

# Truncated Galectin-3 Inhibits Tumor Growth and Metastasis in Orthotopic Nude Mouse Model of Human Breast Cancer<sup>1</sup>

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## ABSTRACT

**Purpose:** The goal of this research was to evaluate a potential therapeutic agent for breast cancer based on galectin-3 that has been implicated in tumorigenicity and metastasis of breast cancer. The hypothesis was that therapy with NH<sub>2</sub>-terminally truncated form of galectin-3 (galectin-3C) will be efficacious for reduction in tumor growth and for inhibition of metastases.

**Experimental Design:** Recombinant human galectin-3 was produced in *Escherichia coli* from which galectin-3C was derived by collagenase enzyme digestion. Toxicity, pharmacokinetic, and organ biodistribution studies were performed in nude mice. For efficacy studies, nude mice bearing orthotopically implanted tumors derived from breast cancer cell line MDA-MB-435 were treated with galectin-3C or a vehicle control i.m. twice daily for 90 days.

**Results:** The maximum tolerated dose of galectin-3C in nude mice was determined to be >125 mg/kg without overt adverse effects. The elimination half-life when administered i.m. was found to be 3.0 h in the serum and 4.3 h in the cellular fraction of the blood. Organ biodistribution studies revealed that galectin-3C localized in the liver, kidneys, and spleen but not in the heart or lungs. We found that the mean tumor volumes and weights were statistically significantly less in mice treated with galectin-3C compared with control mice, and that fewer numbers of mice exhibited lymph node metastases in the treated group compared with the control group.

**Conclusions:** Galectin-3C is not overtly toxic, and is efficacious in reducing metastases and tumor volumes and weights in primary tumors in an orthotopic nude mouse model of human breast cancer.

## INTRODUCTION

Breast cancer is the cancer diagnosed most frequently in women in the United States other than nonmelanoma skin cancer, which has a much lower mortality rate (1–3). Despite the available treatments breast cancer ranks second for women as the cause of death from cancer, and the lifetime risk of breast cancer for women living in the United States is now 1 in 8. Among those factors predictive of risk of recurrence of breast cancer—age, tumor size, hormone receptor status, and histological type and grade—metastatic carcinoma in axillary lymph nodes is more important than any other currently available (4, 5). Metastatic breast cancer has a poor prognosis, especially if the tumor is hormone-independent and is the primary cause of mortality in individuals already affected. Fewer than 10% of patients with metastatic breast cancer survive  $\geq 5$  years (6).

Galectin-3 is a member of a family of mammalian lectins classified together because of the sequence homology within their carbohydrate recognition domains and a characteristic affinity for  $\beta$ -galactoside sugars (7). An early observation in the study of galectins and cancer was that various types of tumor cells express galectins on their surface (8). Experimental evidence also suggested that these galectins could be cross-linked by exogenous glycoprotein ligands resulting in the homotypic aggregation of tumor cells (8). In 1990, Raz *et al.* (9) provided the first direct evidence that galectin-3 expression is associated with the transformation and metastasis of tumor cells *in vivo*. They reported that expression of recombinant galectin-3 in weakly metastatic fibrosarcoma cells resulted in an increased incidence of experimental lung metastases in syngeneic and nude mice. Since that time, mounting evidence has implicated galectin-3 in the growth, differentiation, adhesion, RNA processing, apoptosis, and malignant transformation of tumor cells (10–15).

The process of tumor metastasis requires that cells leave the primary tumor, invade the basement membrane, transverse the bloodstream, interact with a target organ or site, and proliferate to form secondary tumors (16, 17). Interactions of cells with the extracellular matrix and other cells are integral in this process. Recent findings demonstrate that hematogenous cancer metastases originate from intravascular growth of endothelium-attached rather than extravasated cancer cells, highlighting the key role of tumor-endothelial cell interactions in cancer metastases (18). The report that this critical interaction may be mediated by galectin-3 provides a feasible rationale for the pursuit of antiadhesive cancer therapeutics (19).

In this study, we tested the hypothesis that treatment with

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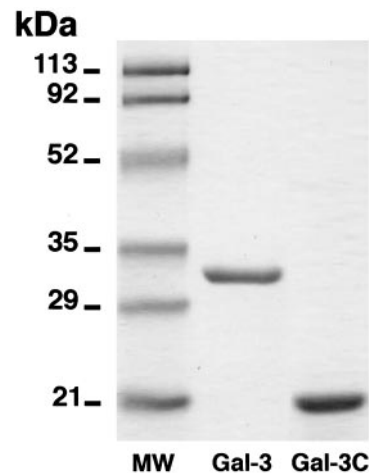
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galectin-3C<sup>3</sup> will be efficacious for breast cancer by inhibition of tumorigenicity, tumor invasion, and metastasis. Galectin-3 contains a single carbohydrate recognition domain located in the COOH terminus and a noncarbohydrate binding NH<sub>2</sub>-terminal domain. The NH<sub>2</sub>-terminal domain promotes the formation of dimers or higher order oligomers of galectin-3, and enables it to cross-link select carbohydrate-containing ligands on cell surfaces and in the extracellular matrix, and thus to modulate cell adhesion and signaling (20, 21). Soluble recombinant galectin-3C retains the ability to bind carbohydrates and competes with endogenous galectin-3 for carbohydrate binding sites. Excess administered galectin-3C that lacks the ability to cross-link carbohydrate-containing ligands should act as a dominant-negative inhibitor of the activity of galectin-3 in promoting cell adhesion and cell signaling. We evaluated the safety, plasma half-life, and efficacy of galectin-3C in an orthotopic implantation model in which transplanted human breast tumors mimic the clinical course of the disease in recipient immunodeficient mice. Our results provide compelling evidence that treatment with galectin-3C could be useful in the treatment and prevention of metastatic breast cancer.

