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Synthetic Inhibitors of Galectin-1 and -3 Selectively Modulate Homotypic Cell Aggregation and TUMOR Cell Apoptosis

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Abstract. *Background: Galectins have emerged as critical regulators of tumor progression and metastasis, by modulating different biological events including homotypic cell aggregation, apoptosis, migration, angiogenesis and immune escape. Therefore, galectin inhibitors might represent novel therapeutic agents for cancer. Materials and Methods: A series of structural analogs of the disaccharide methyl β-lactosaminide were screened as potential galectin inhibitors by examining their capability to block binding of galectin-1 and/or galectin-3 to LGalS3BP in solid-phase assays. To demonstrate any functional role in vitro, oligosaccharides were characterized by their ability to regulate tumor cell apoptosis and LGalS3BP-induced homotypic cell aggregation. Results: Oligosaccharides differentially inhibited binding of each galectin to LGalS3BP. Compounds containing longer oligosaccharide chains were found to be potent inhibitors of both galectins under static conditions. Strikingly, the most active compound in inhibiting homotypic cell aggregation and tumor cell apoptosis was found to be allyl lactoside, which paradoxically exhibited a modest inhibitory capacity for blocking galectin-1 and -3 binding to LGalS3BP. Conclusion: Allyl lactoside represents a novel powerful inhibitor of tumor-associated homotypic cell aggregation and apoptosis. Further investigations are required to remodel selective and potent inhibitors capable of specifically modulating the activity of different members of the galectin family.*

Metastasis is the main cause of death in patients affected by malignant neoplasia (1). Epidemiological studies have indicated that around 60% of these patients are susceptible to metastasis. Hence, identification of successful treatments capable of blocking the metastatic process represents a major goal in basic and clinical oncology.

Accumulating studies indicate that tumor metastasis is a multifactor process initially determined by changes in homotypic and heterotypic cell adhesion, apoptosis, evasion of immune responses, angiogenesis, migration and invasiveness (1). In this regard, several adhesion molecules, such as integrins, cadherins, selectins and proteins belonging to the immunoglobulin superfamily, have been reported to be engaged in this process. In addition, other molecules such as galectins and their glycoconjugate ligands have been described as molecules capable of influencing metastasis (2-4). Galectins, an evolutionarily conserved family of animal lectins, share a consensus amino acid sequence and a carbohydrate recognition domain (CRD) that is responsible for the β-galactoside-binding specificity (2). A typical CRD recognizes glycoconjugates that contain the basic disaccharide *N*-acetyllactosamine: Galβ1,4GlcNAc (LacNAc) (2).

Abbreviations: CRD, carbohydrate-recognition domain; SCLC, small cell lung cancer, OSD, oligosaccharide derivatives; MMT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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Key Words: Galectins, oligosaccharide ligand, apoptosis, cell aggregation.

To date, fifteen galectins have been isolated and sequenced in a wide variety of tissues from different species (2). Galectin-1 is a homodimer composed of subunits of 14.5 kDa containing identical carbohydrate-recognition domains (CRD) (2). On the other hand, galectin-3 is composed of one CRD, localized in the carboxy-terminal group, and an amino-terminal domain that is responsible for the oligomerization of this protein (5).

Galectins are involved in a wide variety of biological processes influencing different steps of tumor progression and metastasis (2, 5-7). Galectin-1 and -3 have been proposed to play a role in apoptosis and cell growth regulation (5, 8, 15). In addition, both galectin-1 and -3 were recently postulated to participate in cell migration and angiogenesis (16-19), and galectin-1 has been shown to contribute to tumor cell evasion from immune responses (3, 4). However, the most important feature of these endogenous lectins is their capacity to promote cell - cell and cell - matrix interactions and influence tumor cell extravasation and metastasis (2, 15). Previous studies have documented the role of galectin-1 and -3 as critical mediators of homotypic adhesion of tumor cells and heterotypic adhesion between cancer cells and leukocytes (15, 20, 21). In addition, clinical studies indicate that high serum levels of galectin-3 in cancer patients directly correlate with a higher incidence of distant metastasis (22). Moreover, Al-Mehdi and colleagues proposed a new model of hematogenous metastasis (23) in which protein-glycan interactions represent a critical reversible event in tumor dissemination (23). Finally, galectin-1 and -3 was shown to mediate tumor cell docking to endothelium in different experimental models (24-26).

