorst, E., Chandler, D.J., Nussio, M., Mamotte, C.D., 2012. Real-time and Label-free Bio-sensing of Molecular Interactions by Surface Plasmon Resonance: A Laboratory Medicine Perspective. Clin. Biochem. Rev. 33, 161–173 https://doi.org/PMID: 23267248.
Hoffmann, M., Hayes, M.R., Pietruszka, J., Elling, L., 2020. Synthesis of the Thomsen-Friedenreich-antigen (TF-antigen) and binding of Galectin-3 to TF-antigen presenting neo-glycoproteins. Glycoconj. J. 37, 457–470. https://doi.org/10.1007/s10719-020-09926-y.
Howard, M.J., 1998. Protein NMR spectroscopy. Curr. Biol. 8, R331–R333. https://doi.org/10.1016/s0960-9822(98)70214-3.
Hsieh, T.-J., Lin, H.-Y., Tu, Z., Huang, B.-S., Wu, S.-C., Lin, C.-H., 2015. Structural Basis Underlying the Binding Preference of Human Galectins-1, -3 and -7 for Galβ1-3/4GlcNAc. PLoS One 10, e0125946. https://doi.org/10.1371/journal.pone.0125946.
Hsieh, T.-J., Lin, H.-Y., Tu, Z., Lin, T.-C., Wu, S.-C., Tseng, Y.-Y., Liu, F.-T., Hsu, S.-T.D., Lin, C.-H., 2016. Dual thio-digalactoside-binding modes of human galectins as the structural basis for the design of potent and selective inhibitors. Sci. Rep. 6, 29457. https://doi.org/10.1038/srep29457.
Hunter, S.A., Cochran, J.R., 2016. Cell-Binding Assays for Determining the Affinity of Protein-Protein Interactions: Technologies and Considerations. Methods Enzymol. 580, 21–44. https://doi.org/10.1016/bs.mie.2016.05.002.
Hyun, J.Y., Pai, J., Shin, I., 2017. The Glycan Microarray Story from Construction to Applications. Acc. Chem. Res. 50, 1069–1078. https://doi.org/10.1021/acs.accounts.7b00043.
Ippel, H., Miller, M.C., Berbís, M.A., Suylen, D., André, S., Hackeng, T.M., Cañada, F.J., Weber, C., Gabius, H.J., Jiménez-Barbero, J., Mayo, K.H., 2015. (1)H, (13)C, and (15)N backbone and side-chain chemical shift assignments for the 36 proline-containing, full length 29 kDa human chimera-type galectin-3. Biomol. NMR Assign. 9, 59–63. https://doi.org/10.1007/s12104-014-9545-3.
Ippel, H., Miller, M.C., Vértesy, S., Zheng, Y., Cañada, F.J., Suylen, D., Umemoto, K., Romanò, C., Hackeng, T., Tai, G., Leffler, H., Kopitz, J., André, S., Kübler, D., Jiménez-Barbero, J., Oscarson, S., Gabius, H.-J., Mayo, K.H., 2016. Intra- and intermolecular interactions of human galectin-3: assessment by full-assignment-based NMR. Glycobiology 26, 888–903. https://doi.org/10.1093/glycob/cww021.
Iurisci, I., Tinari, N., Natoli, C., Angelucci, D., Cianchetti, E., Iacobelli, S., 2000. Concentrations of galectin-3 in the sera of normal controls and cancer patients. Clin. Cancer Res. 6, 1389–1393.
Johannes, L., Jacob, R., Leffler, H., 2018. Galectins at a glance. J. Cell Sci. 131, jcs208884. https://doi.org/10.1242/jcs.208884.
Kakehi, K., Oda, Y., Kinoshita, M., 2001. Fluorescence polarization: analysis of carbohydrate–protein interaction. Anal. Biochem. 297, 111–116. https://doi.org/10.1006/abio.2001.5309.
Kamili, N.A., Arthur, C.M., Gerner-Smidt, C., Tafesse, E., Blenda, A., Dias-Baruffi, M., Stowell, S.R., 2016. Key regulators of galectin-glycan interactions. Proteomics 16, 3111–3125. https://doi.org/10.1002/pmic.201600116.
Kitova, E.N., El-Hawiet, A., Schnier, P.D., Klassen, J.S., 2012. Reliable Determinations of Protein-Ligand Interactions by Direct ESI-MS Measurements. Are we there yet? J. Am. Soc. Mass Spectrom. 23, 431–441. https://doi.org/10.1007/s13361-011-0311-9.
Kitova, E.N., Han, L., Vinals, D.F., Kitov, P.I., Derda, R., Klassen, J.S., 2020. Influence of labeling on the glycan affinities and specificities of glycan-binding proteins. A case study involving a C-terminal fragment of human galectin-3. Glycobiology 30, 49–57. https://doi.org/10.1093/glycob/cwz076.
Kuete, V., Karaosmanoğlu, O., Sivas, H., 2017. Anticancer Activities of African Medicinal Spices and Vegetables. In: Kuete, V. (Ed.), Medicinal Spices and Vegetables from Africa. Elsevier, Oxford, pp. 271–297.
