

Synthetic lactulose amines: novel class of anticancer agents that induce tumor-cell apoptosis and inhibit galectin-mediated homotypic cell aggregation and endothelial cell morphogenesis

Gabriel A. Rabinovich^{1,2}, Albana Cumashi³,
Germán A. Bianco², Domenico Ciavardelli³, Ida Iurisci³,
Maurizia D'Egidio³, Enza Piccolo³, Nicola Tinari³,
Nikolay Nifantiev⁴, and Stefano Iacobelli³

²Division of Immunogenetics, Hospital de Clínicas “José de San Martín,” University of Buenos Aires, Buenos Aires, Argentina; ³Department of Oncology & Neurosciences, University G. D’Annunzio, Medical School & Foundation, 66013 Chieti, Italy; and ⁴Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 119991, Russia

Received on August 25, 2005; revised on October 30, 2005; accepted on November 6, 2005

Galectins, a family of structurally related carbohydrate-binding proteins, contribute to different events associated with cancer biology, including apoptosis, homotypic cell aggregation, angiogenesis and tumor-immune escape. To interfere with galectin–carbohydrate interactions during tumor progression, a current challenge is the design of specific galectin inhibitors for therapeutic purposes. Here, we report the synthesis of three novel low molecular weight synthetic lactulose amines (SLA): (1) *N*-lactulose-octamethylenediamine (LDO), (2) *N,N'*-dilactulose-octamethylenediamine (D-LDO), and (3) *N,N'*-dilactulose-dodecamethylenediamine (D-LDD). These compounds showed a differential ability to inhibit binding of galectin-1 and/or galectin-3 to the highly glycosylated protein 90K in solid-phase assays. In addition, each compound demonstrated selective regulatory effects in different events linked to tumor progression including tumor-cell apoptosis, homotypic cell aggregation, and endothelial cell morphogenesis. Our results suggest that galectin inhibitors with subtle differences in their carbohydrate structures may be potentially used to specifically block different steps of tumor growth and metastasis.

Key words: apoptosis/galectin inhibitors/tumor escape/tumor progression

Introduction

Protein–carbohydrate interactions are critical for the progression and dissemination of cancer cells (Kannagi *et al.*, 2004). Galectins, a family of highly conserved carbohydrate-binding proteins, are characterized by their ability to recognize *N*-acetylglucosamine sequences, which can be displayed on both *N*- and *O*-glycans on cell surface glycoconjugates

(Cooper and Barondes, 1999; Hirabayashi *et al.*, 2002; Stowell *et al.*, 2004). All galectins contain conserved carbohydrate-recognition domains (CRDs) that are responsible for carbohydrate binding (Ahmed and Vasta, 1994; Ahmad *et al.*, 2004). So far, fifteen mammalian galectins have been identified, which can be subdivided into three groups: those containing one CRD (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15); those containing two distinct CRDs in tandem, connected by a linker of up to 70 amino acids (galectin-4, -6, -8, -9, and -12); and galectin-3 which consists of unusual tandem repeats of proline- and glycine-rich short stretches fused onto the CRD (Leffler *et al.*, 2004). Galectins are often overexpressed in cancerous cells and cancer-associated stromal cells (Danguy *et al.*, 2002; van den Brùle *et al.*, 2003; Lahm *et al.*, 2004; Takenaka *et al.*, 2004; van den Brùle *et al.*, 2004). In general, this altered expression correlates with the aggressiveness of the tumors and the acquisition of metastatic phenotype, indicating that galectins might modulate tumor progression and influence disease outcome (Danguy *et al.*, 2002; Greco *et al.*, 2004).

There is increasing evidence that galectins have important functions in several aspects of cancer biology (Liu and Rabinovich, 2005), including tumor transformation (Paz *et al.*, 2001), apoptosis (Perillo *et al.*, 1995; Yang *et al.*, 1996; Akahani *et al.*, 1997; Matarrese *et al.*, 2000, 2005; Rabinovich *et al.*, 2002b), and cell growth regulation (Kim *et al.*, 1999; Wells *et al.*, 1999; Yamaoka *et al.*, 2000; Kopitz *et al.*, 2001). In addition, galectins are also involved in various steps of tumor metastasis, including tumor cell adhesion (Ellerhorst *et al.*, 1999; Levy *et al.*, 2001; van den Brùle *et al.*, 2003), homotypic cell aggregation (Tinari *et al.*, 2001; Glinsky *et al.*, 2003), invasiveness (LeMarer and Hughes, 1996; Hittlet *et al.*, 2003), angiogenesis (Claude *et al.*, 1999; Nangia-Makker *et al.*, 2000) and inflammation (Rabinovich *et al.*, 2002a). Furthermore, recent evidence indicates that galectins may also function as soluble mediators employed by tumor cells to evade immune responses (Rubinstein *et al.*, 2004; Le *et al.*, 2005).

The overall effects of galectins in tumor progression could be the combination of some or all the effects described above, and, in fact, studies using animal models have provided significant support for the role of galectins in tumor growth and metastasis *in vivo* (Bresalier *et al.*, 1998; Honjo *et al.*, 2001; Inufusa *et al.*, 2001; Camby *et al.*, 2002; Song *et al.*, 2002; John *et al.*, 2003; Califice *et al.*, 2004; Rubinstein *et al.*, 2004; Ueda *et al.*, 2004), suggesting that selective inhibition of galectins might have profound implications for cancer therapy. Thus, it is predicted that inhibitors of these carbohydrate-binding proteins (Glinsky *et al.*,

¹To whom correspondence should be addressed; e-mail: gabyrabi@ciudad.com.ar

1996; Andre *et al.*, 2001; Nangia-Makker *et al.*, 2002; Sorme *et al.*, 2002, 2003; Cumpstey *et al.*, 2005; Salameh *et al.*, 2005; Tejler *et al.*, 2005) will find their way into cancer clinical trials, leading to delays in tumor progression and improvements in overall survival (Rabinovich, 2005). Furthermore, the availability of synthetic inhibitors might also contribute as basic research tools to dissect the cellular and molecular mechanisms implicated in the biological functions of galectins.

Here, we report the synthesis of three novel synthetic lactulose amines (SLA) as potent and specific inhibitors of the binding of galectins-1 and -3 to the highly glycosylated protein 90K. These inhibitory agents showed selective modulatory effects in critical steps of tumor progression, including galectin-mediated homotypic cell aggregation, tumor-cell apoptosis and endothelial cell morphogenesis, suggesting their potential contribution to suppress tumor growth and metastasis.

Results

Different SLA selectively inhibit binding of galectin-1 and galectin-3 to the highly glycosylated 90K

The rationale of this study was to compare the inhibitory activity of monomeric and dimeric carbohydrate ligands to assess whether bivalency (as a method to increase local

concentration of galactose-containing ligands near to galectins CRD) may enhance the inhibition of galectin-mediated cell recognition process. In this context, we have synthesized three novel lactulose amine derivatives, which we termed SLA (*N*-lactulose-octamethylenediamine [LDO], *N,N'*-dilactulose-octamethylenediamine [D-LDO], and *N,N'*-dilactulose-dodecamethylenediamine [D-LDD]). They differ each other in the number of lactose residues (1 vs. 2) and the length of the amine chain (8 vs. 12 N) (Figure 1). To determine whether these compounds can interfere in galectin functions, we first assessed the ability of SLA to inhibit the binding of galectins-1 and -3 to microwells coated with 90K (a highly glycosylated protein previously shown to specifically interact with these galectins) (Tinari *et al.*, 2001). Decrease in galectin binding in the presence of SLA was recorded as the inhibitory potency of these compounds. As shown in Figure 2, LDO and D-LDO successfully inhibited binding of galectin-1 (Figure 2A) and galectin-3 (Figure 2B) with IC₅₀ in the order of 20–40 mM. In contrast, the dodecamethylenediamine derivative, D-LDD, showed a reduced ability to inhibit binding of galectin-3 to 90K-coated wells (Figure 2B). Interestingly, at identical concentrations, the three compounds showed a different ability to inhibit binding of galectin-1 (Figure 2A) or galectin-3 (Figure 2B) to 90K. In fact, D-LDD successfully interfered with the binding of galectin-1 to 90K, whereas it failed to inhibit binding of galectin-3 to the glycosylated protein.