In addition to the role of galectins in tumor progression, accumulating evidence indicates the essential contribution of galectin-binding glycoproteins (mainly LGalS3BP) in different events associated with tumor growth and metastasis, mainly homotypic cell aggregation (2, 20, 27). LGalS3BP is a protein initially found in sera of breast cancer patients which has been shown to interact specifically with galectin-1, -3 and -7 (2, 20, 27). Due to particular structural characteristics, this protein can bridge galectins exposed on the surface of tumor cells, thus favoring the formation of homotypic cell aggregates (20, 27). The role of LGalS3BP in neoplastic progression is strongly supported by numerous studies, as the expression levels of this protein in sera and neoplastic tissue from cancer patients tightly correlate with poor prognosis and the occurrence of metastasis (22, 28).

Due to the essential and multifunctional role of galectins and/or their binding glycoconjugates (glycoproteins or glycolipids) in the metastatic process, different candidate inhibitors that could block the interaction between these molecules have been proposed as potential anticancer drugs (5, 29-30). In this regard, compounds named glycoamines were initially isolated from human and mouse serum and then structurally characterized. They consist of penta- or hexasaccharides generally bound to primary or secondary amine group by covalent binding. Some of these compounds were tested for their capacity to inhibit adhesion and aggregation either *in vitro* or *in vivo* (31). Subsequently, Pienta and colleagues have demonstrated that some naturally occurring galectin-binding compounds, such as pectins isolated from citrus, were also effective in reducing the occurrence of metastasis (32).

Given the fact that endogenous sequences similar to oligosaccharide inhibitors are present in different glycoproteins which are involved in different physiological and pathological processes (30), we hypothesized that the introduction of exogenous oligosaccharides may mimic the endogenous ligands for different lectins, thus affecting survival, adhesion and migration of normal or neoplastic cells. Here we report the study of galectin-1 and -3 inhibitory potential of a series of oligosaccharide derivatives (OSDs) **1-11** (Figure 1) containing oligosaccharide fragments related to natural carbohydrate chains of galectin ligands. Compounds **2-11** represent a set of derivatives and structural analogs of disaccharide methyl β-lactosaminide **1** which is used in many investigations as a reference galectin ligand. Compounds **2-11** differ from the lactosaminide **1** due to variation of the structure of aglycon, the substituent at C-2, the sequence of monosaccharide units and the direction of glycoside linkages between them, as well as by the presence of carbohydrate substituents which are typical for natural glycolipid and glycoprotein chains. These synthetic compounds were evaluated for their ability to inhibit binding of galectin-1 and -3 to LGalS3BP and their capacity to modulate homotypic cell aggregation and tumor cell apoptosis.

Materials and Methods

Model oligosaccharides. Oligosaccharide derivatives **1** (33), **2** (34), **3** (35), **4** (36), **5** (37), **6** (38), **7** (39), **8** (40), **9** (37), **10** (34), and **11** (34) were synthesized and purified as described.

Cell cultures. A375 human melanoma cells were cultured as described elsewhere (15). H69 small cell lung carcinoma cells (H69-SCLC, ATCC, Rockville, MD) were grown in RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum and 25 mM HEPES. Previous to all the experiments, SCLC-H69 cells were grown in SITA medium for 24 h (RPMI-1640 supplemented with 30 nM selenium, 5 μg/ml insulin, 10 μg/ml transferrin and 0.25% (w/v) bovine serum albumin). Cell viability was routinely greater than 90%, as judged by trypan blue exclusion.