Kuo, P.L., Hung, J.Y., Huang, S.K., Chou, S.H., Cheng, D.E., Jong, Y.J., Hung, C.H., Yang, C.J., Tsai, Y.M., Hsu, Y.L., Huang, M.S., 2011. Lung cancer-derived galectin-1 mediates dendritic cell anergy through inhibitor of DNA binding 3/IL-10 signaling pathway. J. Immunol. 186, 1521–1530. https://doi.org/10.4049/jimmunol.1002940.
Kupper, C.E., Böcker, S., Liu, H., Adamzyk, C., Kamp, J.V.D., Recker, T., Lethaus, B., Jahnen-Dechent, W., Neuss, S., Müller-Newen, G., Elling, L., 2013. Fluorescent SNAP-Tag Galectin Fusion Proteins as Novel Tools in Glycobiology. Curr. Pharm. Des. 19, 5457–5467. https://doi.org/10.2174/1381612811319300017.
Laaf, D., Bojarová, P., Pelantová, H., Křen, V., Elling, L., 2017. Tailored Multivalent Neo-Glycoproteins: Synthesis, Evaluation, and Application of a Library of Galectin-3-Binding Glycan Ligands. Bioconjug. Chem. 28, 2832–2840. https://doi.org/10.1021/acs.bioconjchem.7b00520.
Laaf, D., Bojarová, P., Elling, L., Křen, V., 2019. Galectin-Carbohydrate Interactions in Biomedicine and Biotechnology. Trends Biotechnol. 37, 402–415. https://doi.org/10.1016/j.tibtech.2018.10.001.
Ladbury, J.E., 2004. Application of Isothermal Titration Calorimetry in the Biological Sciences: Things Are Heating Up! Biotechniques 37, 885–887. https://doi.org/10.2144/04376te01.
Leach, A.R., 2007. 4.05 - Ligand-Based Approaches: Core Molecular Modeling. In: Taylor, J.B., Triggle, D.J. (Eds.), Comprehensive Medicinal Chemistry II. Elsevier, Oxford, pp. 87–118.
Leavitt, S., Freire, E., 2001. Direct measurement of protein binding energetics by isothermal titration calorimetry. Curr. Opin. Struct. Biol. 11, 560–566. https://doi.org/10.1016/s0959-440x(00)00248-7.
Li, P., Liu, S., Lu, M., Bandyopadhyay, G., Oh, D., Imamura, T., Johnson, A.M.F., Sears, D., Shen, Z., Cui, B., Kong, L., Hou, S., Liang, X., Iovino, S., Watkins, S.M., Ying, W., Osborn, O., Wollam, J., Brenner, M., Olefsky, J.M., 2016. Hematopoietic-Derived Galectin-3 Causes Cellular and Systemic Insulin Resistance. Cell 167 (973-984), e912. https://doi.org/10.1016/j.cell.2016.10.025.
Li, X., Tang, X., Lu, J., Yuan, S., 2018. Therapeutic inhibition of galectin3 improves cardiomyocyte apoptosis and survival during heart failure. Mol. Med. Rep. 17, 4106–4112. https://doi.org/10.3892/mmr.2017.8323.
Liu, F.-T., Rabinovich, G.A., 2010. Galectins: regulators of acute and chronic inflammation. Ann. N. Y. Acad. Sci. 1183, 158–182. https://doi.org/10.1111/j.1749-6632.2009.05131.x.
Lo, C.W., Chen, C.S., Chen, Y.C., Hsu, Y.A., Huang, C.C., Chang, C.Y., Lin, C.J., Lin, C.W., Lin, H.J., Liu, F.T., Wan, L., 2018. Allyl Isothiocyanate Ameliorates Obesity by Inhibiting Galectin-12. Mol. Nutr. Food Res. 62, e1700616 https://doi.org/10.1002/mnfr.201700616.
Long, F., Li, W., Chen, W., Liu, D., Chen, Y., Zhou, R., Li, P., 2019. An amperometric biosensor based on $Cu_2O$@Au nanocomposites for the detection of galectin-1 via lactose–galectin interactions. Nanotechnology 30, 485706. https://doi.org/10.1088/1361-6528/ab3cde.
Lü, L., Zhang, L., Wai, M.S.M., Yew, D.T.W., Xu, J., 2012. Exocytosis of MTT formazan could exacerbate cell injury. Toxico.l. In Vitro 26, 636–644. https://doi.org/10.1016/j.tiv.2012.02.006.
Ludwig, A.-K., Michalak, M., Xiao, Q., Gilles, U., Medrano, F.J., Ma, H., FitzGerald, F.G., Hasley, W.D., Melendez-Davila, A., Liu, M., Rahimi, K., Kostina, N.Y., Rodriguez-Emmenegger, C., Möller, M., Lindner, I., Kaltner, H., Cudic, M., Reusch, D., Kopitz, J., Romero, A., Oscarson, S., Klein, M.L., Gabius, H.-J., Percec, V., 2019. Design–functionality relationships for adhesion/growth-regulatory galectins. Proc. Natl. Acad. Sci. U. S. A. 116, 2837–2842. https://doi.org/10.1073/pnas.1813515116.
Luo, H., Liu, B., Zhao, L., He, J., Li, T., Zha, L., Li, X., Qi, Q., Liu, Y., Yu, Z., 2017. Galectin-3 mediates pulmonary vascular remodeling in hypoxia-induced pulmonary