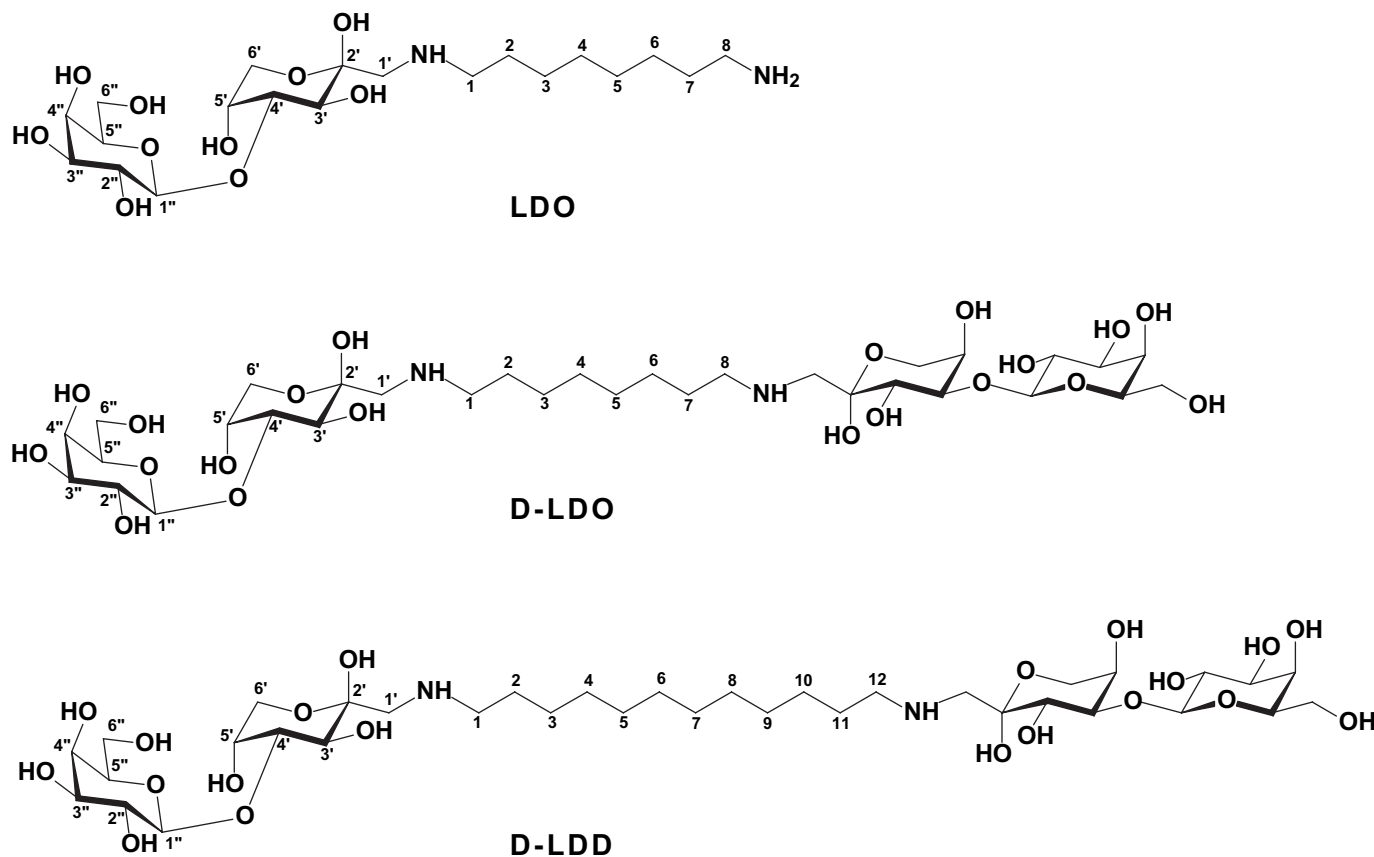


Fig. 1. Chemical structure of synthetic lactulose-amines: LDO, D-LDO, and D-LDD. These compounds differ each other in the number of lactose residues (1 vs. 2) and the length of the amine chain (8 vs. 12 N).

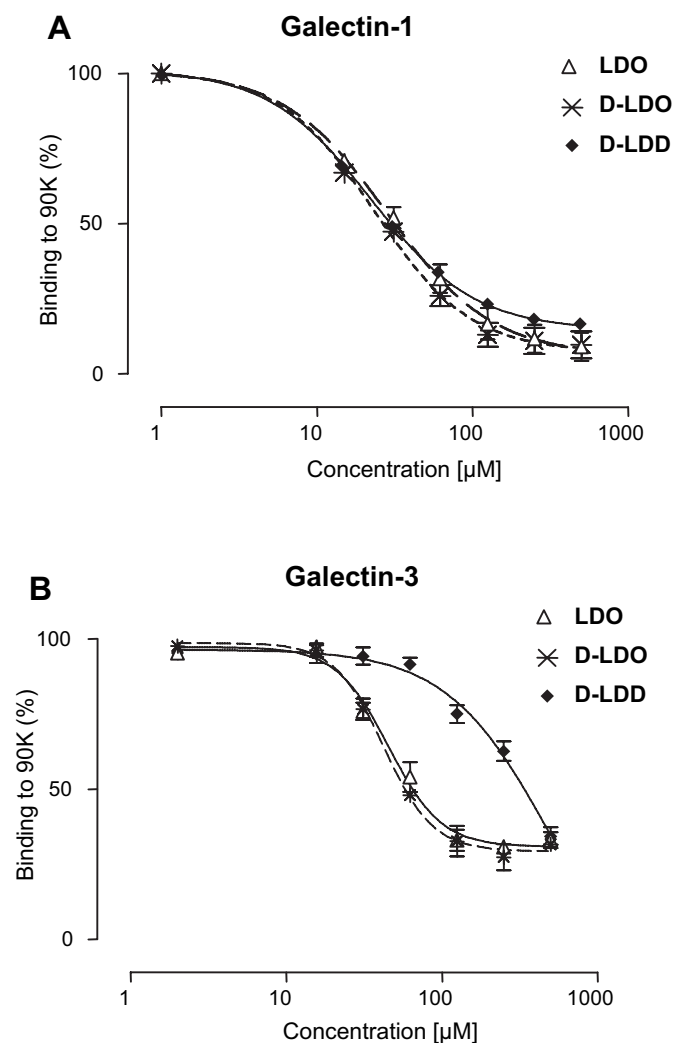


Fig. 2. Ability of LDO (Δ), D-LDO (\times), and D-LDD (\blacklozenge) to inhibit binding of galectin-1 (**A**) or galectin-3 (**B**) to immobilized 90K in solid-phase assays. Results are shown as percentages of inhibition. The data were fit by nonlinear regression to the formula for single-site competitive inhibition: $Y = 100 / (1 + 10^{(X - \log(\text{IC}_{50}))})$, where Y = binding with the inhibitor as a percentage of binding without the inhibitor and X = logarithm of the inhibitor concentration.

These findings indicate that individual SLA with subtle differences in their chemical structure may differentially inhibit the binding of galectin-1 or galectin-3 to specific glycoconjugate ligands present on the cell surface or extracellular matrix.

SLA inhibit homotypic tumor-cell aggregation

It has been reported that 90K (a galectin-binding protein) promotes homotypic cell aggregation by bridging galectin-1 or galectin-3 molecules on the surface of adjacent cells (Tinari *et al.*, 2001). Because SLA inhibited galectin-1 and galectin-3 binding to 90K, we have undertaken this model to evaluate whether SLA can disrupt the interactions between 90K and galectins and inhibit homotypic cell aggregation, a critical event involved in tumor progression and metastasis. As shown in Figure 3A, A375 melanoma