Recombinant proteins. The plasmid pKK-233-2 with cDNA for human galectin-3 was a gift from Dr. Fu-Tong Liu (University of California, Davis). The construct encoding for galectin-1 was obtained as follows: the cDNA encoding full length human galectin-1 was amplified by PCR from IMAGE clone 4722280 using primers 5'-GCCAG CCATGGCTTGTGGTC-3' and 5'-GGCAAGCTTTCAGTCAAAG GC-3'. The purified product was inserted into NcoI/HindIII digested vector pKK-233-2. These plasmids were used to transform *Escherichia*

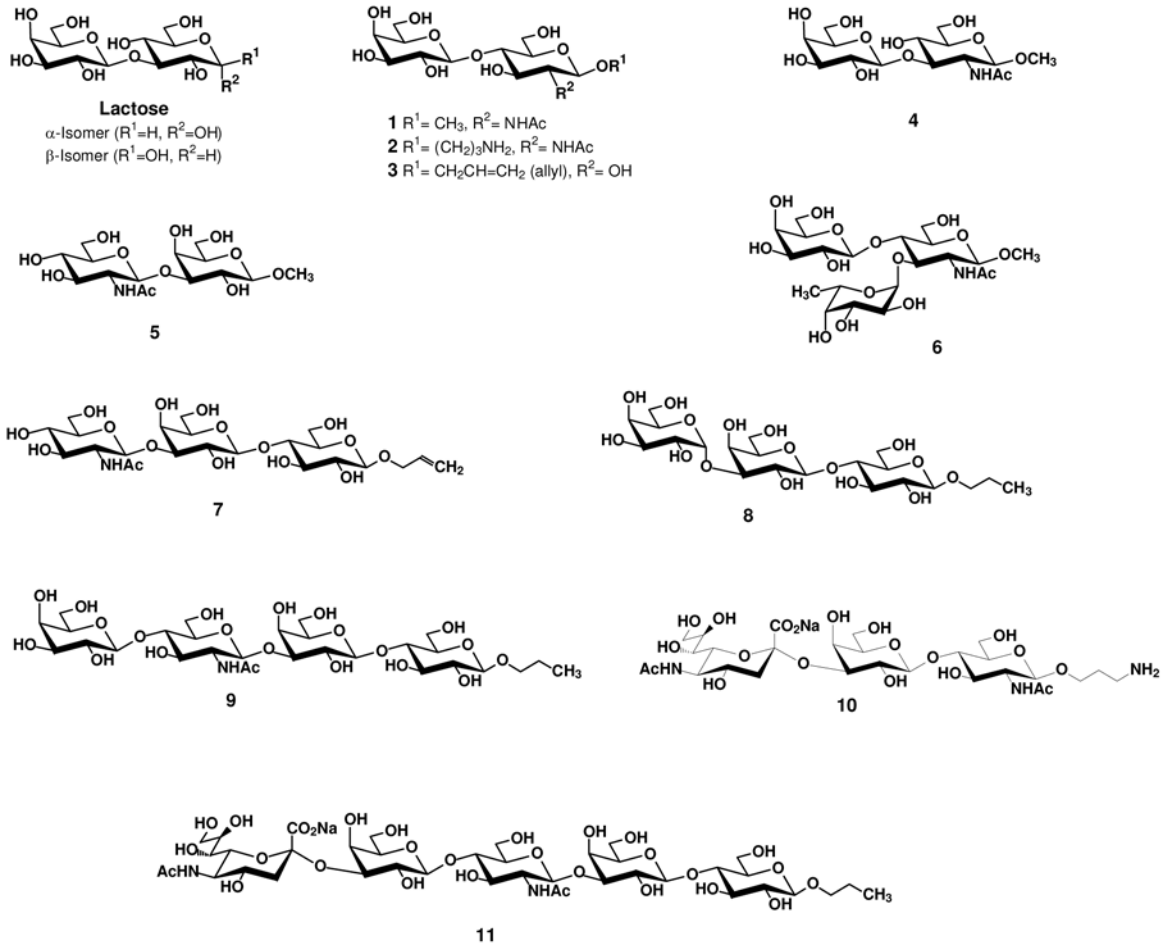

Figure 1. *Chemical structures of the oligosaccharide galectin inhibitors studied.*