arterial hypertension. J. Am. Soc. Hypertens. 11 (673-683), e673 https://doi.org/10.1016/j.jash.2017.07.009.
Mahanti, M., Pal, K.B., Sundin, A.P., Leffler, H., Nilsson, U.J., 2020. Epimers Switch Galectin-9 Domain Selectivity: 3*N*-Aryl Galactosides Bind the C-Terminal and Gulosides Bind the N-Terminal. ACS Med. Chem. Lett. 11, 34–39. https://doi.org/10.1021/acsmedchemlett.9b00396.
Marchiori, M.F., Pires Souto, D.E., Oliveira Bortot, L., Francisco Pereira, J., Kubota, L.T., Cummings, R.D., Dias-Baruffi, M., Carvalho, I., Campo, V.L., 2015. Synthetic 1,2,3-triazole-linked glycoconjugates bind with high affinity to human galectin-3. Bioorg. Med. Chem. 23, 3414–3425. https://doi.org/10.1016/j.bmc.2015.04.044.
Marin-Royo, G., Gallardo, I., Martínez-Martínez, E., Gutiérrez, B., Jurado-López, R., López-Andrés, N., Gutiérrez-Tenorio, J., Rial, E., Bartolomé, M.A.V., Nieto, M.L., Cachofeiro, V., 2018. Inhibition of galectin-3 ameliorates the consequences of cardiac lipotoxicity in a rat model of diet-induced obesity. Dis. Model. Mech. 11 https://doi.org/10.1242/dmm.032086.
Marshall, D., LaBerge, J.M., Firetag, B., Miller, T., Kerlan, R.K., 2013. The Changing Face of Percutaneous Image-guided Biopsy: Molecular Profiling and Genomic Analysis in Current Practice. J. Vasc. Interv. Radiol. 24, 1094–1103. https://doi.org/10.1016/j.jvir.2013.04.027.
Martinez-Bosch, N., Barranco, L.E., Orozco, C.A., Moreno, M., Visa, L., Iglesias, M., Oldfield, L., Neoptolemos, J.P., Greenhalf, W., Earl, J., Carrato, A., Costello, E., Navarro, P., 2018. Increased plasma levels of galectin-1 in pancreatic cancer: potential use as biomarker. Oncotarget 9, 32984–32996. https://doi.org/10.18632/oncotarget.26034.
Martínez-Martínez, E., Calvier, L., Fernández-Celis, A., Rousseau, E., Jurado-López, R., Rossoni, L.V., Jaisser, F., Zannad, F., Rossignol, P., Cachofeiro, V., López-Andrés, N., 2015. Galectin-3 Blockade Inhibits Cardiac Inflammation and Fibrosis in Experimental Hyperaldosteronism and Hypertension. Hypertension 66, 767–775. https://doi.org/10.1161/HYPERTENSIONAHA.115.05876.
Martínez-Martínez, E., Calvier, L., Rossignol, P., Rousseau, E., Fernández-Celis, A., Jurado-López, R., Laville, M., Cachofeiro, V., López-Andrés, N., 2016. Galectin-3 inhibition prevents adipose tissue remodelling in obesity. Int. J. Obes. 40, 1034–1038. https://doi.org/10.1038/ijo.2016.19.
Martos-Maldonado, M.C., Quesada-Soriano, I., García-Fuentes, L., Vargas-Berenguel, A., 2020. Multivalent Lactose–Ferrocene Conjugates Based on Poly (Amido Amine) Dendrimers and Gold Nanoparticles as Electrochemical Probes for Sensing Galectin-3. Nanomaterials 10, 203. https://doi.org/10.3390/nano10020203.
Mende, M., Bordoni, V., Tsouka, A., Loeffler, F.F., Delbianco, M., Seeberger, P.H., 2019. Multivalent glycan arrays. Faraday Discuss. 219, 9–32. https://doi.org/10.1039/C9FD00080A.
Meng, X.Y., Zhang, H.X., Mezei, M., Cui, M., 2011. Molecular docking: a powerful approach for structure-based drug discovery. Curr. Comput. Aided Drug Des. 7, 146–157. https://doi.org/10.2174/157340911795677602.
Morris, G.M., Lim-Wilby, M., 2008. Molecular docking. Methods Mol. Biol. 443, 365–382. https://doi.org/10.1007/978-1-59745-177-2_19.
Mukherjee, R., Kim, S.W., Park, T., Choi, M.S., Yun, J.W., 2015. Targeted inhibition of galectin 1 by thiodigalactoside dramatically reduces body weight gain in diet-induced obese rats. Int. J. Obes. 39, 1349–1358. https://doi.org/10.1038/ijo.2015.74.
Nguyen, H.H., Park, J., Kang, S., Kim, M., 2015. Surface Plasmon Resonance: A Versatile Technique for Biosensor Applications. Sensors 15, 10481–10510. https://doi.org/10.3390/s150510481.
Nielsen, M.I., Stegmayr, J., Grant, O.C., Yang, Z., Nilsson, U.J., Boos, I., Carlsson, M.C., Woods, R.J., Unverzagt, C., Leffler, H., Wandall, H.H., 2018. Galectin binding to cells and glycoproteins with genetically modified glycosylation reveals galectin–glycan specificities in a natural context. J. Biol. Chem. 293, 20249–20262. https://doi.org/10.1074/jbc.RA118.004636.