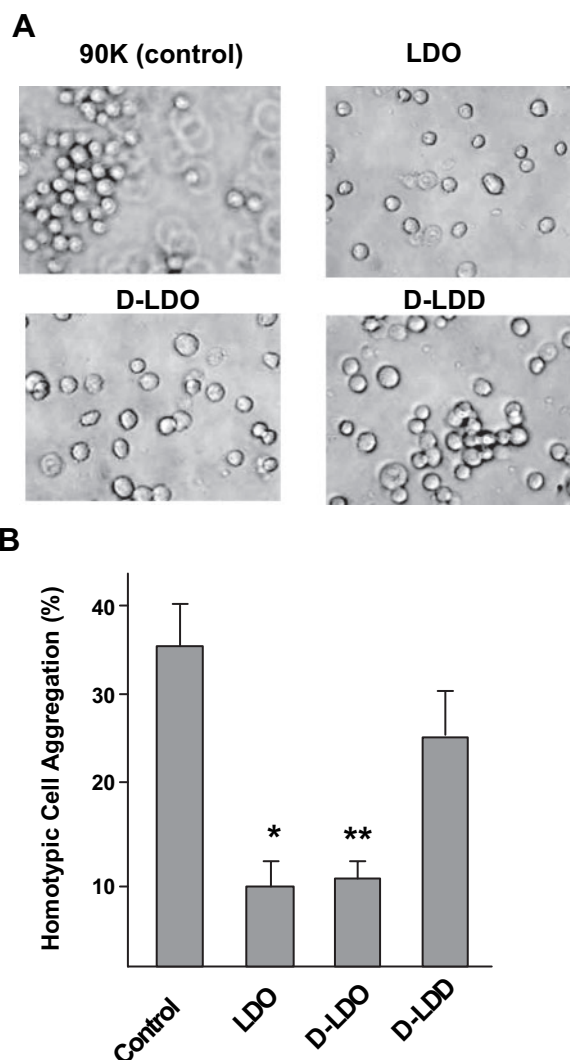


Fig. 3. Inhibition of 90K-induced homotypic aggregation of A375 human melanoma cells by SLA. (**A**) Digital images of 400 \times phase contrast pictures of A375 human melanoma cells showing homotypic cell aggregation when incubated with 10 $\mu\text{g}/\text{ml}$ of 90K in the presence of 0.25 mM each of LDO, D-LDO, or D-LDD. (**B**) Percentage of homotypic cell aggregation in the presence of 90K (positive control) in the absence or presence of different SLA (LDO, D-LDO, and D-LDD). Cells were agitated for 1 h in the presence of 10 $\mu\text{g}/\text{ml}$ 90K with or without different SLA added at final concentrations of 0.25 mM or with lactose (10 mM), and the percentage of aggregation was determined as described in *Materials and methods*. 90K-induced homotypic cell aggregation was partially inhibited by anti-galectin-1 or anti-galectin-3 antibodies (data not shown). Error bars represent the SD of triplicate measurements of at least three independent experiments. * $P < 0.05$; ** $P < 0.01$ versus untreated control.

cells formed aggregates in the presence of 90K, whereas in its absence, most of the cells remained as single cells in suspension. This effect was inhibited by addition of lactose (10 mM) and was partially abrogated by anti-galectin-1 or anti-galectin-3 blocking antibodies (Tinari *et al.*, 2001 and data not shown). Interestingly, both LDO and D-LDO strongly inhibited the homotypic cell aggregation, whereas D-LDD had no significant effect (Figure 3A and B). Importantly, LDO, D-LDO, and D-LDD had no effect when added alone to A375 cells in the absence of 90K (data not shown).

SLA promote tumor-cell apoptosis

The most extensively studied function of galectins that is relevant to tumor progression is the regulation of apoptosis (Yang *et al.*, 1996; Akahani *et al.*, 1997; Hoyer *et al.*, 2004; Rubinstein *et al.*, 2004; Takenaka *et al.*, 2004; Endharti *et al.*, 2005). To determine whether the observed anti-adhesive effect of SLA resulted in tumor-cell apoptosis, we evaluated annexin V binding to SLA-treated SCLC-H69, a human small cell lung carcinoma cell line, which synthesizes substantial amounts of galectin-1 and galectin-3 (Figure 4A). Control cultures (media alone) exhibited about 8% of cells undergoing apoptosis (data not shown). However, when cultures were exposed to D-LDO or D-LDD, a higher percentage of cells underwent apoptosis, and this frequency increased in a dose-dependent manner to $31.6 \pm 0.8\%$ and $44.1 \pm 1.1\%$ for D-LDO, and to $24.5 \pm 1.3\%$ and $47.4 \pm 2.2\%$ for D-LDD, when cells were exposed to SLA inhibitors at concentrations of 0.5 and 1 mM, respectively (Figure 4B; $P < 0.001$ vs. controls). It should be mentioned that a modest increase in the percentage of apoptotic cells (16.4 and 24.4%) was observed after treatment with LDO at the concentrations tested (0.5 and 1 mM, respectively). Furthermore, the same concentrations of SLA were not effective in promoting apoptosis to the immortalized nontumorigenic cell line MCF10A or to resting T lymphocytes which do not express galectin-1 or galectin-3 (Joo *et al.*, 2001; Fuertes *et al.*, 2004) (data not shown). Collectively, these data demonstrate that D-LDO and D-LDD promote apoptosis of SCLC-H69 tumor cells, indicating that different SLA not only have different specificities but also have selective effectiveness on different biological effects.

SLA inhibit endothelial cell morphogenesis

It has been reported that galectin-3 is a pro-angiogenic factor for human umbilical vein endothelial cells (HUVEC) (Nangia-Makker *et al.*, 2000). Furthermore, galectin-1 has been shown to modulate heterotypic interactions between tumor and endothelial cells, suggesting its potential role in promoting angiogenesis (Clausse *et al.*, 1999). Therefore, we investigated whether SLA could affect endothelial cell morphogenesis (a typical feature of angiogenesis) measured by capillary tube formation. HUVEC, which express high levels of galectin-1 and galectin-3 (Figure 5A) were plated on matrigel and stimulated with bovine crude endothelial cell growth factor (ECGF) to form tube-like structures, a process involving migration, invasion, and differentiation of these cells (Figure 5B; positive control). This effect was inhibited by using 10 mM lactose (data not shown). As shown in Figure 5C, in the presence of D-LDO, the formation of tubes was severely impaired, and this effect was partially prevented by addition of anti-galectin-1 or anti-galectin-3 antibodies (Figure 5F). Interestingly, the inhibitory effect seemed to be specific for D-LDO, because neither LDO (Figure 5D) nor D-LDD (Figure 5E) affected tubulogenesis. A summary of the results obtained in three independent experiments is shown in Figure 5F. These results confirm the specificity and selectivity of each synthetic inhibitor on different biological events linked to tumor progression and metastasis.

Discussion

Galectins have emerged as promising molecular targets for cancer therapy, and galectin inhibitors have the potential to be used as anti-tumor and anti-metastatic agents (Danguy *et al.*, 2002; Lahm *et al.*, 2004; Liu and Rabinovich, 2005). In fact, the galectin-3 C-terminal domain fragment significantly suppresses tumor growth and inhibits metastasis in a mouse model of human breast cancer (John *et al.*, 2003). In addition, peptides specific to the galectin-3 CRD significantly inhibit the adhesion of a human breast carcinoma cell line to endothelial cells *in vitro* (Zou *et al.*, 2005) and administration of an anti-galectin-3 antibody specifically inhibits liver metastasis by adenocarcinoma cell lines (Inufusa *et al.*, 2001).

In addition, we have recently demonstrated that tumors can overwhelm T cell effector functions through galectin-1-mediated mechanisms suggesting a critical role for this protein in tumor-immune privilege (Rubinstein *et al.*, 2004). Blockade of the inhibitory effects of galectin-1 within tumor tissue, using antisense melanoma transfectants, resulted in reduced tumor mass and enhanced tumor rejection in a syngeneic model of murine melanoma (Rubinstein *et al.*, 2004). These effects were accompanied by the generation of a potent tumor-specific T-cell-mediated response. Interestingly, recent clinical studies identified galectin-1 as a link between tumor hypoxia and tumor-immune privilege (Le *et al.*, 2005).

Challenges for the future will be to employ potent and selective small inhibitors of galectins, and, 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 metastasis of human breast carcinoma xenografts growing in the mammary fat pads of nude mice (Glinsky *et al.*, 1996). The treated animals had no apparent toxicity from chronic daily injection of synthetic glycoamines up to 17 weeks of treatment. However, the molecular mechanisms involved in this anti-metastatic effect have not been clearly identified. More recently, other studies (Nangia-Makker *et al.*, 2002) 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 (Nangia-Makker *et al.*, 2002).

In addition, recent findings described the synthesis of Wedglike glycodendrimers with two, four, and eight lactose moieties using 3,5 di-(2-aminoethoxy) benzoic acid as the branching unit (Andre *et al.*, 2001). These compounds were tested in solid-phase competition assays with lactose maxiclusters and various *N*-glycans branching profiles (miniclusters) and successfully inhibited the binding of galectin-1 to this glycosylated matrix with a relative inhibitory potency of 150 regarding free lactose (Andre *et al.*, 2001). Furthermore, during the past few years, Nilsson and colleagues designed a variety of efficient, stable, and high affinity galectin inhibitors, including low micromolar inhibitors of galectin-3 based on 3'-derivatization of *N*-acetylglucosamine