## MATERIALS AND METHODS

**Production of Galectin-3C.** Intact recombinant galectin-3 was produced in *Escherichia coli* B121/DE3 containing the pET3c plasmid with the human galectin-3 coding DNA. The organisms were lysed by sonication and the galectin-3 protein purified by affinity chromatography on lactosyl-Sepharose (20). The purified galectin-3 was dialyzed to remove lactose and exhaustively digested with *Clostridium histolyticum* collagenase type VII (Sigma, St. Louis, MO), and the resulting galectin-3C purified again on lactosyl-Sepharose (21). Purified galectin-3C, corresponding to amino acids 108–250 containing the carbohydrate recognition domain, was dialyzed against water, lyophilized, and 0.5 mg of lactose added per mg of protein. Approximately 5 µg each of galectin-3 and galectin-3C were analyzed by SDS-PAGE on a precast 12% polyacrylamide gel (BioRAD, Hercules, CA) and stained with EZBlue, a Coomassie blue protein stain (Sigma-Aldrich, St. Louis, MO) that enables detection of as little as 5 ng of protein (see Fig. 1). The X-ray crystal structure of galectin-3C in complex with lactose and *N*-acetyllactosamine was determined previously (22).

To produce <sup>35</sup>S-labeled galectin-3C for pharmacokinetic studies, the pET3c plasmid with the human galectin-3 coding DNA was transfected into *Escherichia coli* B834 (Novagen, Madison, WI), which is a methionine auxotroph derived from BL21/DE3. The *E. coli* were adapted for growth on M9 minimal medium supplemented with 50 µg/ml ampicillin and 40 µg/ml methionine (M9-Met) by passage on M9-Met plates three times. To produce <sup>35</sup>S galectin-3, a colony from the last plate was inoculated into 0.5 liters of M9-Met broth supplemented with 1.0 mCi <sup>35</sup>S-Met. The bacteria were cultured, induced with isopropyl thiogalactoside, and harvested as described previously



**Fig. 1** Purified recombinant galectin-3 and galectin-3C analyzed by SDS-PAGE with 12% polyacrylamide. Exhaustive collagenase digestion of galectin-3 was performed to produce galectin-3C, which begins at amino acid Gly<sup>108</sup>. Both proteins exhibited a purity level of >99% based on the sensitivity level of the Coomassie stain used.

(20). For bacterial lysis, 5 ml of 25% sucrose in 50 mM Tris HCl (pH 8.0) with 50 mM NaCl, 20 mM EDTA, and 8 mg lysozyme were added to the pellet. After 10 min on ice, 16 ml of water was added and the sample kept on ice another 30 min. The sample was centrifuged at 12,000 rpm for 30 min and the supernatant applied to lactosyl-Sepharose. The <sup>35</sup>S galectin-3 was eluted, dialyzed and treated with collagenase to generate <sup>35</sup>S galectin-3C as described above.

**Animal Studies.** All of the animal experiments including the MTD study, assessment of metastasis, and tumor volume measurements were performed at AntiCancer, Inc. (San Diego, CA), under the direction of Dr. Robert M. Hoffman. Female athymic CD-1 nude mice between 4 and 5 weeks of age were bred and maintained in a HEPA-filtered environment with cages, food, and bedding sterilized by autoclaving. The breeding pairs were obtained from the Charles River Laboratories (Wilmington, MA). The animal diets were obtained from Harlan Teklad (Madison, WI). Ampicillin (Sigma) at a concentration of 5% (v/v) was added to the autoclaved drinking water.

**Study of MTD.** The study comprised four dose groups with each group consisting of 5 mice. The s.c. doses were 1 mg/kg, 5 mg/kg, 25 mg/kg, and 125 mg/kg administered as a single bolus. In addition, a vehicle-treated control group consisting of 5 mice was evaluated. Animals were observed for 5 days total after injection at which time body weight and viability were determined.

**Pharmacokinetic and Biodistribution Analyses.** Analyses of the pharmacokinetic and biodistribution characteristics of galectin-3C were determined for s.c., i.v., and i.m. routes of administration of galectin-3C into nude mice. Groups of 3 or 5 mice (~0.025 kg/mouse) were each injected with 150 µg/mouse (1 mg/ml; 6 mg/kg) of <sup>35</sup>S-labeled galectin-3C and unlabeled galectin-3C that were mixed together to achieve a specific activity of approximately 25–35 cpm/µg. For the i.m. route, the animals were sacrificed and blood samples were obtained by

<sup>3</sup>The abbreviations used are: galectin-3C NH<sub>2</sub>-terminally truncated galectin-3; MTD, maximum tolerated dose; GFP, green fluorescent protein.

terminal cardiac puncture at four time points: 2, 4, 8, and 12 h after injection. In addition, blood samples were obtained from one control group of 5 animals 1 h after injection of vehicle only (1 mg/ml lactose in PBS). Serum samples from 200 or 300  $\mu$ l of blood were analyzed for radioactivity in triplicate. The mean of the radioactivity detected in the samples from the vehicle control group was subtracted from all of the samples.