coli (BL21) and the recombinant proteins were purified (15). Recombinant LGalS-3BP was produced and purified essentially as previously described (15).

Solid-phase binding assays. Binding assays were performed essentially as described elsewhere (20). Briefly, microtiter plates were coated with the LGalS3BP (5 μg/ml, 100 μl/well) overnight at 4˚C. After 2 hours saturation in PBS-1% BSA-0.05% Tween-20 at room temperature, plates were incubated with recombinant galectin-1 or recombinant galectin-3 (5 μg/ml, 100 μl/well) in the presence or absence of oligosaccharides (ranging from 15.6 to 1000 to μM). After washing with PBS with Tween 0.05% (washing buffer), plates were incubated with anti-galectin-1 and anti-galectin-3 antibodies, at room temperature for 1 h (polyclonal rabbit anti-galectin-1 or monoclonal M3/38 anti-galectin-3, gifts from Dr. Rabinovich GA) followed by peroxidase conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) for galectin-1 (diluted 1:1,000) or peroxidase-conjugated mouse anti-rat (Sigma) IgG for galectin-3 (diluted 1:10,000) at room temperature for 45 minutes. Binding of galectins to LGal3SBP was detected using 3,3,5,5,tetramethylbenzidine (100 μl/well) substrate (15 minutes shaking) and 1 M H_2SO_4 (100 μl/well) to stop the reaction. Absorbance was determined using a microplate reader (450 nm; Bio-Rad Model 550v; Bio-Rad, San Jose, CA, USA) and values were converted to protein concentrations based on a standard curve. In all the experiments, lactose (ranging from 15.6 to 2,000 to μM) was used as control for binding assays. For each compound, the concentration required to inhibit by 50% the binding of galectins to LGalS3BP (IC_{50}) was calculated. The potency of each compound respect to potency of lactose was calculated as following: Relative Potency to Lactose=IC_{50} Compoud/ IC_{50} Lactose.

Cell aggregation assay. Confluent A375 human melanoma cells were harvested using 0.02% EDTA (ethylenediaminetetraacetic acid) and single-cell suspensions (1×10^6 cells/ml in PBS) were incubated with 10 μg/ml of recombinant LGalS3BP alone or in the presence of oligosaccharides (each at 0.5 mM). Aliquots containing 0.5 ml cell suspension were placed in polypropylene tubes and agitated at 100 rpm at 37˚C for 1 h. Homotypic cell aggregation was then stopped by the addition of 50 μl of 10% paraformaldehyde. The number of single cells in suspension was counted, and the extent of the aggregation was calculated using the following equation: 1-(Nt/Nc) ×100, were Nt (test) and Nc (control) represent the number of single cells in the presence or absence of oligosaccharide derivatives.

Table I. *Inhibitory effect of oligosaccharides on LGalS3BP binding to galectin-1 and galectin-3.*

Oligosaccharide inhibitor	LGalS3BP binding to			
	Galectin-1		Galectin-3	
	IC_{50} (μM)	Relative to lactose	IC_{50} (μM)	Relative to lactose
Lactose	921±49	1.0	1017±69	1.0
1	391±21	2.4	676±12	1.5
2	282±76	3.3	401±41	2.5
3	614±20	1.5	799±20	1.3
4	426±37	2.2	269±30	3.8
9	240±83	3.8	203±25	5.0
10	146±63	6.3	213±46	4.8
11	310±31	3.0	243±40	4.2