Nieminen, J., Kuno, A., Hirabayashi, J., Sato, S., 2007. Visualization of Galectin-3 Oligomerization on the Surface of Neutrophils and Endothelial Cells Using Fluorescence Resonance Energy Transfer. J. Biol. Chem. 282, 1374–1383. https://doi.org/10.1074/jbc.M604506200.
Noll, A.J., Gourdine, J.-P., Yu, Y., Lasanajak, Y., Smith, D.F., Cummings, R.D., 2016. Galectins are human milk glycan receptors. Glycobiology 26, 655–669. https://doi.org/10.1093/glycob/cww002.
Noresson, A.L., Aurelius, O., Öberg, C.T., Engström, O., Sundin, A.P., Håkansson, M., Stenström, O., Akke, M., Logan, D.T., Leffler, H., Nilsson, U.J., 2018. Designing interactions by control of protein–ligand complex conformation: tuning arginine–arene interaction geometry for enhanced electrostatic protein–ligand interactions. Chem. Sci. 9, 1014–1021. https://doi.org/10.1039/C7SC04749E.
Numata, K., 2020. How to define and study structural proteins as biopolymer materials. Polym. J. 52, 1043–1056. https://doi.org/10.1038/s41428-020-0362-5.
Öberg, C.T., Leffler, H., Nilsson, U.J., 2011. Inhibition of Galectins with Small Molecules. Chimia (Aarau) 65, 18–23. https://doi.org/10.2533/chimia.2011.18.
Oinam, L., Changarathil, G., Raja, E., Ngo, Y.X., Tateno, H., Sada, A., Yanagisawa, H., 2020. Glycome profiling by lectin microarray reveals dynamic glycan alterations during epidermal stem cell aging. Aging Cell 19, e13190. https://doi.org/10.1111/acel.13190.
Oyelaran, O., Gildersleeve, J.C., 2009. Glycan arrays: recent advances and future challenges. Curr. Opin. Chem. Biol. 13, 406–413. https://doi.org/10.1016/j.cbpa.2009.06.021.
Pacholski, C., Rosencrantz, S., Rosencrantz, R.R., Balderas-Valadez, R.F., 2020. Plasmonic biosensors fabricated by galvanic displacement reactions for monitoring biomolecular interactions in real time. Anal. Bioanal. Chem. 412, 3433–3445. https://doi.org/10.1007/s00216-020-02414-0.
Papazafiropoulou, A., Tentolouris, N., 2009. Matrix metalloproteinases and cardiovascular diseases. Hippokratia 13, 76–82.
Parsonage, G., Trebilcock, E., Toscano, M.A., Bianco, G.A., Ilarregui, J.M., Buckley, C.D., Rabinovich, G.A., 2006. Roles of galectins in chronic inflammatory microenvironments. Futur. Rheumatol. 1, 441–454. https://doi.org/10.2217/17460816.1.4.441.
Patel, B., Kishor, C., Houston, T.A., Shatz-Azoulay, H., Zick, Y., Vinik, Y., Blanchard, H., 2020. Rational Design and Synthesis of Methyl-beta-D-galactomalonyl Phenyl Esters as Potent Galectin-8N Antagonists. J. Med. Chem. 63, 11573–11584. https://doi.org/10.1021/acs.jmedchem.0c00602.
Paz, H., Joo, E.J., Chou, C.H., Fei, F., Mayo, K.H., Abdel-Azim, H., Ghazarian, H., Groffen, J., Heisterkamp, N., 2018. Treatment of B-cell precursor acute lymphoblastic leukemia with the Galectin-1 inhibitor PTX008. J. Exp. Clin. Cancer Res. 37, 67. https://doi.org/10.1186/s13046-018-0721-7.
Peterson, K., Kumar, R., Stenström, O., Verma, P., Verma, P.R., Håkansson, M., Kahl-Knutsson, B., Zetterberg, F., Leffler, H., Akke, M., Logan, D.T., Nilsson, U.J., 2018. Systematic Tuning of Fluoro-galectin-3 Interactions Provides Thiodigalactoside Derivatives with Single-Digit nM Affinity and High Selectivity. J. Med. Chem. 61, 1164–1175. https://doi.org/10.1021/acs.jmedchem.7b01626.
Pietrzyk, A.J., Bujacz, A., Mak, P., Potempa, B., Niedziela, T., 2015. Structural studies of *Helix aspersa* agglutinin complexed with GalNAc: A lectin that serves as a diagnostic tool. Int. J. Biol. Macromol. 81, 1059–1068. https://doi.org/10.1016/j.ijbiomac.2015.09.044.
Pietrzyk-Brzezinska, A.J., Bujacz, A., 2020. H-type lectins – Structural characteristics and their applications in diagnostics, analytics and drug delivery. Int. J. Biol. Macromol. 152, 735–747. https://doi.org/10.1016/j.ijbiomac.2020.02.320.
Primo, E.N., Kogan, M.J., Verdejo, H.E., Bollo, S., Rubianes, M.D., Rivas, G.A., 2018. Label-Free Graphene Oxide-Based Surface Plasmon Resonance Immunosensor for the Quantification of Galectin-3, a Novel Cardiac Biomarker. ACS Appl. Mater. Interfaces 10, 23501–23508. https://doi.org/10.1021/acsami.8b03039.