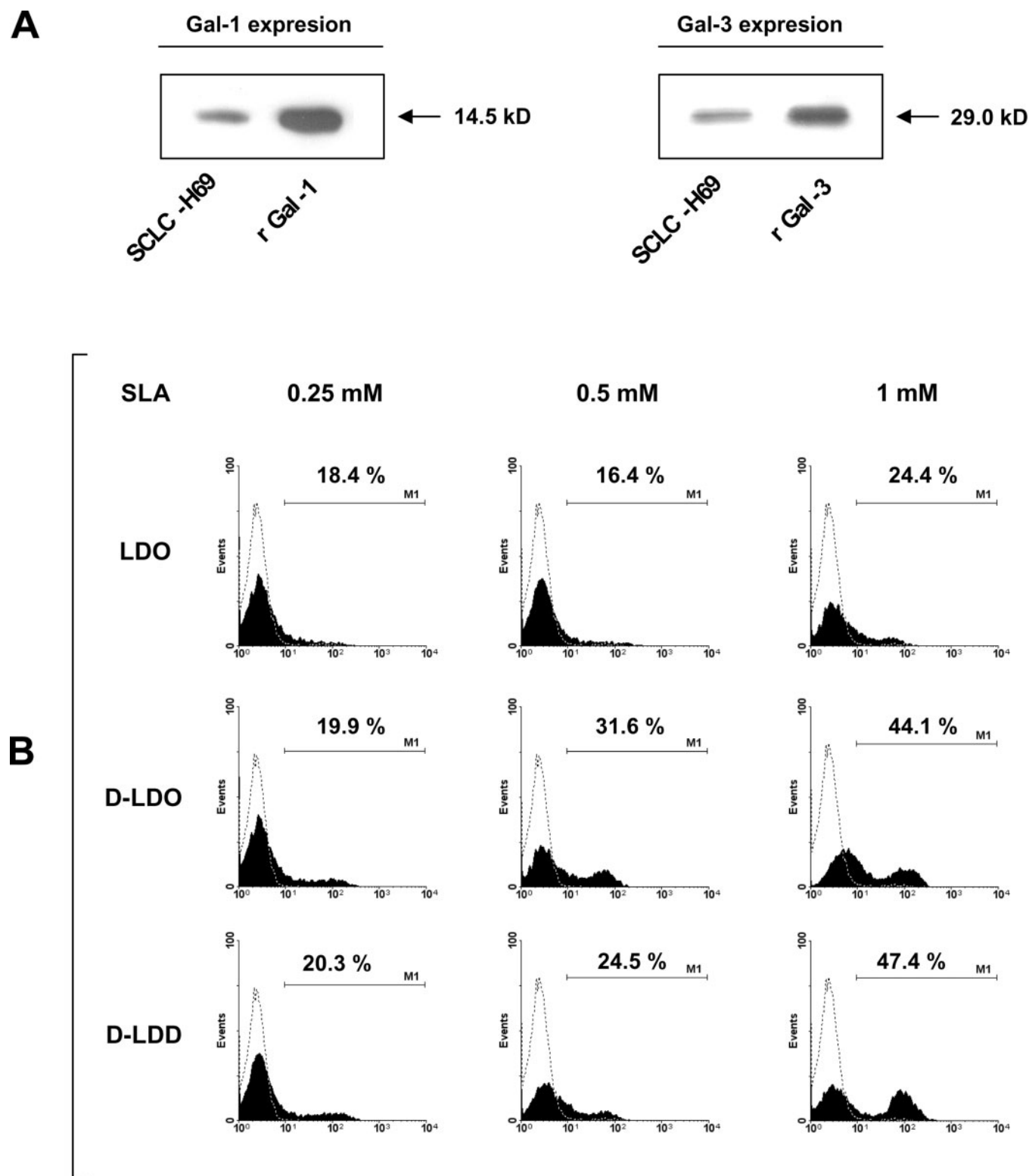


Fig. 4. Effects of SLA on apoptosis of H69-SCLC tumor cells. **(A)** Expression of galectins-1 and -3 in SCLC-H69 tumor cells was assessed by western blot analysis using specific polyclonal antibodies. rGal-1 and -3 were run as controls. **(B)** To evaluate apoptosis, cells were grown in SITA medium for 24 h before addition of the indicated concentrations of SLA for further 24 h. The percentage of cells undergoing apoptosis was determined by annexin V binding using a FITC-annexin-V staining kit. The same concentrations of SLA were not effective in promoting apoptosis to the immortalized nontumorigenic cell line MCF10A or human resting T lymphocytes (data not shown). Data represent the mean \pm SD of at least three independent experiments performed in triplicates. The percentage of apoptotic cells detected in control cultures (no added SLA) was $8.0 \pm 0.8\%$.

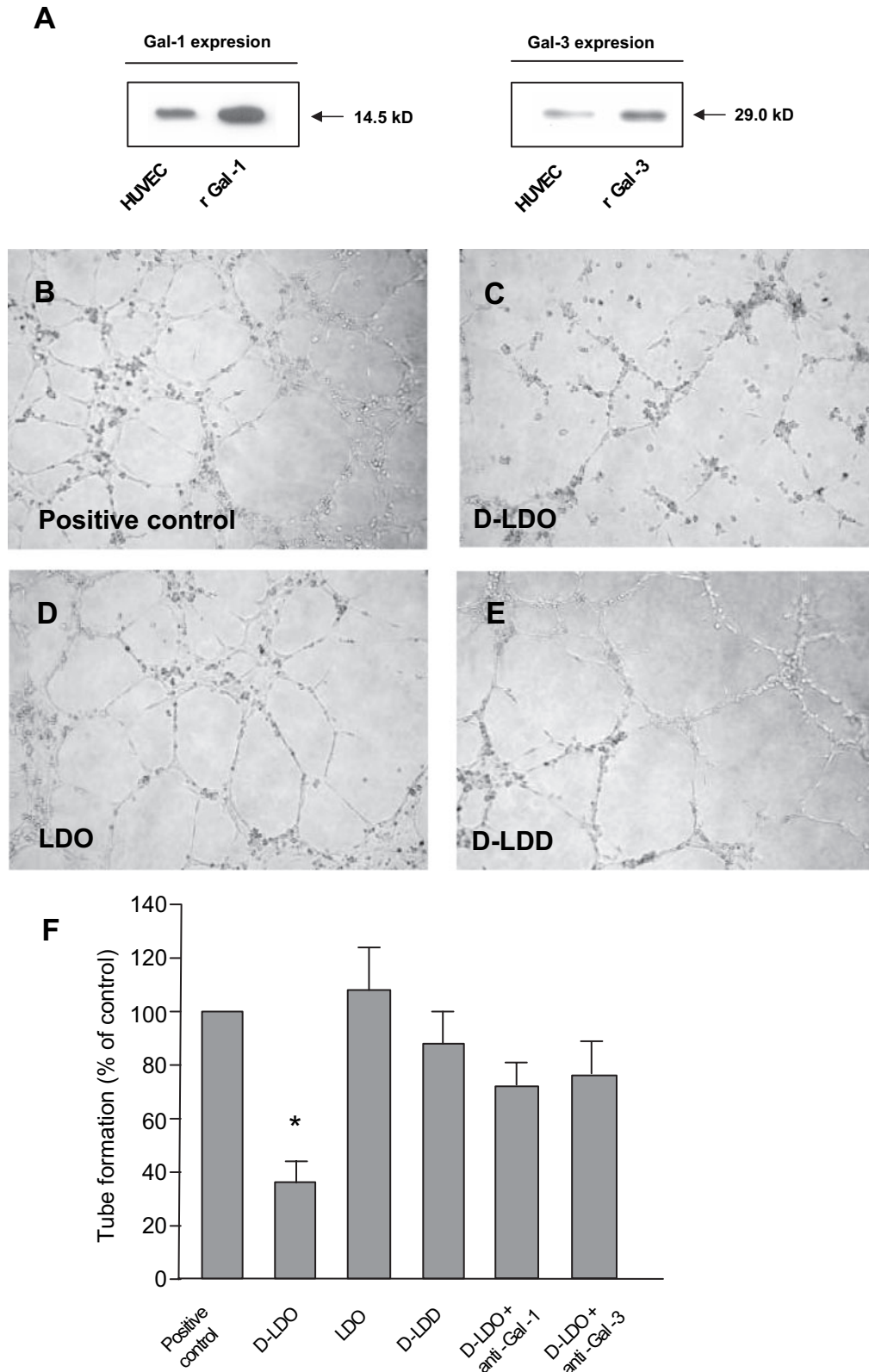


Fig. 5. Effect of SLA on endothelial cell morphogenesis. (A) Expression of galectins-1 and -3 in HUVEC was assessed by western blot analysis using specific polyclonal antibodies. rGal-1 and -3 were run as controls. (B–E) The ability of SLA to modulate *in vitro* angiogenesis was evaluated by measuring the extensiveness of endothelial cells forming a capillary tube-like network structure. HUVECs were plated on Matrigel-coated chamber slides and treated with 50 µg/ml bovine ECGF (positive control) in the absence (B) or presence of 0.1 mM D-LDO (C), 0.1 mM LDO (D), or 0.1 mM D-LDD (E). Tube formation was assessed after 18 h. Results are expressed as percentages of tube formation (means ± SD) (F). D-LDO-induced inhibition of tubulogenesis was partially prevented in the presence of anti-galectin-1 or anti-galectin-3 antibodies (F) demonstrating the relevance of galectins in these processes. Representative digital images of 400× phase contrast pictures are shown. Similar results were obtained from two independent experiments. * $P < 0.01$ versus untreated control.