For a direct comparison of i.v. *versus* i.m. administration of galectin-3C, two groups of 3 mice each were injected with the mixture of  $^{35}$ S-labeled galectin-3C and unlabeled galectin-3C either i.v. or i.m. At 8 h after injection, serum and blood cell samples from 200  $\mu$ l of blood were analyzed for radioactivity in triplicate. In addition, the organ biodistribution of galectin-3C was determined in mice injected s.c. and i.v. with the mixture of  $^{35}$ S-labeled galectin-3C and unlabeled galectin-3C. At 8 h after injection, the heart, lungs, liver, kidneys, and spleen were removed, and the amount of radioactivity was measured.

The following formula based on the exponential decay law was used to calculate the amount of radioactivity present in the galectin-3C at the time of injection based on the specific activity previously determined per  $\mu$ g ( $\ln N/N_0 = -\lambda t$ ; Ref. 23). In this formula  $N$  is the number of undecomposed atoms at elapsed time  $t$ ,  $N_0$  is number of atoms originally present (when  $t = 0$ ), and  $\lambda$  is the decay constant. The half-life ( $T_{1/2}$ ) of  $^{35}$ S that is 87.2 days was used to characterize the rate of decay ( $\lambda = 0.693/T_{1/2}$ ).

**Establishment of Orthotopic Implant Mouse Model of Human Breast Cancer.** Breast cancer cell line MDA-MB-435, which expresses galectin-3 (24, 25), was transfected with a plasmid expressing GFP as described previously (26), and cells were injected into the subcutis of nude mice to form solid tumors (27). Anesthetized test animals were transplanted by surgical orthotopic implantation using fragments harvested from the s.c. growing tumors. An incision  $\sim$ 0.5 cm long was made on the second right mammary gland, and two fragments of 1 mm<sup>3</sup> of MDA-MB-435-GFP tumor tissue were sutured into the fat pad of the gland with a sterile nylon 8–0 surgical suture. All of the surgical and animal manipulations and procedures were conducted under HEPA-filtered laminar flow hoods.

**Treatment with Galectin-3C.** The orthotopically transplanted animals used for the study were selected to establish groups of similar mean tumor size and body weight. Groups for each of the cohort conditions were randomly chosen. Administration of the treatments was begun when tumors reached palpable sizes. The dosing schedule for the vehicle control and galectin-3C groups was i.m. injection twice a day of 125  $\mu$ g (5 mg/kg) with an approximately 6–8-h interval for 90 days. Galectin-3C was injected as a solution of concentration 1 mg/ml in PBS containing 1 mg/ml lactose, and the vehicle control was PBS containing 1 mg/ml lactose. The addition of lactose to the solution increased the stability over time of the carbohydrate recognition domain of solubilized galectin-3C.

**Analysis of Mice.** The primary tumors were measured with a pair of calipers once a week after initiation of treatment through the end of the study, as well as by computer imaging of the relative diameters of the fluorescent tumors. Parameters determined at the end of 90 days were volume of primary tumor based on caliper measurements and calculated as ( $w^2 \times \text{length}/2$ ), number of metastases based on tumor histology, and animal

weight. Whole body imaging of the GFP expression by the MDA-MB-435 was carried out in a light box illuminated by blue-light fiber optics (Lighttools Research, Encinitas, CA) and imaged using a thermoelectrically cooled C5810 3-chip Hamamatsu CCD camera (Hamamatsu Photonic Systems, Bridgewater, NJ) as described previously (26).

**Assessment of Metastasis.** At autopsy, tissue samples from the axillary lymph node, the liver, and the lungs were collected, fixed in 10% formalin, embedded in paraffin, and sectioned, and then processed through standard procedures of H&E staining for subsequent microscopic examination.

**Statistical Methods Used in Efficacy Evaluation.** Animal body weight comparisons were performed by Student's  $t$  test. The primary tumor volume and weight were evaluated by the Mann-Whitney  $t$  test. The incidence of metastasis in the lymph node, the lung, and the liver were evaluated by the  $\chi^2$ /Fisher-exact test. All of the tests were two-sided with  $\alpha = 0.05$ . Also, to conduct a more sensitive analysis capable of describing the trajectory through time and to factor out clustering because of cage and animal to animal variation, tumor volume growth over time was analyzed using a repeated measures statistical model for log tumor volume. Specifically, SAS Proc Mixed was used to fit a linear mixed model with random effects for cage, and mouse-specific slopes and intercepts. The fitted model was quadratic in treatment day (28).

## RESULTS

### Toxicity Analyses

To determine the MTD, five groups of mice (4 in each) were injected s.c. with 1, 5, 25, or 125 mg/kg of galectin-3C, or with vehicle only. In the MTD study, animals were observed for 5 days total after injection with galectin-3C or vehicle only after which the body weight and viability were determined. The mean body weights for the vehicle-treated control and the galectin-3C-treated groups remained statistically identical ( $P > 0.10$ ;  $t$  test) for the five groups at 5 days of observation indicating that none of the doses of galectin-3C that we tested affected the normal physiological growth of the mice. No overt abnormal signs were observed. Hence, we concluded that galectin-3C can be injected into nude mice at a dose as high as 125 mg/kg without acute toxic effects. In addition, the lack of chronic toxicity was evidenced by the fact that all of the animals survived the 90-day course of treatment in the efficacy study described below, and the tumor-free mean body weight changes of the test and control groups were statistically identical ( $P = 0.303$ ).