Pugliese, G., Iacobini, C., Pesce, C.M., Menini, S., 2015. Galectin-3: an emerging all-out player in metabolic disorders and their complications. Glycobiology 25, 136–150. https://doi.org/10.1093/glycob/cwu111.
Qi, W., Zhang, Y., Kochovski, Z., Wang, J., Lu, Y., Chen, G., Jiang, M., 2018. Self-assembly of Human Galectin-1 via dual supramolecular interactions and its inhibition of T-cell agglutination and apoptosis. Nano Res. 11, 5566–5572. https://doi.org/10.1007/s12274-018-2169-7.
Rauthu, S.R., Shiao, T.C., André, S., Miller, M.C., Madej, É., Mayo, K.H., Gabius, H.J., Roy, R., 2015. Defining the Potential of Aglycone Modifications for Affinity/Selectivity Enhancement against Medically Relevant Lectins: Synthesis, Activity Screening, and HSQC-Based NMR Analysis. Chembiochem 16, 126–139. https://doi.org/10.1002/cbic.201402474.
Restuccia, A., Tian, Y.F., Collier, J.H., Hudalla, G.A., 2015. Self-Assembled Glycopeptide Nanofibers as Modulators of Galectin-1 Bioactivity. Cell. Mol. Bioeng. 8, 471–487. https://doi.org/10.1007/s12195-015-0399-2.
Restuccia, A., Fettis, M.M., Hudalla, G.A., 2016. Glycomaterials for immunomodulation, immunotherapy, and infection prophylaxis. J. Mater. Chem. B 4, 1569–1585. https://doi.org/10.1039/C5TB01780G.
Restuccia, A., Fettis, M.M., Farhadi, S.A., Molinaro, M.D., Kane, B., Hudalla, G.A., 2018. Evaluation of self-assembled glycopeptide nanofibers modified with *N,N′*-diacetyllactosamine for selective galectin-3 recognition and inhibition. ACS Biomater. Sci. Eng. 4, 3451–3459. https://doi.org/10.1021/acsbiomaterials.8b00611.
Rich, R.L., Myszka, D.G., 2007. Higher-throughput, label-free, real-time molecular interaction analysis. Anal. Biochem. 361, 1–6. https://doi.org/10.1016/j.ab.2006.10.040.
Richards, S.-J., Gibson, M.I., 2021. Toward glycomaterials with selectivity as well as affinity. JACS Au 1, 2089–2099. https://doi.org/10.1021/jacsau.1c00352.
Rillahan, C.D., Paulson, J.C., 2011. Glycan microarrays for decoding the glycome. Annu. Rev. Biochem. 80, 797–823. https://doi.org/10.1146/annurev-biochem-061809-152236.
Robinson, B.S., Arthur, C.M., Evavold, B., Roback, E., Kamili, N.A., Stowell, C.S., Vallecillo-Zúniga, M.L., Van Ry, P.M., Dias-Baruffi, M., Cummings, R.D., Stowell, S.R., 2019. The Sweet-Side of Leukocytes: Galectins as Master Regulators of Neutrophil Function. Front. Immunol. 10, 1–17. https://doi.org/10.3389/fimmu.2019.01762.
Robinson-Bennett, B., Han, A., 2005. 30 Role of Immunohistochemistry in Elucidating Lung Cancer Metastatic to the Ovary from Primary Ovarian Carcinoma. In: Hayat, M. A. (Ed.), Molecular Genetics, Gastrointestinal Carcinoma, and Ovarian Carcinoma. Elsevier, Oxford, pp. 537–545.
Rodrigues, J.G., Balmaña, M., Macedo, J.A., Poças, J., Fernandes, Â., de Freitas-Junior, J. C.M., Pinho, S.S., Gomes, J., Magalhães, A., Gomes, C., Mereiter, S., Reis, C.A., 2018. Glycosylation in cancer: Selected roles in tumour progression, immune modulation and metastasis. Cell. Immunol. 333, 46–57. https://doi.org/10.1016/j.cellimm.2018.03.007.
Rosencrantz, R.R., Nguyen, V.H., Park, H., Schulte, C., Böker, A., Schnakenberg, U., Elling, L., 2016. Lectin binding studies on a glycopolymer brush flow-through biosensor by localized surface plasmon resonance. Anal. Bioanal. Chem. 408, 5633–5640. https://doi.org/10.1007/s00216-016-9667-9.
Rosencrantz, S., Tang, J.S.J., Schulte-Osseili, C., Böker, A., Rosencrantz, R.R., 2019. Glycopolymers by RAFT Polymerization as Functional Surfaces for Galectin-3. Macromol. Biosci. 220, 1900293. https://doi.org/10.1002/macp.201900293.
Roy, R., Cao, Y., Kaltner, H., Kottari, N., Shiao, T.C., Belkhadem, K., André, S., Manning, J.C., Murphy, P.V., Gabius, H.-J., 2017. Teaming up synthetic chemistry and histochemistry for activity screening in galectin-directed inhibitor design. Histochem. Cell Biol. 147, 285–301. https://doi.org/10.1007/s00418-016-1525-5.
Rupp, B., 2009. Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology. Garland Science, Taylor and Francis Group, New York.