with an inhibitory potency of about 50 times better than *N*-acetyllactosaminide (Sorme *et al.*, 2002), *O*-galactosyl aldoximes (Tejler *et al.*, 2005) and a collection of thiodigalactoside derivatives (Cumpstey *et al.*, 2005; Salameh *et al.*, 2005). In this regard, we found that different SLA successfully inhibit galectin-1 and galectin-3 binding to 90K-coated matrix in binding inhibition assays with IC₅₀ in the order of 20–40 mM. More importantly, these inhibitory agents successfully interfere with galectin-mediated homotypic cell aggregation, tumor-cell apoptosis, and endothelial cell morphogenesis; three critical events linked to tumor metastasis. Interestingly, each SLA differentially inhibits galectin-1- or galectin-3-mediated effects suggesting subtle differences in the specificity and/or mechanisms of action of each compound. Although we have focused here on the effects of SLA on galectins-1 and -3, further studies should be required to explore the effects of these inhibitors on other galectins involved in tumor biology, including galectins-7, -8, and -9.

Although galectin inhibitors have emerged as promising candidates as anticancer drugs, it is of vital importance to point out that inhibition of galectin expression might not be beneficial for all tumor types, specially for those that have down-regulated expression of certain galectins (Pacis *et al.*, 2000; Ueda *et al.*, 2004). In these cases, introducing galectins by gene therapy or by specific induction of the gene might prove to be an effective therapy. In this regard, galectin expression can be also modulated by differentiating, chemotherapeutic and antimetastatic agents, as has been clearly demonstrated for galectin-1 (Lu *et al.*, 2000; Rabinovich *et al.*, 2002c).

In addition to the role of galectins in tumor biology, these carbohydrate-binding proteins have also been described as potent regulators of the inflammatory response (Rabinovich *et al.*, 2002a, 2004). Therefore, potent specific inhibitors of galectins would also be valuable as novel immunomodulatory agents and basic research tools for elucidating the precise mechanisms involved in the biological functions of galectins. At present, we are focusing our efforts in improving the procedure to obtain SLA at a larger scale to inject *in vivo* and test their modulatory effect in several experimental models of cancer including the B16 melanoma (Rubinstein *et al.*, 2004).

In conclusion, our results suggest that SLA demonstrate promising activity and could serve as a basis for further structure optimization to get effective and safe pharmacological inhibitors. In addition, the increased understanding of the role of galectins in cancer and inflammation and the mechanisms of action of galectin inhibitors at different steps of tumor progression should provide more insights into how the regulation of galectin expression and activity might be exploited for therapeutic purposes.

Materials and methods

Reagents and chemical analysis

All anhydrous solvents and reagents were obtained from Aldrich or Fluka. Thin-layer chromatography (TLC) separation was performed on silica gel 60 F-250 (Macherey-Nagel); spots were detected by heating after spraying the

plates with anisaldehyde and ninhydrin. ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AC 300 instruments. δ are given in ppm and referenced to internal Si (Me)₄ (δ = 0.00 ppm). J values are given in Hertz. Assignments of signals were made from analysis of ¹³C spectra of lactulose and amines and from comparative data available for other Amadori compounds. Chemical shifts assignments of signals were made by bidimensional total correlation spectroscopy (2D-TOCSY) and bidimensional rotational overhauser effect spectroscopy (2D-ROESY) NMR spectra. 2D-TOCSY spectra were collected using a Bruker AMX 400 spectrometer with 16, 36, 56 ms mixing time at *T* = 310 K. 2D-ROESY spectra were obtained using the same spectrometer and with 284 ms acquisition time at the same temperature. ESI mass spectra were obtained on a Micromass ZMD instrument operating in positive ion mode with the following experimental parameters: capillary voltage, 2800 V; cone voltage, 66 V; source temperature, 100°C; and desolvation temperature, 150°C.

Synthesis of LDO, D-LDO, and D-LDD

Anhydrous lactose (997.8 mg, 2.76 mmol) was dissolved in 22 mL of DMF/methanol (3/8 = v/v) under a nitrogen flow. Acetic acid (0.238 mL, 4.15 mmol) and 1,8-diaminooctane for LDO and D-LDO (158.7 mg, 1.10 mmol) or 1,12-diaminododecane for D-LDD (221.9 mg, 1.10 mmol) were then added and the reaction mixtures heated at 60°C under reflux for 1 h; the solvent was evaporated under reduced pressure and the residue dissolved in water. The glycoamine fraction was separated from starting lactose by ion-exchange chromatography on an Amberlite CG120 column. The column was eluted with water (200 mL) followed by 100 mL of 0.5 M NH₄OH. SLA compounds were purified by preparative HPLC on a C18 Spherisorb 10 OBS column that was eluted with 0.01 M NH₄⁺COO⁻/CH₃OH (95:5), pH 5.5 and were obtained as a slightly yellow solid by precipitation in cold acetone-water (9:1).

LDO, 45.2 mg, yield: 3.5% TLC: *R*_f = 0.18, ¹H NMR (300 MHz, D₂O): δ (ppm) 4.65 (d, 1H, *J* = 7.4 Hz, H-1''), 4.29 (s, 1H, H-5'), 4.23 (dd, 1H, *J* = 9.9, 3.2 Hz, H-4'), 4.12 (d, 1H, *J* = 12.2 Hz, H-6'_a), 4.03 (s, 1H, H-4''), 3.9–3.6 (m, 7H, H-3', H-6'_b, H-2'', H-3'', H-5', H-6'_{a,b}), 3.38 (s, 2H, H-1'_{a,b}), 3.1–2.7 (m, 4H, CH₂-1, and -8), 1.53 (s, 4H, CH₂-2, and -7), 1.41 (s, 8H, CH₂-3, -4, -5, -6). Electrospray mass spectrometry (ESI-MS): *m/z* = 469.2 (M + H⁺), 491.3 (M + Na⁺). Calculated for C₂₀H₄₀N₂O₁₀, 468.27.

D-LDO, 69.6 mg, yield: 8%, TLC: *R*_f = 0.22, ¹H NMR (400 MHz, D₂O): δ (ppm) 4.56 (d, 2H, *J* = 7.9 Hz, H-1''), 4.23 (s, 2H, H-5'), 4.13 (dd, 2H, *J* = 8.0, 2.8 Hz, H-4'), 4.02 (d, 2H, *J* = 12.0 Hz, H-6'_a), 3.93 (s, 2H, H-4''), 3.87 (d, 2H, *J* = 8.0 Hz, H-3'), 3.83 (s, 4H, H-6''_{a,b}), 3.81 (d, 2H, *J* = 12.0 Hz, H-6'_b), 3.70 (d, 2H, *J* = 4 Hz, H-5''), 3.67 (d, 2H, *J* = 3.2 Hz, H-3''), 3.61 (d, 2H, *J* = 7.6 Hz, H-2''), 3.35 (s, 4H, H-1'_{a,b}), 3.11 (s, 4H, CH₂-1 and -8), 1.78 (s, 4H, CH₂-2 and -7), 1.43 (s, 8H, CH₂-3, -4, -5, -6), ¹³C-NMR: (77.5 MHz, D₂O): δ (ppm) 105.6 (C-1''), 98.7 (C-2'), 80.5 (C-4'), 78.8 (C-3''), 74.1 (C-2''), 72.0 (C-4''), 71.9 (C-3'), 69.9 (C-5'), 66.9 (C-6'), 64.5 (C-6''), 56.1 (C-1'), 31.2 (C-1, 2, 7, 8), 28.8 (C-3, C-6), 28.1 (C-4, C-5). ESI-MS: *m/z* = 793.1 (M + H⁺), 814.9 (M + Na⁺). Calculated for C₃₂H₆₀N₂O₂₀, 792.37.

D-LDD, 59.6 mg, yield 6.5%, TLC: $R_f = 0.28$, $^1\text{H-NMR}$ (300 MHz, D_2O): δ (ppm) 4.63 (d, 2H, $J = 7.3$ Hz, H-1''), 4.30 (s, 2H, H-5'), 4.21 (dd, 2H, $J = 9.6, 2.7$ Hz, H-4'), 4.09 (d, 2H, $J = 12.0$ Hz, H-6'_a), 4.01 (s, 2H, H-4''), 3.80 (d, 2H, $J = 12.0$ Hz, H-6'_b), 3.75 (d, 2H, $J = 3.4$ Hz, H-3''), 3.40 (s, 4H, H1'_{a,b}), 3.19 (t, 4H, $J = 7.8$ Hz, CH_2 -1 and -12), 1.81 (s, 4H, CH_2 -2 and -11), 1.29 (s, 16H, CH_2 -3, -4, -5, -6, -7, -8, -9, -10). ESI-MS: $m/z = 849.2$ ($\text{M} + \text{H}^+$), 871.1 ($\text{M} + \text{Na}^+$). Calculated for $\text{C}_{36}\text{H}_{68}\text{N}_2\text{O}_{20}$, 848.44.