### Pharmacokinetic and Biodistribution Analyses

We produced  $^{35}$ S-labeled galectin-3C for analysis of pharmacokinetic and biodistribution characteristics of i.v. and i.m. administration in nude mice. For i.m. administration of galectin-3C  $T_{1/2} = 3.0$  h (Fig. 2A), and for the portion of the dose bound to the cellular fraction of the blood the  $T_{1/2} = 4.3$  h (Fig. 2B). We also compared the 8-h distribution of galectin-3C into serum *versus* blood cell compartments for galectin-3C-administered i.v. and i.m. (Fig. 2C). For i.v. administration, the quantity of galectin-3C associated with blood cells was greater than that in serum at 8 h ( $P = 0.033$ ;  $t$  test). For i.m., there was

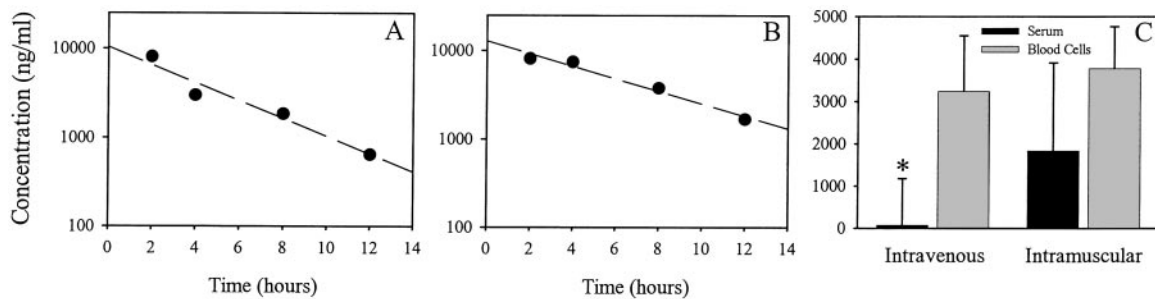


Fig. 2 Pharmacokinetic analysis of the i.m. administration of galectin-3C determined at 2–12 h. Mice were injected with  $^{35}\text{S}$ -labeled galectin-3C, and at the indicated time points the animals were sacrificed, and serum (A) and blood cell (B) levels of radioactivity were determined. Data are presented as the mean of the ng/ml detected in 3 mice at each time point. The correlation coefficients ( $r^2$ ) for the linear regression lines shown in A and B are 0.95 and 0.97, respectively. Pharmacokinetic analysis of the i.v. and i.m. administration of galectin-3C are presented as the mean of the ng/ml at 8 h (C); bars,  $\pm$ SD. \*,  $P = 0.033$  when compared with ng/ml associated with blood cells ( $t$  test).

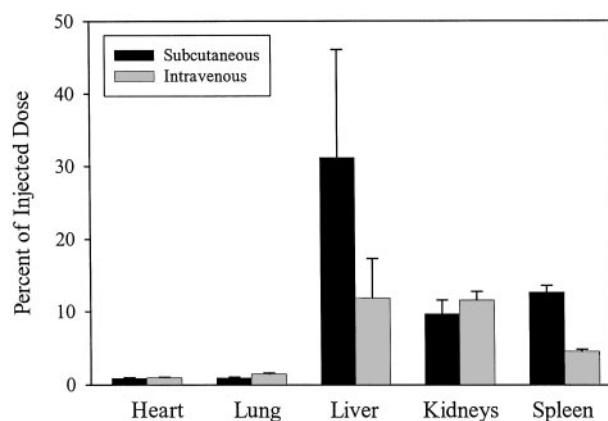


Fig. 3 Organ biodistribution analysis of the s.c. and i.v. administration of galectin-3C. Mice were injected with  $^{35}\text{S}$ -labeled galectin-3C, and at 8 h after injection the animals were sacrificed, and the heart, lungs, liver, kidneys, and spleen were removed, and the amount of associated radioactivity was determined. Background radioactivity was determined by measuring radioactive counts associated with organs from mice injected with vehicle only. Background counts for each organ were subtracted from the presented data. Each sample was analyzed in triplicate and the data are given as the mean of the percentage of injected dose detected in the organ; bars,  $\pm$ SD.

not a statistically significant difference in the amount of galectin-3C localized to either compartment.

We next investigated the organ biodistribution of galectin-3C. Two groups of 5 mice were injected either s.c. or i.v. with the mixture of  $^{35}\text{S}$ -labeled galectin-3C and unlabeled galectin-3C, and a third group of 5 mice was injected i.v. with lactose in PBS vehicle. At 8 h after injection, the heart, lungs, liver, kidneys, and spleen were removed, and the amount of radioactivity was measured. As shown in Fig. 3, the liver, kidney, and spleen were sources of galectin-3C-associated radioactivity above the background vehicle level, whereas neither the heart nor lungs localized any galectin-3C. These data suggest that in addition to serum and blood cells, certain organs may localize galectin-3C and serve as reservoirs for eventual systemic release or may function in removal of galectin-3C from circulation.