Cell death assay. SCLC-H69 cells were plated at a density of 1×10^5 cells/ well in 48-well plates in SITA medium for 24 hours and treated alone or with different oligosaccharide derivates (each at 0.5 mM) for further 24 hours. After treatments, floating cells were collected and centrifuged at 1,000×g for 5 minutes. To evaluate tumor cell death, 1 μl of a mixture of ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml) (1/1 v/v) was added to a 200 μl cell suspension and the percentage of cells undergoing apoptosis was determined using fluorescence microscopy as described elsewhere (15). Cell death was confirmed by measuring the frequency of subdiploid nuclei following propidium iodide incorporation.

Statistical analysis. Comparison between groups was performed by using Student's t- test. Only *p*-values ≤0.05 were considered as statistically significant.

Results

Selective inhibition of galectin-1 and -3 binding to LGalS3BP by synthetic oligosaccharide derivatives. We first evaluated the ability of compounds **1-11** to inhibit the binding of galectin-1 and -3 to LGalS3BP, a highly glycosylated protein previously shown to interact with these lectins (20), in solid-phase assays. In all cases, the inhibitory effect of lactose was considered as a positive control.

As shown in Table I, the oligosaccharide derivates may be classified into different groups according to their inhibitory potency on galectin binding to LGalS3BP. Oligosaccharides **1-4** and **11** exhibited modest inhibitory activities with at least one of the galectins, whereas compounds **9** and **10** were found to be stronger inhibitors of galectin-1 and -3 in solid-phase binding assays. Compounds **5** and **6** showed no activity (hence data sot shown), while the trisaccharides **7** and **8** were only capable of slightly interfering with the binding of galectin-3 to LGalS3BP (Figure 2 and Table I). Elongation of the lactosaminide chain by 3'-sialylation (**1→10**) or the substitution of methyl aglycon by $-(CH_2)_3NH_2$ group (**1→2**) increased the ability to inhibit both galectins, while the transfer from (**1→4**)-linked lactosaminide **1** to its (**1→3**)-linked iso-lactosaminide isomer **2**, or the attachment of lactoside block to **1** from the reducing end (**1→9**) enhanced the ability to inhibit galectin-3 but not galectin-1 (Table I). These findings indicate that individual OSDs with subtle differences in their chemical structure may differentially inhibit the binding of galectin-1 or galectin-3 to specific glycoconjugate ligands.

Interestingly, some of the compounds tested in these assays were more potent than the natural disaccharide lactose, which is traditionally used as a competitor of galectin binding in *in vitro* assays. Relatively low activity of lactose could be due to the domination of its α-isomer (Figure 1) in aqueous solution, while it is the β-isomer that generates lactoside and lactosaminide fragments in the chains of natural galectin ligands.

Effect of oligosaccharids derivatives on LGalS3BP-induced homotypic aggregation in A375 melanoma cells. We previously demonstrated that LGalS3BP is capable of inducing homotypic aggregation of tumor cell lines, a critical event involved in tumor progression and metastasis, by mediating specific interactions of galectin-1 and-3 present on tumor cells (20, 41). Moreover, in a recent study, we described the use of synthetic lactulose amines (SLA) as specific inhibitors that prevent this effect on A375 cells (15).

In an attempt to characterize the functional properties of OSDs, we next screened the functional activity of oligosaccharides on LGalS3BP-induced homotypic aggregation in A375 human melanoma cells. As shown in Figure 3, only three of the compounds tested (oligosaccharides **2**, **3**, **11**) significantly inhibited homotypic cell aggregation (**2**, **3** $p<0.005$; 11 $p<0.01$) at a final concentration of 0.5 mM. Among the chemical features that characterize these active compounds, it is worthwhile mentioning that the longer chains of **3** and **11** oligosaccharides, which considerably improved their ability to block galectin carbohydrate interactions in solid-phase assay, were not always effective inhibitors in rolling conditions (Figures 2 and 3).