Rustiguel, J.K., Kumagai, P.S., Dias-Baruffi, M., Costa-Filho, A.J., Nonato, M.C., 2016a. Recombinant expression, purification and preliminary biophysical and structural studies of C-terminal carbohydrate recognition domain from human galectin-4. Protein Expr. Purif. 118, 39–48. https://doi.org/10.1016/j.pep.2015.09.026.

Rustiguel, J.K., Soares, R.O.S., Meisburger, S.P., Davis, K.M., Malzbender, K.L., Ando, N., Dias-Baruffi, M., Nonato, M.C., 2016b. Full-length model of the human galectin-4 and insights into dynamics of inter-domain communication. Sci. Rep. 6, 33633. https://doi.org/10.1038/srep33633.

Ruvolo, P.P., 2016. Galectin 3 as a guardian of the tumor microenvironment. Biochim. Biophys. Acta, Mol. Cell Res. 1863, 427–437. https://doi.org/10.1016/j.bbamcr.2015.08.008.

Ryu, W.-S., 2017. Virus Structure, Molecular Virology of Human Pathogenic Viruses. Elsevier, Oxford, pp. 21–29.

Salameh, B.A., Cumpstey, I., Sundin, A., Leffler, H., Nilsson, U.J., 2010. 1H-1,2,3-triazol-1-yl thiodigalactoside derivatives as high affinity galectin-3 inhibitors. Bioorg. Med. Chem. 18, 5367–5378. https://doi.org/10.1016/j.bmc.2010.05.040.

Salmaso, V., Moro, S., 2018. Bridging Molecular Docking to Molecular Dynamics in Exploring Ligand-Protein Recognition Process: An Overview. Front. Pharmacol. 9, 923. https://doi.org/10.3389/fphar.2018.00923.

Sciacchitano, S., Lavra, L., Morgante, A., Ulivieri, A., Magi, F., De Francesco, G.P., Bellotti, C., Salehi, L.B., Ricci, A., 2018. Galectin-3: One Molecule for an Alphabet of Diseases, from A to Z. Int. J. Mol. Sci. 19, 379. https://doi.org/10.3390/ijms19020379.

Sethi, A., Sanam, S., Munagalasetty, S., Jayanthi, S., Alvala, M., 2020. Understanding the role of galectin inhibitors as potential candidates for SARS-CoV-2 spike protein: *in silico* studies. RSC Adv. 10, 29873–29884. https://doi.org/10.1039/D0RA04795C.

Shams-Ud-Doha, K., Kitova, E.N., Kitov, P.I., St-Pierre, Y., Klassen, J.S., 2017. Human Milk Oligosaccharide Specificities of Human Galectins. Comparison of Electrospray Ionization Mass Spectrometry and Glycan Microarray Screening Results. Anal. Chem. 89, 4914–4921. https://doi.org/10.1021/acs.analchem.6b05169.

Sindrewicz, P., Li, X., Yates, E.A., Turnbull, J.E., Lian, L.-Y., Yu, L.-G., 2019. Intrinsic tryptophan fluorescence spectroscopy reliably determines galectin-ligand interactions. Sci. Rep. 9, 11851. https://doi.org/10.1038/s41598-019-47658-8.

Sindrewicz, P., Yates, E.A., Turnbull, J.E., Lian, L.-Y., Yu, L.-G., 2020. Interaction with the heparin-derived binding inhibitors destabilizes galectin-3 protein structure. Biochem. Biophys. Res. Commun. 523, 336–341. https://doi.org/10.1016/j.bbrc.2019.12.054.

Song, X., Xia, B., Stowell, S.R., Lasanajak, Y., Smith, D.F., Cummings, R.D., 2009. Novel Fluorescent Glycan Microarray Strategy Reveals Ligands for Galectins. Chem. Biol. 16, 36–47. https://doi.org/10.1016/j.chembiol.2008.11.004.

Sörme, P., Kahl-Knutsson, B., Huflejt, M., Nilsson, U.J., Leffler, H., 2004. Fluorescence polarization as an analytical tool to evaluate galectin–ligand interactions. Anal. Biochem. 334, 36–47. https://doi.org/10.1016/j.ab.2004.06.042.

Stegmayr, J., Lepur, A., Kahl-Knutson, B., Aguilar-Moncayo, M., Klyosov, A.A., Field, R. A., Oredsson, S., Nilsson, U.J., Leffler, H., 2016. Low or No Inhibitory Potency of the Canonical Galectin Carbohydrate-binding Site by Pectins and Galactomannans. J. Biol. Chem. 291, 13318–13334. https://doi.org/10.1074/jbc.M116.721464.

Stegmayr, J., Zetterberg, F., Carlsson, M.C., Huang, X., Sharma, G., Kahl-Knutson, B., Schambye, H., Nilsson, U.J., Oredsson, S., Leffler, H., 2019. Extracellular and intracellular small-molecule galectin-3 inhibitors. Sci. Rep. 9, 2186. https://doi.org/10.1038/s41598-019-38497-8.

Stowell, S.R., Arthur, C.M., McBride, R., Berger, O., Razi, N., Heimburg-Molinaro, J., Rodrigues, L.C., Gourdine, J.-P., Noll, A.J., von Gunten, S., Smith, D.F., Knirel, Y.A., Paulson, J.C., Cummings, R.D., 2014. Microbial glycan microarrays define key features of host-microbial interactions. Nat. Chem. Biol. 10, 470–476. https://doi.org/10.1038/nchembio.1525.

Stryker, Z.I., Rajabi, M., Davis, P.J., Mousa, S.A., 2019. Evaluation of Angiogenesis Assays. Biomedicines 7, 37. https://doi.org/10.3390/biomedicines7020037.

Sun, W., Li, L., Li, L.J., Yang, Q.Q., Zhang, Z.R., Huang, Y., 2017. Two birds, one stone: dual targeting of the cancer cell surface and subcellular mitochondria by the galectin-3-binding peptide G3-C12. Acta Pharmacol. Sin. 38, 806–822. https://doi.org/10.1038/aps.2016.137.

Sundblad, V., Morosi, L.G., Geffner, J.R., Rabinovich, G.A., 2017. Galectin-1: A Jack-of-All-Trades in the Resolution of Acute and Chronic Inflammation. J. Immunol. 199, 3721–3730. https://doi.org/10.4049/jimmunol.1701172.