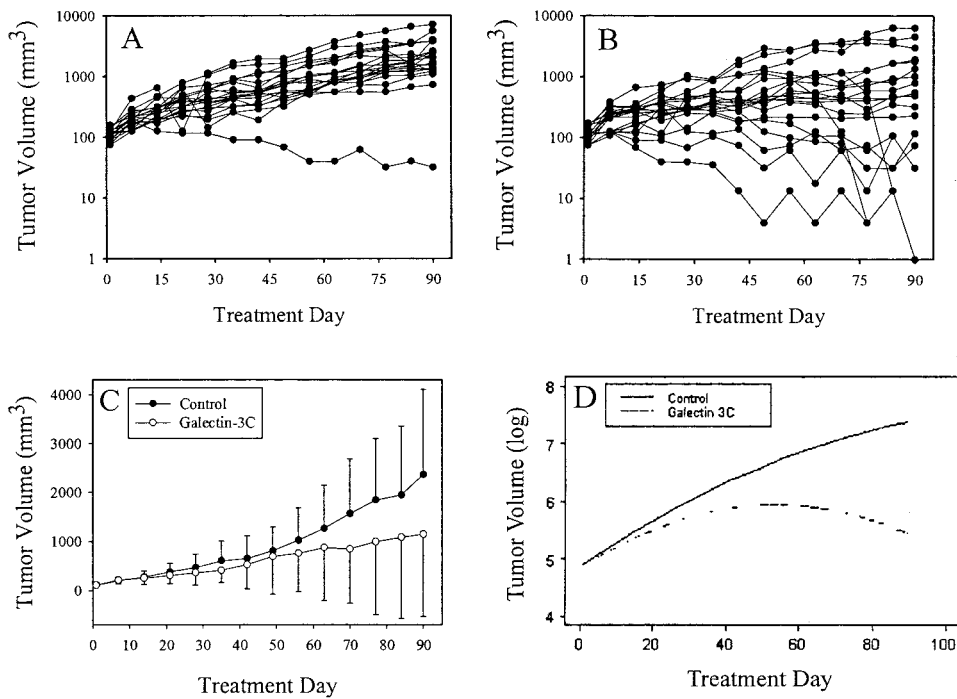
### Efficacy Study of Galectin-3C

#### Assessment of Primary Tumor Volume and Weight.

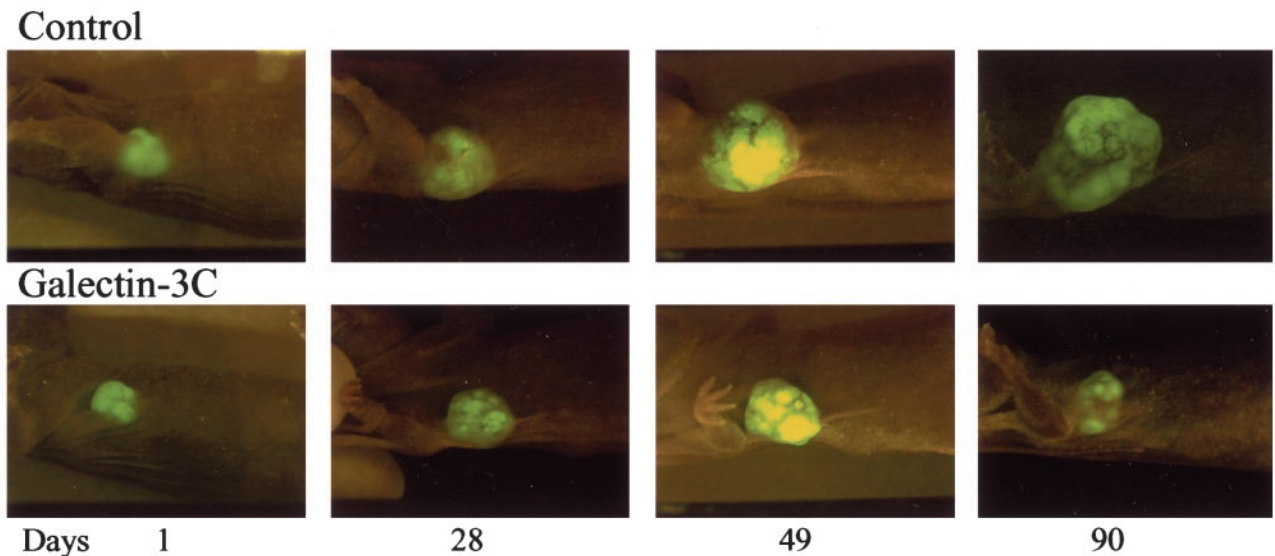
To test the efficacy of galectin-3C the growth of tumors was assessed by both caliper measurements and whole-body fluorescence imaging of the primary tumors. The tumor volumes over time as measured with a pair of calipers for each animal in the control group are presented in Fig. 4A, and for each animal in the treatment group in Fig. 4B. For the control group, the tumor growth curves followed what was observed in this model for 19 of 20 mice, *i.e.*, a continuous nearly exponential growth to an average tumor size of  $\sim 20$  times the initial size over 3 months. In 1 mouse from both the control and the galectin-3C treated groups the tumor decreased in size from the beginning of treatment and as such these may be classified as “nontakes.” For the treated group, 19 of 20 growth curves were similar to the controls over the first 30–40 days of the study, albeit rising at a slightly slower rate. After about the midpoint of the study most of the tumors in the treated mice grew much slower than in the control group or even decreased in size. In 2 treated mice the tumor had disappeared at the end of the study although it was about 10 times the original size at day 40–60. Only 5 of the treated mice had growth curves approximately similar to the control group by the end of the study.