Oligosaccharide derivates induced apoptosis in human small cell lung carcinoma. Previous work has demonstrated that galectin-3 may protect cells from apoptosis induced by UV and chemotherapeutic agents (42, 43). Moreover, we and others reported that overexpression of galectin-3 in tumor cells protects these cells from anoikis, a process that links cell adhesion and apoptosis (2, 44). Therefore, we evaluated whether the anti-adhesive effect of oligosaccharide derivatives may result in tumor cell apoptosis. For this purpose, ethidium bromide/acridine orange staining was used to monitor

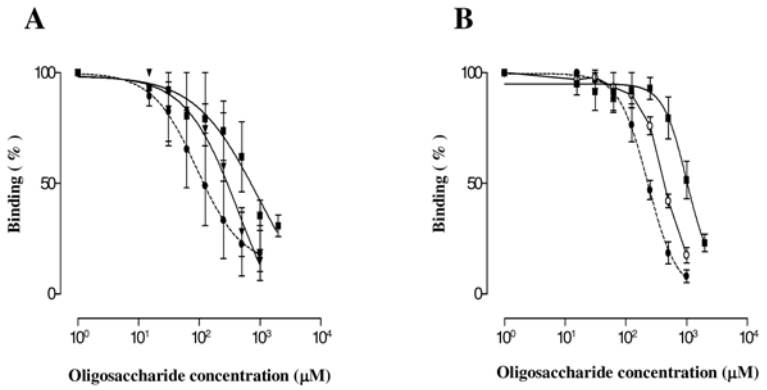

Figure 2. *Inhibitory effect of representative oligosaccharide derivates on galectin-1 (A) (examples: 4, 10, lactose) and galectin-3 (B) (examples: 2, 10, lactose) binding to immobilized LGalS3BP in solid-phase assay. Results are shown as percentages of residual binding. The data were fit by nonlinear regression to the formula for single site competitive inhibition: Y=100/1+10 (X-log(IC_{50})), where Y=binding with the inhibitor as a percentage of binding without the inhibitor and X=logarithm of the inhibitor concentration in μM.*

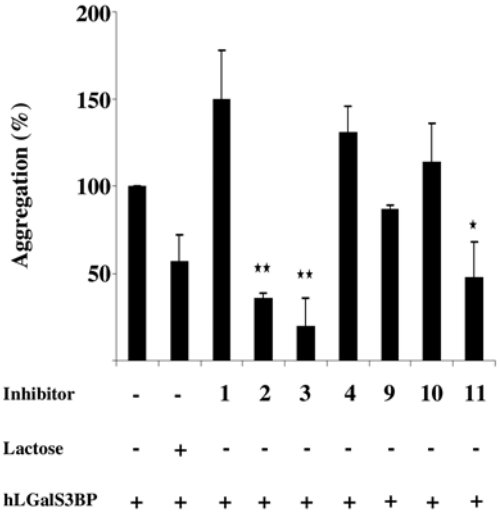

Figure 3. *Effect of oligosaccharide derivates on LGalS3BP-induced homotypic aggregation of A375 human melanoma cells. Inhibition of LGalS3BP (10 μg/ml) induced homotypic cell aggregation as induced by lactose (10 mM, positive control) and different oligosaccharides (0.5 mM) is shown. Cells were agitated for 1 h in the presence of 10 μg/ml LGalS3BP with or without different OSDs added at final concentrations of 0.5 mM, or with lactose (10 mM), and the percentage of aggregation was determined as described in Materials and Methods. Error bars represent the SD of triplicate measurements. *p<0.01; **p<0.005 versus untreated control.*