Cell cultures

H69 small cell lung carcinoma cells (H69-SCLC) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 25 mM HEPES. Previous to all the experiments, H69-SCLC cells were grown in SITA medium for 24 h (RPMI 1640 supplemented with 30 nM selenium, 5 $\mu\text{g}/\text{ml}$ insulin, 10 $\mu\text{g}/\text{ml}$ transferrin, and 0.25% [w/v] bovine serum albumin). Cell viability was routinely >90%, as judged by trypan blue exclusion. A375 human melanoma cells were cultured as previously described (Tinari *et al.*, 2001). HUVEC were isolated from human umbilical cord veins by collagenase digestion as described (Nangia-Makker *et al.*, 2000). The endothelial cells were grown on gelatin-coated dishes in medium 199 containing 10% fetal bovine serum (Gibco, Gaithersburg, MD), supplemented with 12 U/mL heparin and 50 $\mu\text{g}/\text{ml}$ bovine crude ECGF at 37°C under 5% CO_2 . HUVECs were used for experiments at passages two to five.

Preparation and purification of recombinant proteins

The plasmid pKK-233-2 with cDNA for human galectin-3 was a gift from Dr. F.-T. 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 polymerase chain reaction (PCR) from IMAGE clone 4722280 using primers 5'-GCCAGCCATGGCTTGTGGTC-3' and 5'-GGCAAGCTTTCAGTCAAAGGC-3'. The purified product was inserted into NcoI/HindIII-digested vector pKK-233-2. These plasmids were used to transform *Escherichia coli* (BL21), and the recombinant proteins were purified as described (Cho and Cummings, 1995). Recombinant 90K was produced and purified essentially as previously reported (Silvestri *et al.*, 1998).

Solid-phase binding assays

Binding assays were performed essentially as described (Tinari *et al.*, 2001). Briefly, microtiter plates were coated with the 90K (100 $\mu\text{l}/\text{well}$, 5 $\mu\text{g}/\text{ml}$) overnight at 4°C. After 2-h saturation in phosphate-buffered saline (PBS)-1% BSA-0.05% Tween 20 at room temperature, plates were incubated with recombinant galectin-1 (rGal-1) or rGal-3 (100 $\mu\text{l}/\text{well}$, 5 $\mu\text{g}/\text{ml}$) in the presence or absence of SLA (ranging from 1000 to 15.6 mM). After washing with PBS, 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) followed by peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) for galectin-1 (diluted 1:1000) or peroxidase-conjugated mouse anti-rat (Sigma) IgG for galectin-3 (diluted 1:10,000) at room temperature for 45 min.

Binding of galectins to 90K was detected using 3,3',5,5'-tetramethylbenzidine (100 $\mu\text{l}/\text{well}$) substrate (15 min in shaking) and 1 M H_2SO_4 (100 $\mu\text{l}/\text{well}$) to stop the reaction. Absorbance was determined using a microplate reader (450 nm; Bio-Rad Model 550, Bio-Rad, San Jose, CA), and values were converted to protein concentrations based on a standard curve. In all the experiments, lactose was used as control for binding assays. For each compound, the concentration required to inhibit by 50% the binding of galectins to 90K (IC₅₀) was calculated. The lactose relative potency of each compound was calculated as well.

Cell aggregation assays

Confluent A375 human melanoma cells were harvested using 0.02% ethylenediaminetetraacetic acid and single-cell suspensions (1×10^6 cells/mL in PBS) were incubated with 10 $\mu\text{g}/\text{ml}$ of recombinant 90K alone or in the presence of SLA (each at 0.25 mM). Aliquots containing 0.5 mL cell suspensions 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 mL of 10% paraformaldehyde. The number of single cells in suspension was counted, and the extent of the aggregation was calculated using the following equation: $1 - (N_t/N_c) \times 100$, where N_t (test) and N_c (control) represent the number of single cells in the presence or absence of SLA. To demonstrate the relevance of galectins in this process, in another set of experiments, we added anti-galectin-1 or anti-galectin-3 blocking antibodies previously described (Rubinstein *et al.*, 2004).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis

Western blot analysis of galectin-1 and galectin-3 expression was performed in HUVEC and H69-SCLC cells essentially as described (Rubinstein *et al.*, 2004). Briefly, cells were washed three times with PBS and lysed with 1% 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate (Sigma) in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4 (Tris-buffered saline), in the presence of a mixture of protease inhibitors (Sigma). Protein concentration of lysates was measured with the Micro BCA kit (Pierce, Rockford, IL). The same amount of proteins from different samples was loaded onto the gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ), and equal loading was confirmed by Ponceau S staining. Blocked PVDF membranes were incubated with a rabbit anti-galectin-1 (diluted 1:5000) or a rabbit anti-galectin-3 (diluted 1:3000) polyclonal antibodies. Bound antibodies were detected with peroxidase-labeled anti-rabbit immunoglobulin G (Bio-Rad) and chemiluminiscent detection with the enhanced chemiluminescence western blotting detection reagent (Amersham Biosciences). As controls, rGals-1 and -3 were run in parallel. No bands were observed in western blots incubated with normal rabbit sera.

Cell death assays

H69-SCLC cells were plated at a concentration of 1×10^5 cells/well in 48-well plates in SITA medium for 24 h and treated with different concentration of SLA in the absence or

presence of anti-galectin antibodies for further 24 h. After treatments, floating cells were collected and centrifuged at $1000 \times g$ for 5 min. Tumor cell death was evaluated using the FITC-annexin V binding assay (Immunotech, Marseille, France) according to the manufacturer's recommended protocol, and 10,000 events were acquired in a FACSCalibur flow cytometer. Apoptosis was confirmed by fluorescence microscopy by adding 1 mL of a mixture of ethidium bromide (100 $\mu\text{g}/\text{mL}$) and acridine orange (100 $\mu\text{g}/\text{mL}$) (1/1 v/v) to a 200- μL cell suspension.

In vitro capillary tube formation assays

The ability of SLA to inhibit angiogenesis *in vitro* (endothelial cell morphogenesis) was evaluated in a capillary tube formation assay using HUVEC cultured on a synthetic basement membrane matrix. Chamber slides (Gibco) were coated with growth factor-depleted Matrigel (Becton & Dickinson, Franklin Lakes, NJ) on ice. Matrigel was allowed to solidify for 30 min at 37°C. HUVEC (5×10^4 cells) resuspended in 100 μL of M199 medium containing 10% fetal bovine serum and 50 $\mu\text{g}/\text{mL}$ bovine ECGF were seeded onto each Matrigel-coated well. SLA were dissolved in PBS and added at a final concentration of 0.1 mM. To demonstrate the relevance of galectins in these processes, in another set of experiments, we added lactose (10 mM) or anti-galectin-1/ anti-galectin-3 blocking antibodies (Rubinstein *et al.*, 2004). After 18 h incubation at 37°C and 5% CO₂ humidified atmosphere, cultures were visualized. For each individual well, four photographs were taken from different locations within the well to cover the central area, and the tube network was quantitated and expressed as the percentage of tube formation per field. The final results were pooled from two separate experiments.

Statistical analysis

Comparison between groups was made by analysis of variance (ANOVA). Statistical analysis of apoptosis data was performed by using Student's *t*-test or one-way ANOVA using the Statview program for Macintosh. Only *p* values of <0.05 were considered as statistically significant.

Acknowledgments

The authors thank Marta A. Toscano for kind support. This work was supported by grants from Ministero della Salute, Ricerca Finalizzata 2002 and AIRC (to S.I.) and by grants from Mizutani Foundation for Glycoscience (Japan), The National Agency for Promotion of Science and Technology (PICT 2003-05-13787, Argentina), University of Buenos Aires (M091), Sales Foundation (Argentina) and Antorchas Foundation, Argentina (to G.A.R.). G.A.R. is an independent researcher of CONICET.