A comparison of tumor growth over time in an untreated and galectin-3C treated mouse as visualized by fluorescence imaging is shown in Fig. 5. The images of the treated animal in Fig. 5 show growth followed by regression of the tumor, whereas the tumor in the control animal showed steady growth throughout the course of the 90-day study.

**Statistical Analysis of Galectin-3C Efficacy.** The mean tumor volumes for each group are shown in Fig. 4C. At day 90, the mean of the tumor volumes in the mice treated with galectin-3C was significantly less than that in the control group ( $P = 0.003$ ). In addition, the mean weight of the tumors derived from the mice in the galectin-3C-treated group at autopsy was less than the control group by a factor of  $\sim 2$ -fold ( $1.25 \text{ g} \pm 1.65$  versus  $2.34 \text{ g} \pm 1.47$ , respectively;  $P = 0.007$ ;  $\chi^2$ ), a finding that coincides with the observation of larger tumors in the control group. Only 1 of the control mice had a tumor  $< 700 \text{ mm}^3$ , whereas 13 treated mice had tumors less than that size ( $P < 0.001$ ).



*Fig. 4* Effect of galectin-3C treatment on the growth of primary orthotopic xenograph tumors in individual nude mice injected with either vehicle control (A) or galectin-3C (B). The primary tumors were measured with calipers once per week after initiation of the treatment through the end of the 90-day treatment period. Tumor volume was calculated by the formula  $W^2 \times L/2$ , where  $W$  is the smallest dimension. C represents the primary tumor growth as the means of the vehicle control and galectin-3C-treated groups at each time point; bars,  $\pm$ SD. D shows an analysis of tumor growth over time using a repeated measures model. In this model, a SAS Proc Mixed analysis was used to fit a linear mixed model with random effects for cage, mouse slope, intercept, and quadratic in treatment day. The model shows steady growth for the control group but regressive growth for the galectin-3C group.



*Fig. 5* Examples of GFP fluorescence visualization of tumors in live mice from treated and control groups at different time points. The tumors, grown in the orthotopic model, derived from MDA-MB-435 breast cancer cells stably transfected with a plasmid expressing GFP. The fluorescent tumor was visualized in the live mouse under UV light. Images of a representative animal from the vehicle-only control group and the galectin-3C-treated group taken at days 1, 28, 49, and 90 are presented.

In addition to the statistical data analyses of the efficacy data described above, an analysis of tumor volume growth over time was performed using a repeated measures model for tumor volume, with cage in random statement. Specifically, this was a SAS Proc Mixed analysis to fit a linear mixed model with random effects for cage, mouse slope, and intercept, and the fitted model was quadratic in treatment day. The model was

applied to the vehicle control and galectin-3C treatment data for tumor volume, which showed a significant difference at day 90 when tested with the Mann-Whitney U test. The model allowed us to sensitize the analysis by factoring out nontreatment parameters such as cage and mouse variation, tumor volume trajectory differences within a group, and residual unexplained variation. As shown in Fig. 4D, the log of the mean tumor

Table 1 Incidence of metastases in lymph node, lung, and liver

Treatment group	Number of mice	Lymph node	<i>P</i> <sup>a</sup>	Lung	<i>P</i>	Liver	<i>P</i>
Vehicle control	20	11	-	3	-	2	-
Galectin-3C	20	4	0.022	4	1.0	0	0.487

<sup>a</sup> All of the treated groups compared with vehicle control by the  $\chi^2$ /Fisher's exact test.

Table 2 Number of mice with or without metastases in treatment groups as a function of tumor volume

Treatment group	Metastatic assessment	Small tumors (0–1500 mm <sup>3</sup> )	Medium tumors (1501–2999 mm <sup>3</sup> )	Large tumors (>2999 mm <sup>3</sup> )
Vehicle control	Metastases	5 (LN only)	2 (LN only)	4 <sup>a</sup>
Vehicle control	No metastases	3	5	1
Galectin-3C	Metastases	0	2 (2 lung; 0 liver) <sup>b</sup>	2 (2 lung; 0 liver) <sup>b</sup>
Galectin-3C	No metastases	15 <sup>c</sup>	1	0

<sup>a</sup> All 4 mice had lymph node metastases; 2 of those had liver and lung metastases, and 1 of those had lung metastases.

<sup>b</sup> Both mice had lymph node and lung but not liver metastases.

<sup>c</sup> Fifteen percent (3 of 20) of the galectin-3C-treated mice had tumor volumes of 0 at the end of the study.

volume in the group of mice treated with galectin-3C is statistically significantly less than that in the vehicle control group during >50% of the treatment period. At day 45 of treatment, the slope of the tumor volume curve for the galectin-3C group is significantly different from that of the vehicle control group ( $P = 0.045$ ; SAS type 3 F test). After day 45, treatment with galectin-3C resulted in increased differences between the slopes and trajectories of the two lines representing the mean tumor volumes of the two groups of mice, with an overall regression of tumor growth in treated mice.

**Histopathology of Primary Tumors.** Histopathological examination of the tumors after treatment was done by H&E staining (data not shown). The medium and large tumors (>1500 mm<sup>3</sup>) of the control group and the treated group showed dense large irregular cells typical of this model. The small tumors showed areas of tumor cells, generally smaller than in large tumors, interrupted by areas of necrosis. For the smallest tumors the areas of necrosis were predominant. There was no apparent qualitative difference between histopathological pictures of tumors of the same size from the treated or control mice. Thus, although the galectin-3C treatment appeared to induce necrosis in the primary tumors, there was no apparent difference between this necrotic effect and what was observed in the few small tumors of the control group.