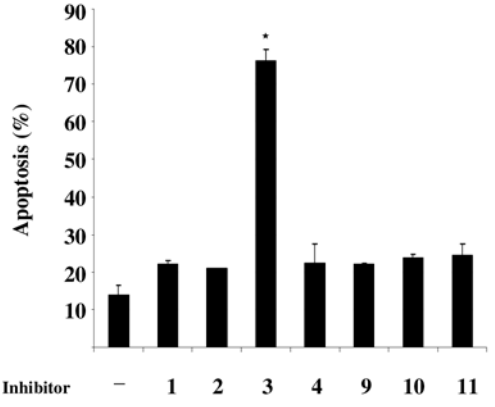

Figure 4. *Effect of oligosaccharide inhibitor (0.5 mM) on apoptosis of small cell lung carcinoma cells. Cells were grown in SITA medium for 24 h before addition of the indicated concentrations of oligosaccharides for further 24 h. The percentage of cells undergoing apoptosis was determined by acridine orange/ethidium bromide staining using fluorescence microscopy and further confirmed by propidium iodide staining of subdiploid nuclei (data not shown). Data represent the mean±SD of three independent experiments performed in triplicates. The percentage of apoptotic cells detected in control cultures (no added OSD) was 13.6±2.6.*

apoptosis in OSD-treated SCLC-H69. As shown in Figure 4, control cultures exhibited 13.6±2.6% of cells undergoing apoptosis. However, when cells were exposed to compound **3** at a concentration of 0.5 mM, the percentage of apoptotic cells increased to 76±2.9% (p<0.0005). Remarkably, this rate of apoptosis was comparable to that induced by the chemotherapeutic agent etoposide (data not shown). Furthermore, no significant effect on tumor cell apoptosis was observed when other oligosaccharides were used.

Collectively, our results demonstrate that allyl lactoside **3**, but not the other oligosaccharides, promoted apoptosis of SCLC-H69 cells, indicating that these synthetic compounds may have selectivity for different biological processes.

Discussion

Accumulating evidence indicates that galectins, particularly galectin-1 and -3, play a key role in tumor progression and

metastasis (2, 8-11, 15, 20, 21). Up-regulated expression of galectins is well documented in different tumor types, including astrocytoma, glioblastoma, melanoma, lung, prostate, breast and ovary carcinomas (2). In particular, gene and protein expression profile analysis has led to the identification of galectin-1 and -3 as typical proteins whose expression is up regulated in a plethora of tumors and metastatic lesions, as compared with their non-transformed and/or non-invasive counterparts (5). In general, galectin expression is associated with poor prognosis and the acquisition of a metastatic phenotype (2). Indeed, studies using animal models have provided significant support to the role of galectins in tumor growth and metastasis *in vivo* (2, 3, 45), suggesting that selective inhibition of galectins might have profound implications for cancer therapy. Thus, it is predicted that inhibition of these glycan-binding proteins will find its way into cancer clinical trials (2, 15, 46-51). Furthermore, the availability of synthetic inhibitors might also serve as basic research tools to dissect the mechanisms implicated in the biological functions of galectins.

Challenges for the future will be employing these potent and selective inhibitors of different members of the galectin family; in fact, molecules with such properties have already been developed. Pioneer studies reported the effects of two synthetic low molecular weight glycoamine analogs (Fru-D-Leu and Lac-L-Leu) on the metastatic potential of human breast carcinoma xenografts growing in the mammary fat pads of nude mice (46). More recently, other studies (47) examined the effects of modified citrus pectin, a water-soluble polysaccharide fiber derived from citrus fruit that specifically inhibits galectin-3 in tumor growth and metastasis. Interestingly, the authors found that citrus pectin, given orally, inhibits carbohydrate-mediated tumor growth, angiogenesis and metastasis by disrupting the interactions between galectin-3 and its specific carbohydrate ligands (47). In addition, recent findings described the synthesis of wedge-like glycodendrimers with two, four and eight lactose moieties using 3,5 di-(2-aminoethoxy) benzoic acid as the branching unit (2). These compounds successfully inhibited the binding of galectin-1 to a highly glycosylated matrix. Furthermore, during recent years, Nilsson and colleagues designed a variety of efficient and stable galectin inhibitors with high affinity, including low micromolar inhibitors of galectin-3 based on 3'-derivatization of *N*-acetyllactosamine with an inhibitory potenty of ~50 times greater than *N*-acetyllactosamine (52), *O*-galactosyl aldoximes (51) and a collection of thiodigalactoside derivatives (49, 50). We found that lactulose amine derivatives may also inhibit galectin-1 and galectin-3 binding to LGalS3BP in binding inhibition assays with IC_{50}s in the order of 20-40 μM (15).