Abbreviations

2D-ROESY, bidimensional rotational overhauser effect spectroscopy; 2D-TOCSY, bidimensional total correlation spectroscopy; CRD, carbohydrate-recognition domains; D-LDD, *N,N'*-dilactulose-dodecamethylenediamine; D-LDO,

N,N'-dilactulose-octamethylenediamine; ECGF, endothelial cell growth factor; ESI-MS, electrospray mass spectrometry; HUVEC, human umbilical vein endothelial cells; LDO, *N*-lactulose-octamethylenediamine; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; rGal, recombinant galectin; SLA, synthetic lactulose amines; TLC, thin-layer chromatography.

References

- Ahmad, N., Gabius, H.-J., Sabesan, S., Oscarson, S., and Brewer, C.F. (2004) Thermodynamic binding studies of bivalent oligosaccharides to galectin-1 and galectin-3, and the carbohydrate recognition domain of galectin-3. *Glycobiology*, **14**, 817–825.
- Ahmed, H. and Vasta, G.R. (1994) Galectins: conservation of functionally and structurally relevant amino acid residues defines two types of carbohydrate recognition domains. *Glycobiology*, **4**, 545–548.
- Akahani, S., Nangia-Makker, P., Inohara, H., Kim, H.R., and Raz, A. (1997) Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res.*, **57**, 5272–5276.
- Andre, S., Pieters, R.J., Vrasidas, I., Kaltner, H., Kuwabara, I., Liu, F.-T., Liskamp, R.M., and Gabius, H.-J. (2001) Wedgelike glycodendrimers as inhibitors of binding of mammalian galectins to glycoproteins, lactose, maiclusters, and cell surface glycoconjugates. *ChemBiochem*, **2**, 822–830.
- Bresalier, R.S., Mazurek, N., Sternberg, L.R., Byrd, J.C., Yunker, C.K., Nangia-Makker, P., and Raz, A. (1998) Metastasis of human colon cancer is altered by modifying expression of the β -galactoside-binding protein galectin-3. *Gastroenterology*, **115**, 287–296.
- Califice, S., Castronovo, V., Bracke, M., and van den Br ule, F. (2004) Dual activities of galectin-3 in human prostate cancer: tumor suppression of nuclear galectin-3 vs tumor promotion of cytoplasmic galectin-3. *Oncogene*, **23**, 7527–7536.
- Camby, I., Belot, N., Lefranc, F., Sadeghi, N., de Launoit, Y., Kaltner, H., Musette, S., Darro, F., Danguy, A., Salmon, I., Gabius, H.J., and Kiss, R. (2002) Galectin-1 modulates human glioblastoma cell migration into the brain through modifications to the actin cytoskeleton and levels of expression of small GTPases. *J. Neuropathol. Exp. Neurol.*, **61**, 585–596.
- Cho, M. and Cummings, R.D. (1995) Galectin-1, a β -galactoside-binding lectin in Chinese hamster ovary cells. I. Physical and chemical characterization. *J. Biol. Chem.*, **270**, 5198–5206.
- Clausse, N., van den Br ule, F., Waltregny, D., Garnier, F., and Castronovo, V. (1999) Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell-cell adhesion. *Angiogenesis*, **3**, 317–325.
- Cooper, D.N. and Barondes, S.H. (1999) God must love galectins: he made so many of them. *Glycobiology*, **9**, 979–984.
- Cumpstey, I., Sundin, A., Leffler, H., and Nilsson, U.J. (2005) C2-symmetrical thiodigalactoside bis-benzamido derivatives as high-affinity inhibitors of galectin-3: efficient lectin inhibition through double arginine-arene interactions. *Angew. Chem. Int. Ed. Engl.*, **44**, 5110–5112.
- Danguy, A., Camby, I., and Kiss, R. (2002) Galectins and cancer. *Biochim. Biophys. Acta*, **1572**, 285–293.
- Ellerhorst, J., Nguyen, T., Cooper, D.N., Lotan, D., and Lotan, R. (1999) Differential expression of endogenous galectin-1 and galectin-3 in human prostate cancer cell lines and effects of overexpressing galectin-1 on cell phenotype. *Int. J. Oncol.*, **14**, 217–224.
- Endharti, A.T., Zhou, Y.W., Nakashima, I., and Suzuki, H. (2005) Galectin-1 supports survival of naive T cells without promoting cell proliferation. *Eur. J. Immunol.*, **35**, 86–97.
- Fuertes, M.B., Molinero, L.L., Toscano, M.A., Ilarregui, J.M., Rubinstein, N., Fainboim, L., Zwirner, N., and Rabinovich, G.A. (2004) Regulated expression of galectin-1 during T-cell activation involves Lck and Fyn kinases and signaling through MEK1/ERK, p38 MAP kinase and p70S6 kinase. *Mol. Cell. Biochem.*, **267**, 177–185.
- Glinksy, G.V., Price, J.E., Glinksy, V.V., Mossine, V.V., Kiriakova, G., and Metcalf, J.B. (1996) Inhibition of human breast cancer metastasis in nude mice by synthetic glycoamines. *Cancer Res.*, **56**, 5319–5324.