**Assessment of Metastasis.** At autopsy, tissue samples from the axillary lymph node, the liver, and the lungs were collected and processed through standard procedures of H&E staining for microscopic examination. Metastasis to the axillary lymph node, the lung, and the liver was assessed microscopically. Tables 1 and 2 show the results of this assessment. Statistical analyses were carried out using the  $\chi^2$ /Fisher exact test. Eleven of 20 mice had axillary lymph node metastasis in the control group whereas only 4 of the 20 mice developed axillary lymph node metastasis in the galectin-3C group by the end of the study ( $P = 0.022$ ). The incidence of metastases in the liver and the lung between the galectin-3C group and the control group was not different. In the control group, 5 of the 8 small tumors had associated metastases, whereas 0 of the 15 small

tumors in the galectin-3C group had associated metastases. The trend to reduced metastases in the treated group did not appear to exist in connection with the larger tumors (>1500 mm<sup>3</sup>).

## DISCUSSION

The results of this study show that galectin-3C when administered as a single agent significantly regressed the growth and inhibited metastasis of breast tumors in a mouse orthotopic model. Using a treatment dose that was 25-fold less than the highest nontoxic acute dose, the mean volume of the tumors in the treated group was less than that of the vehicle-only control group beginning on day 14 until the end of the study, with the difference reaching statistical significance by day 63 using a *t* test to compare the groups. Only 1 animal in the control group had a tumor <700 mm<sup>3</sup>, whereas 13 animals in the treated group had tumors less than this size. Furthermore, tumor growth slowed and tumor volume regressed over time in some individual mice in the treated group, and there was no primary tumor observed in 3 treated animals (15%) at necropsy at the end of the 90-day study. In addition, the mean tumor weight of the galectin-3C-treated group at autopsy was less than the control group by a factor of ~2-fold, a finding that coincides with the observation of larger tumors in the control group. Taken together, these data support the conclusion that treatment of breast tumor-bearing mice with galectin-3C significantly reduced the progression of tumor growth. This conclusion was additionally substantiated by fitting the data to a SAS Proc Mixed model (28), which revealed a regression in tumor growth over time in treated mice.

This study tested the efficacy of galectin-3C that was affinity purified after digestion of recombinant human galectin-3 with bacterial collagenase to produce a single polypeptide product containing the COOH-terminal carbohydrate recognition domain. A previous NH<sub>2</sub>-terminal sequence analysis of the products of incomplete collagenase digestion of galectin-3 with *C. histiolyticum* collagenase type VII revealed two polypeptides with ~80% galectin-3C that began with Gly<sup>108</sup> and 20% that began with Ser<sup>96</sup> (29). However, after exhaustive digestion with

this enzyme SDS-PAGE analysis clearly reveals a single protein product that corresponds to an NH<sub>2</sub>-terminally truncated form of recombinant galectin-3 that begins with Gly<sup>108</sup>.

The MDA-MB-435 human breast cancer cells that were used in the study had been modified to express GFP to enable direct observation of the primary tumor in living animals and metastases on autopsy (26). GFP expression did not appear to affect tumor growth, as the rate of growth of the tumors in the control animals was similar to that observed previously after orthotopic implantation in nude mice of MDA-MB-435 cells not expressing GFP (30). Also, when rat breast cancer cells were modified to express GFP they retained the same growth rate and morphology *in vitro*, and when injected orthotopically into the mammary fat pads of rats they retained the same metastatic phenotype as the unmodified cells (31). The rate of metastasis to the lymph nodes in the vehicle only control animals in our study was 55% (11 of 20), which is similar to the 77.5% average rate of lymph node metastasis observed previously after injection of MDA-MB-435 cells in an orthotopic nude mouse model (32). Our observation of 15% of control animals (3 of 20) with metastases in the lungs also was similar to an average of 16% observed previously (32). The percentage of animals with metastasis from orthotopic implantation of this cell line can vary; for example, another study reported 71% of animals with lymph node metastases and 53% with lung metastases (33).

Treatment with galectin-3C reduced the percentage of animals with metastases from 55% (11 of 20) in the control group to 20% (4 of 20) in the treated group, a decrease of 2.75-fold. None of the 15 animals in the treated group with tumors <1500 mm<sup>3</sup> in size had metastases, whereas 62.5% (5 of 8) of the control group that had tumors of this size had metastases. However, with regard to tumors >1500 mm<sup>3</sup>, only 1 of 5 animals in the treated group was free of metastases compared with 6 of 12 animals in the control group which had tumors of this volume in the absence of metastases. Apparently animals in the treated group that had metastatic tumors were not responding to galectin-3C, as their primary tumors were correspondingly large as well.

We found that the half-life of galectin-3C in the serum after i.m. injection was 3.0 h and that in the cellular fraction of the blood was 4.3 h. Within the cellular fraction, it is likely that galectin-3C is binding to the ABO blood antigens expressed by mice (34), because galectin-3 has been shown to bind to human blood group A-related oligosaccharides, and human blood group B and O erythrocytes (35). Organ biodistribution data showed that galectin-3C localized in the liver, kidney, and spleen but not in the heart or lungs. This result is similar to the reported biodistribution of <sup>125</sup>I-labeled murine galectin-3 in these organs of mice after i.v. injection (36). It is worth noting that 2 of the 11 mice with metastases in the untreated group had liver metastases, whereas none of the 4 mice with metastases in the treated group showed evidence of liver metastases. Although this result was not statistically significant, it is tempting to speculate that localization of galectin-3C in the liver may have prevented metastatic tumor formation in this organ. Conversely, 3 untreated mice and 4 treated mice had lung metastases, suggesting that the lungs were more susceptible to metastases because of the relative lack of galectin-3C localization in this organ.