In the present study, we show that structural variations of oligosaccharides themselves might be responsible for the different potency by which these compounds inhibit the binding of galectin-1 and -3 to LGalS3BP. In addition, we found that some of these inhibitors were specific for either galectin-1, or galectin-3. This finding is of critical importance considering recent observations on the divergent functions of galectin-1 and -3 on cell adhesion, immune cell regulation and apoptosis (5, 53), and the importance of specifically targeting individual galectins, without interfering with the functions of other members of this family.

Interaction of galectins with β-galactoside-decorated glycoproteins has been described as an important mechanism responsible for the homotypic aggregation of tumor cells and emboli formation (3, 19). This phenomenon can protect tumor cells in the circulation, thus leading to increased invasiveness and metastasis (1, 3). In addition, we and others have demonstrated that galectin-3 overexpression can favour tumor cell anoikis, a phenomenon which links cell adhesion and cell death (5, 12, 32).

We found previously that synthetic lactulose amines, which block the interactions between galectins and LGalS3BP, have promising therapeutic effects since they are capable of blocking tumor cell aggregation and angiogenesis, and of triggering tumor cell apoptosis (15). Here we compared the series of oligosaccharides that are homologous to normal components of serum using *in vitro* assays. We demonstrate that oligosaccharide derivatives can differentially inhibit binding of galectin-1 or -3 and interrupt two different steps that are critical for tumor metastasis, namely homotypic cell aggregation and tumor cell apoptosis. Some of these compounds display a potent inhibitory effect on LGalS3BP-induced homotypic aggregation of A375 cells, under rolling conditions at 37˚C resembling the prevailing circumstances in the bloodstream; this process has critical importance during the intravascular phase of the metastatic dissemination. On the other hand, some oligosaccharides showed the capacity to modulate apoptosis of tumor cells. This effect might be, at least in part, associated with the interruption of galectin binding to the tumor cell surface as has been suggested (44). Interestingly, apoptosis induced by some oligosaccharides is comparable to that promoted by antiblastic chemotherapeutic drugs in MTT assays. Furthermore, the observation that these compounds are similar to serum glycoproteins is of particular interest for clinical applications. Remarkably, the ability to interfere with multiple pathways involved in cancer progression suggests that glycomimetics might also affect other galectin-independent mechanisms. This possibility warrants future studies.

However, before galectin-1-based therapeutic agents can be extrapolated to clinical settings, a more thorough understanding of the mechanisms involved in galectin functions is required. In this regard a number of questions remain to be addressed: i) To what extent is there functional redundancy and specificity of action within the galectin family? ii) What is the rational explanation for the different

functions exerted by galectin-1 and -3 within different environmental contexts? iii) What are the levels of galectin-1 and -3 attained *in vivo* during tumor dissemination? All these questions should be experimentally addressed before galectins can be used as anticancer targets in clinical settings.

In summary, our results suggest that oligosaccharide derivatives, especially allyl lactoside **3**, demonstrate promising activity and could serve as a basis for further structure optimization and site-specific modifications to obtain effective and safe pharmacological inhibitors. In addition, a comparative evaluation of the antitumor activity of different galectin inhibitors is essential to fully validate the concept of galectins as potential targets for anticancer therapy.

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