Suthahar, N., Meijers, W.C., Silljé, H.H.W., Ho, J.E., Liu, F.-T., de Boer, R.A., 2018. Galectin-3 Activation and Inhibition in Heart Failure and Cardiovascular Disease: An Update. Theranostics 8, 593–609. https://doi.org/10.7150/thno.22196.

Talaga, M.L., Fan, N., Fueri, A.L., Brown, R.K., Bandyopadhyay, P., Dam, T.K., 2016. Multitasking Human Lectin Galectin-3 Interacts with Sulfated Glycosaminoglycans and Chondroitin Sulfate Proteoglycans. Biochemistry 55, 4541–4551. https://doi.org/10.1021/acs.biochem.6b00504.

Tang, Z., He, J., Chen, J., Niu, Y., Zhao, Y., Zhang, Y., Yu, C., 2018. A sensitive sandwich-type immunosensor for the detection of galectin-3 based on N-GNRs-Fe-MOFs@AuNPs nanocomposites and a novel AuPt-methylene blue nanorod. Biosens. Bioelectron. 101, 253–259. https://doi.org/10.1016/j.bios.2017.10.026.

Tavares, M.R., Bláhová, M., Sedláková, L., Elling, L., Pelantová, H., Konefal, R., Etrych, T., Křen, V., Bojarová, P., Chytil, P., 2020. High-Affinity *N*-(2-Hydroxypropyl)methacrylamide Copolymers with Tailored *N*-Acetyllactosamine Presentation Discriminate between Galectins. Biomacromolecules 21, 641–652. https://doi.org/10.1021/acs.biomac.9b01370.

Than, N.G., Romero, R., Goodman, M., Weckle, A., Xing, J., Dong, Z., Xu, Y., Tarquini, F., Szilagyi, A., Gal, P., Hou, Z., Tarca, A.L., Kim, C.J., Kim, J.-S., Haidarian, S., Uddin, M., Bohn, H., Benirschke, K., Santolaya-Forgas, J., Grossman, L.I., Erez, O., Hassan, S.S., Zavodszky, P., Papp, Z., Wildman, D.E., 2009. A primate subfamily of galectins expressed at the maternal–fetal interface that promote immune cell death. Proc. Natl. Acad. Sci. 106, 9731–9736. https://doi.org/10.1073/pnas.0903568106.

Thaysen-Andersen, M., Mysling, S., Højrup, P., 2009. Site-Specific Glycoprofiling of N-Linked Glycopeptides Using MALDI-TOF MS: Strong Correlation between Signal Strength and Glycoform Quantities. Anal. Chem. 81, 3933–3943. https://doi.org/10.1021/ac900231w.

Thijssen, V.L., Heusschen, R., Caers, J., Griffioen, A.W., 2015. Galectin expression in cancer diagnosis and prognosis: A systematic review. Biochim. Biophys. Acta 1855, 235–247. https://doi.org/10.1016/j.bbcan.2015.03.003.

Varinská, L., Fáber, L., Petrovová, E., Balážová, L., Ivančová, E., Kolář, M., Gál, P., 2020. Galectin-8 favors VEGF-induced angiogenesis: *In vitro* study in human umbilical vein endothelial Cells and *In vivo* study in chick chorioallantoic membrane. Anticancer Res. 40, 3191–3201. https://doi.org/10.21873/anticanres.14300.

Vašíček, T., Spiwok, V., Červený, J., Petrásková, L., Bumba, L., Vrbata, D., Pelantová, H., Křen, V., Bojarová, P., 2020. Regioselective 3-O-Substitution of Unprotected Thiodigalactosides: Direct Route to Galectin Inhibitors. Chem. Eur. J. 26, 9620–9631. https://doi.org/10.1002/chem.202002084.

Verschuere, T., Van Woensel, M., Fieuws, S., Lefranc, F., Mathieu, V., Kiss, R., Van Gool, S.W., De Vleeschouwer, S., 2013. Altered galectin-1 serum levels in patients diagnosed with high-grade glioma. J. Neuro-Oncol. 115, 9–17. https://doi.org/10.1007/s11060-013-1201-8.

Vivian, J.T., Callis, P.R., 2001. Mechanisms of Tryptophan Fluorescence Shifts in Proteins. Biophys. J. 80, 2093–2109. https://doi.org/10.1016/S0006-3495(01)76183-8.

Vrbata, D., Filipová, M., Tavares, M.R., Červený, J., Vlachová, M., Šírová, M., Pelantová, H., Petrásková, L., Bumba, L., Konefał, R., Etrych, T., Křen, V., Chytil, P., Bojarová, P., 2022. Glycopolymers Decorated with 3-O-Substituted Thiodigalactosides as Potent Multivalent Inhibitors of Galectin-3. J. Med. Chem. in press. https://doi.org/10.1021/acs.jmedchem.1c01625.

Wang, W.-H., Lin, C.-Y., Chang, M.R., Urbina, A.N., Assavalapsakul, W., Thitithanyanont, A., Chen, Y.-H., Liu, F.-T., Wang, S.-F., 2020. The role of galectins in virus infection - A systemic literature review. J. Microbiol. Immunol. Infect. 53, 925–935. https://doi.org/10.1016/j.jmii.2019.09.005.

Wartchow, C.A., Podlaski, F., Li, S., Rowan, K., Zhang, X., Mark, D., Huang, K.-S., 2011. Biosensor-based small molecule fragment screening with biolayer interferometry. J. Comput. Aided Mol. Des. 25, 669. https://doi.org/10.1007/s10822-011-9439-8.