- Glinsky, V.V., Glinsky, G.V., Glinskii, O.V., Huxley, V.H., Turk, J.R., Mossine, V.V., Deutscher, S.L., Pienta, K.J., and Quinn, T.P. (2003) Intravascular metastatic cancer cell homotypic aggregation at the sites of primary attachment to the endothelium. *Cancer Res.*, **63**, 3805–3811.
- Greco, C., Vona, R., Cosimelli, M., Matarrese, P., Straface, E., Scordati, P., Giannarelli, D., Casale, V., Assisi, D., Mottolise, M., and others. (2004) Cell surface overexpression of galectin-3 and the presence of its ligand 90K in the blood plasma as determinants in colon neoplastic lesions. *Glycobiology*, **14**, 783–792.
- Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W.E., and others. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim. Biophys. Acta*, **1572**, 232–254.
- Hittelet, A., Legendre, H., Nagy, N., Bronckart, Y., Pector, J.C., Salmon, I., Yeaton, P., Gabius, H.J., Kiss, R., and Camby, I. (2003) Upregulation of galectins-1 and -3 in human colon cancer and their role in regulating cell migration. *Int. J. Cancer*, **103**, 370–379.
- Honjo, Y., Nangia-Makker, P., Inohara, H., and Raz, A. (2001) Down-regulation of galectin-3 suppresses tumorigenicity of human breast carcinoma cells. *Clin. Cancer Res.*, **7**, 661–668.
- Hoyer, K.K., Pang, M., Gui, D., Shintaku, I.P., Kuwabara, I., Liu, F.-T., Said, J.W., Baum, L.G., and Teittel, M.A. (2004) An anti-apoptotic role for galectin-3 in diffuse large B-cell lymphomas. *Am. J. Pathol.*, **164**, 893–902.
- Inufusa, H., Nakamura, M., Adachi, T., Aga, M., Kurimoto, M., Nakatani, Y., Wakano, T., Miyake, M., Okuno, K., Shiozaki, H., and Yasutomi, M. (2001) Role of galectin-3 in adenocarcinoma liver metastasis. *Int. J. Oncol.*, **19**, 913–919.
- John, C.M., Leffler, H., Kahl-Knutsson, B., Svensson, I., and Jarvis, G.A. (2003) Truncated galectin-3 inhibits tumor growth and metastasis in orthopic nude mouse model of human breast cancer. *Clin. Cancer Res.*, **9**, 2374–2383.
- Joo, H.G., Goedegebuure, P.S., Sadanaga, N., Nagoshi, M., von Bernstorff, W., and Eberlein, T.J. (2001) Expression and function of galectin-3, a β -galactoside-binding protein in activated T lymphocytes. *J. Leukoc. Biol.*, **69**, 555–564.
- Kannagi, R., Izawa, M., Koike, T., Miyazaki, K., and Kimura, N. (2004) Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. *Cancer Sci.*, **95**, 377–384.
- Kim, H.R., Lin, H.M., Biliran, H., and Raz, A. (1999) Cell cycle arrest and inhibition of anoikis by galectin-3 in human breast epithelial cells. *Cancer Res.*, **59**, 4148–4154.
- Kopitz, J., von Reizenstein, C., Andre, S., Kaltner, H., Uhl, J., Ehemann, V., Cantz, M., and Gabius, H.-J. (2001) Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3. *J. Biol. Chem.*, **276**, 35917–35923.
- Lahm, H., Andre, S., Hoeflich, A., Kaltner, H., Siebert, H.C., Sordat, B., von der Lieth, C.W., Wolf, E., and Gabius, H.-J. (2004) Tumor galectinology: insights into the complex network of a family of endogenous lectins. *Glycoconj. J.*, **20**, 227–238.
- Le, Q.T., Shi, G., Cao, H., Nelson, D.W., Wang, Y., Chen, E.Y., Zhao, S., Kong, C., Richardson, D., O'Byrne, K.J., and others. (2005) Galectin-1: a link between tumor hypoxia and tumor immune privilege. *J. Clin. Oncol.*, **23**. Epub ahead of print.
- Leffler, H., Carlsson, S., Hedlund, M., Qian, Y., and Poirier, F. (2004) Introduction to galectins. *Glycoconj. J.*, **19**, 433–440.
- LeMarer, N. and Hughes, R.C. (1996) Effects of the carbohydrate-binding protein galectin-3 on the invasiveness of human breast carcinoma cells. *J. Cell. Physiol.*, **168**, 51–58.
- Levy, Y., Arbel-Goren, R., Hadari, Y.R., Eshhar, S., Ronen, D., Elhanany, E., Geiger, B., and Zick, Y. (2001) Galectin-8 functions as a matricellular modulator of cell adhesion. *J. Biol. Chem.*, **276**, 31285–31295.
- Liu, F.-T. and Rabinovich, G.A. (2005) Galectins as modulators of tumour progression. *Nat. Rev. Cancer*, **5**, 29–41.
- Lu, Y., Lotan, D., and Lotan, R. (2000) Differential regulation of constitutive and retinoic acid-induced galectin-1 gene transcription in murine embryonal carcinoma and myoblastic cells. *Biochim. Biophys. Acta*, **1491**, 13–19.
- Matarrese, P., Fusco, O., Tinari, N., Natoli, C., Liu, F.-T., Semeraro, M.L., Malorni, W., and Iacobelli, S. (2000) Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. *Int. J. Cancer*, **85**, 545–554.
- Matarrese, P., Tinari, N., Mormone, E., Bianco, G.A., Toscano, M.A., Ascione, B., Rabinovich, G.A., and Malorni, W. (2005) Galectin-1 sensitizes resting human T lymphocytes to Fas (CD95)-mediated cell death via mitochondrial hyperpolarization, budding, and fission. *J. Biol. Chem.*, **280**, 6969–6985.
- Nangia-Makker, P., Honjo, Y., Sarvis, R., Akahani, S., Hogan, V., Pienta, K.J., and Raz, A. (2000) Galectin-3 induces endothelial cell morphogenesis and angiogenesis. *Am. J. Pathol.*, **156**, 899–909.
- Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Breasler, R., and Raz, A. (2002) Inhibition of human cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin. *J. Natl. Cancer Inst.*, **94**, 854–1862.
- Pacis, R.A., Pilat, M.J., Pienta, K.J., Wojno, K., Raz, A., Hogan, V., and Cooper, C.R. (2000) Decreased galectin-3 expression in prostate cancer. *Prostate*, **44**, 118–123.
- Paz, A., Haklai, R., Elad-Sfadia, G., Ballan, E., and Kloog, Y. (2001) Galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation. *Oncogene*, **20**, 7486–7493.
- Perillo, N.L., Pace, K.E., Seilhamer, J.J., and Baum, L.G. (1995) Apoptosis of T cells mediated by galectin-1. *Nature*, **378**, 736–739.
- Rabinovich, G.A. (2005) Galectin-1 as a potential cancer target. *Br. J. Cancer*, **92**, 1188–1192.
- Rabinovich, G.A., Baum, L.G., Tinari, N., Paganelli, R., Natoli, C., Liu, F.-T., and Iacobelli, S. (2002a) Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol.*, **23**, 313–320.
- Rabinovich, G.A., Ramhorst, R.E., Rubinstein, N., Corigliano, A., Daroqui, M.C., Kier-Joffe, E.B., and Fainboim, L. (2002b) Induction of allogenic T-cell hyporesponsiveness by galectin-1-mediated apoptotic and non-apoptotic mechanisms. *Cell Death Differ.*, **9**, 661–670.
- Rabinovich, G.A., Rubinstein, N., Matar, P., Rozados, V., Gervasoni, S., and Scharovsky, G.O. (2002c) The antimetastatic effect of a single low dose of cyclophosphamide involves modulation of galectin-1 and Bcl-2 expression. *Cancer Immunol. Immunother.*, **50**, 597–603.
- Rabinovich, G.A., Toscano, M.A., Ibarregui, J.M., and Rubinstein, N. (2004) Shedding light on the immunomodulatory properties of galectins: novel regulators of innate and adaptive immune responses. *Glycoconj. J.*, **19**, 565–573.
- Rubinstein, N., Alvarez, M., Zwirner, N.W., Toscano, M.A., Ibarregui, J.M., Bravo, A., Mordoh, J., Fainboim, L., Podhajcer, O.L., and Rabinovich, G.A. (2004) Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T-cell-mediated rejection: a potential mechanism of tumor-immune privilege. *Cancer Cell*, **5**, 241–251.
- Salameh, B.A., Leffler, H., and Nilsson, U.J. (2005) 3-(1,2,3-Triazol-1-yl)-1-thio-galactosides as small, efficient and hydrolytically stable inhibitors of galectin-3. *Bioorg. Med. Chem. Lett.*, **15**, 3344–3346.
- Silvestri, B., Calderazzo, F., Coppola, V., Rosato, A., Iacobelli, S., Natoli, C., Ullrich, A., Sures, I., Azam, M., Brakebush, C., and others. (1998) Differential effect on TCR:CD3 stimulation of a 90-kD glycoprotein (gp90/Mac-2BP), a member of the scavenger receptor cysteine-rich domain protein family. *Clin. Exp. Immunol.*, **113**, 394–400.
- Song, Y.K., Billiar, T.R., and Lee, Y.J. (2002) Role of galectin-3 in breast cancer metastasis: involvement of nitric oxide. *Am. J. Pathol.*, **160**, 1069–1075.
- Sorme, P., Qian, Y., Nyholm, P.G., Leffler, H., and Nilsson, U.J. (2002) Low micromolar inhibitors of galectin-3 based on 3'-derivatization of N-acetyllactosamine. *Chembiochem*, **3**, 183–189.
- Sorme, P., Kahl-Knutsson, B., Wellmar, U., Magnusson, B.G., Leffler, H., and Nilsson, U.J. (2003) Design and synthesis of galectin inhibitors. *Methods Enzymol.*, **363**, 157–169.
- Stowell, S.R., Dias-Baruffi, M., Penttilä, L., Renkonen, O., Nyame, A.K., and Cummings, R.D. (2004) Human galectin-1 recognition of poly-N-acetyllactosamine and chimeric polysaccharides. *Glycobiology*, **14**, 157–167.
- Takenaka, Y., Fukumori, T., and Raz, A. (2004) Galectin-3 and metastasis. *Glycoconj. J.*, **19**, 543–549.
- Tejler, J., Leffler, H., and Nilsson, U.J. (2005) Synthesis of O-galactosyl aldoximes as potent LacNAc-mimetic galectin-3 inhibitors. *Bioorg. Med. Chem. Lett.*, **15**, 2343–2345.
- Tinari, N., Kuwabara, I., Hufflejt, M.E., Shen, P.F., Iacobelli, S., and Liu, F.-T. (2001) Glycoprotein 90K/MAC-2BP interacts with galectin-1 and mediates galectin-1-induced cell aggregation. *Int. J. Cancer*, **91**, 167–172.

- Ueda, S., Kuwabara, I., and Liu, F.-T. (2004) Suppression of tumor growth by galectin-7 gene transfer. *Cancer Res.*, **64**, 5672–5676.
- van den Brule, F., Califice, S., and Castronovo, V. (2004) Expression of galectins in cancer: a critical review. *Glycoconj. J.*, **19**, 537–542.
- van den Brule, F., Califice, S., Garnier, F., Fernandez, P.L., Berchuk, A., and Castronovo, V. (2003) Galectin-1 accumulation in the ovary carcinoma peritumoral stroma is induced by ovary carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and fibronectin. *Lab. Invest.*, **83**, 377–386.
- Wells, V., Davies, D., and Mallucci, L. (1999) Cell cycle arrest and induction of apoptosis by β -galactoside-binding protein (β -GBP) in human mammary cancer cells. *Eur. J. Cancer*, **35**, 978–983.
- Yamaoka, K., Mishima, K., Nagashima, Y., Asai, A., Sanai, Y., and Kirino, T. (2000) Expression of galectin-1 mRNA correlates with the malignant potential of human gliomas and expression of antisense galectin-1 inhibits the growth of 9 glioma cells. *J. Neurosci. Res.*, **59**, 722–730.
- Yang, R.Y., Hsu, D.K., and Liu, F.-T. (1996) Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc. Natl. Acad. Sci. U. S. A.*, **93**, 6737–6742.
- Zou, J., Glinsky, V.V., Landon, L.A., Matthews, L., and Deutscher, S.L. (2005) Peptides specific to the galectin-3 carbohydrate recognition domain inhibit metastasis-associated cancer cell adhesion. *Carcinogenesis*, **26**, 309–318.