Treatment with galectin-3C appeared to be well-tolerated, as toxicity was not observed from single s.c. injections of doses up to 125 mg/kg. This result is consistent with the finding that galectin-3 null mutant mice were viable and had no overt abnormalities (37). The apparent lack of toxicity of galectin-3C is important in considering its potential utility as an anticancer drug, because undesirable side effects can limit the efficacy of anticancer agents.

We tested galectin-3C because we thought that it should effectively compete with endogenous galectin-3 for both intracellular and extracellular carbohydrate binding sites, and prevent its homophilic cross-linking and other types of protein-protein binding interactions. This hypothesis is supported by the fact that galectin-3C produced by exhaustive digestion with *C. histolyticum* collagenase type VII loses the cooperativity in binding to laminin that is possessed by galectin-3 (20). Also reports from two different laboratories show that truncation of galectin-3 by other enzymes to remove 62 (38) or 111 (39) amino acid residues from the NH<sub>2</sub> terminus leads to the loss of the ability of galectin-3 to self-associate and, thereby, to hemagglutinate RBCs.

Galectin-3 can be found on the plasma membrane, and depending on the cell type is limited to the cytoplasm, or found in both the cytoplasm and nucleus (40–42). The variability in the intra- and extracellular localization of galectin-3 is not well understood, but it appears to be important in terms of biological function. Galectin-3 can be secreted by a nonclassical mechanism and internalized by cells (43, 44). Galectin-3 is similar to a number of other proteins that exhibit unconventional intercellular transfer in that it is secreted despite the absence of a signal peptide. These proteins are internalized by cells, and are able to directly access the cytoplasm and the nucleus by a process that does not involve classical endocytosis (45). Although our study did not address the localization of galectin-3C, it is likely that galectin-3C was internalized by cells based on the subcellular localization of comparable mutants of hamster galectin-3 (42).

Extracellularly, galectin-3 mediates aggregation of tumor cells and the binding of tumor cells to extracellular matrix (46). Galectin-3 has been shown to bind to a number of substrates (35, 47–53), including several such as tumor-associated Thomsen-Friedenreich antigen, tumor laminin, and carcinoembryonic antigen that are implicated in tumorigenicity and metastasis (24, 47, 54, 55).

The regression of tumor growth that we observed may be the result of inhibition of the antiapoptosis activity of galectin-3 that appears to have more than one mechanism, and appears to function both intra- and extracellularly. Intracellular galectin-3 has been found to inhibit apoptosis due to a variety of agents after it was first reported that transfection of T cells with galectin-3 cDNA conferred resistance to apoptosis induced by anti-Fas antibody and staurosporine (56). Galectin-3 has been shown to protect breast cancer cells from apoptosis induced by genistein (57), tumor necrosis factor  $\alpha$  (58), active oxygen species (58), nitric oxide (59), alteration of mitochondrial membrane potential, caspase activation (58), and loss of cell anchorage (anoikis; Refs. 58, 60). The expression of galectin-3 within breast cancer cells was reported to inhibit cisplatin-induced apoptosis, and this antiapoptotic effect was abrogated by substitution of alanine for glycine in the NWGR motif that is

expressed by galectin-3 and conserved in the Bcl-2 family (61). Contact of mammary epithelial cells with the extracellular matrix was found to suppress expression of caspase-1 that induces apoptosis through an integrin-dependent pathway (62). In general, differentiation and tumorigenicity of breast epithelium are thought to be regulated to some degree by the interaction with extracellular matrix and integrins (63, 64).

Studies of some types of cancer have found a positive correlation between the expression of galectin-3, and tumorigenicity and metastasis such as in colon (65, 66) and thyroid cancer (67); others studies such as those of breast cancer have found a negative correlation (68, 69). In prostate cancer, loss of the nuclear expression of galectin-3 was associated with malignancy (70). Correlation of galectin-3 expression and tumorigenicity has been complicated by its expression in normal epithelial cells that give rise to some cancers, and possibly by our lack of knowledge of the significance of the variable localization of galectin-3. A study that focused on analysis of the extracellular expression of galectin-3 found that the concentrations in the sera of 99 patients with breast, gastrointestinal, lung, ovarian cancer, melanoma, or non-Hodgkin's lymphoma were elevated compared with the concentrations in sera of normal patients (71). In addition, patients with metastatic disease had higher levels than individuals that had localized tumors.

In summary, the results indicate that galectin-3C has minimal overt toxicity and that it could have utility in treatment of breast cancer by shrinking tumors and reducing tumor growth over time, and by inhibiting metastasis. These findings are particularly significant because of the poor prognosis of metastatic breast cancer. Our results highlight the growing body of data that indicate galectin-3 facilitates the growth and metastasis of breast and certain other types of cancer. We plan additional studies of galectin-3C and other molecules that specifically inhibit the carbohydrate binding of galectin-3 as potential treatments for these cancers.

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