Wöhrle, J., Krämer, S.D., Meyer, P.A., Rath, C., Hügle, M., Urban, G.A., Roth, G., 2020. Digital DNA microarray generation on glass substrates. Sci. Rep. 10, 5770. https://doi.org/10.1038/s41598-020-62404-1.

Wolfenden, M., Cousin, J., Nangia-Makker, P., Raz, A., Cloninger, M., 2015. Glycodendrimers and Modified ELISAs: Tools to Elucidate Multivalent Interactions of Galectins 1 and 3. Molecules 20, 7059–7096. https://doi.org/10.3390/molecules20047059.

Xue, H., Liu, L., Zaho, Z., Zhang, Z., Guan, Y., Cheng, H., Zhou, Y., Tai, G., 2016. The N-terminal tail coordinates with carbohydrate recognition domain to mediate galectin-3 induced apoptosis in T cells. Oncotarget 8, 49824–49838. https://doi.org/10.18632/oncotarget.17760.

Yip, K.M., Fischer, N., Paknia, E., Chari, A., Stark, H., 2020. Atomic-resolution protein structure determination by cryo-EM. Nature 587, 157–161. https://doi.org/10.1038/s41586-020-2833-4.

Yola, M.L., Atar, N., 2020. Amperometric galectin-3 immunosensor-based gold nanoparticle-functionalized graphitic carbon nitride nanosheets and core–shell Ti-MOF@COFs composites. Nanoscale 12, 19824–19832. https://doi.org/10.1039/D0NR05614F.

Yu, L., Ruifrok, W.P.T., Meissner, M., Bos, E.M., van Goor, H., Sanjabi, B., van der Harst, P., Pitt, B., Goldstein, I.J., Koerts, J.A., van Veldhuisen, D.J., Bank, R.A., van Gilst, W.H., Silljé, H.H.W., de Boer, R.A., 2013. Genetic and Pharmacological Inhibition of Galectin-3 Prevents Cardiac Remodeling by Interfering with Myocardial Fibrogenesis. Circ. Heart Fail. 6, 107–117. https://doi.org/10.1161/CIRCHEARTFAILURE.112.971168.

Zetterberg, F.R., Peterson, K., Johnsson, R.E., Brimert, T., Håkansson, M., Logan, D.T., Leffler, H., Nilsson, U.J., 2018. Monosaccharide derivatives with low-nanomolar lectin affinity and high selectivity based on combined fluorine–amide, phenyl–arginine, sulfur–π, and halogen bond interactions. ChemMedChem 13, 133–137. https://doi.org/10.1002/cmdc.201700744.

Zhang, S., Moussodia, R.-O., Murzeau, C., Sun, H.-J., Klein, M.L., Vértesy, S., André, S., Roy, R., Gabius, H.-J., Percec, V., 2015. Dissecting molecular aspects of cell interactions using glycodendrimersomes with programmable glycan presentation and engineered human lectins. Angew. Chem. Int. Ed. 54, 4036–4040. https://doi.org/10.1002/anie.201410882.

Zhang, T., Zheng, Y., Zhao, D., Yan, J., Sun, C., Zhou, Y., Tai, G., 2016. Multiple approaches to assess pectin binding to galectin-3. Int. J. Biol. Macromol. 91, 994–1001. https://doi.org/10.1016/j.ijbiomac.2016.06.058.

Zhang, H., Laaf, D., Elling, L., Pieters, R.J., 2018. Thiodigalactoside-BSA-Conjugates as High-Potency Inhibitors of Galectin-3: An Outstanding Example of Multivalent Presentation of Small Molecule Inhibitors. Bioconjug. Chem. 29, 1266–1275. https://doi.org/10.1021/acs.bioconjchem.8b00047.

Zhang, H., Ippel, H., Miller, M.C., Wong, T.J., Griffioen, A.W., Mayo, K.H., Pieters, R.J., 2019. Hybrid ligands with calixarene and thiodigalactoside groups: galectin binding and cytotoxicity. Org. Chem. Front. 6, 2981–2990. https://doi.org/10.1039/C9QO00810A.

Zhang, K., Pintilie, G.D., Li, S., Schmid, M.F., Chiu, W., 2020. Resolving Individual-Atom of Protein Complex using Commonly Available 300-kV Cryo-electron Microscopes. bioRxiv. https://doi.org/10.1101/2020.08.19.256909, 2008.2019.256909.

Zhao, Y., Tong, L., Li, Y., Pan, H., Zhang, W., Guan, M., Li, W., Chen, Y., Li, Q., Li, Z., Wang, H., Yu, X.-F., Chu, P.K., 2016. Lactose-Functionalized Gold Nanorods for

Sensitive and Rapid Serological Diagnosis of Cancer. ACS Appl. Mater. Interfaces 8, 5813–5820. https://doi.org/10.1021/acsami.5b11192.

Zheng, H., Handing, K.B., Zimmerman, M.D., Shabalin, I.G., Almo, S.C., Minor, W., 2015. X-ray crystallography over the past decade for novel drug discovery – where are we heading next? Expert Opin. Drug Discovery 10, 975–989. https://doi.org/10.1517/17460441.2015.1061991.

Zhou, C., Reesink, H.L., Putnam, D.A., 2019. Selective and Tunable Galectin Binding of Glycopolymers Synthesized by a Generalizable Conjugation Method. Biomacromolecules 20, 3704–3712. https://doi.org/10.1021/acs.biomac.9b00759.