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TITLE: Efficacy of Galectin-3C in Mouse Model of Metastatic Breast Cancer

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# Efficacy of Galectin-3C in Mouse Model of Metastatic Breast Cancer

## Abstract

The goal of this research is to evaluate a potential therapeutic agent for breast cancer based on the lectin galectin-3. The hypothesis to be tested is that therapy with an N-terminally truncated form of galectin-3 (galectin-3C) will be efficacious for inhibition of metastases. The overall purpose of the research is to determine the efficacy, safety and mechanism of action of galectin-3C in treatment of metastatic breast cancer. To this end, recombinant galectin-3 was produced from which galectin-3C was derived by collagenase enzyme digestion and affinity chromatography. For ELISA detection of injected galectin-3C in nude mice, chickens were immunized with galectin-3C but the response yielded only low affinity antibody. An alternative detection strategy was developed by metabolically labeling galectin-3 with $^{35}$S methionine prior to collagenase cleavage. The maximum tolerated dose of galectin-3C in nude mice at 5 days was determined to be a minimum of 125 mg/kg without adverse effects. The pharmacokinetic analysis of the intravenous administration of $^{35}$S-labeled galectin-3C into nude mice, although not complete, indicated an elimination half-life of galectin-3C of 8.4 hours. The data indicate that galectin-3C is well-tolerated in mice and that a sufficient concentration of galectin-3C is sustained in serum to warrant subsequent efficacy testing.
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(5) INTRODUCTION

The goal of this research is the evaluation of a potential therapeutic agent for breast cancer based on a lectin, that acts directly to reduce metastases. Soluble recombinant N-terminally truncated galectin-3 (galectin-3C) should effectively compete with endogenous galectin-3 for carbohydrate binding sites in the extracellular matrix and cell-cell adhesions important in tumor invasion and metastasis. The non-carbohydrate binding domain of galectin-3 promotes multimerization of the protein, and enables it to crosslink cancer cells to the matrix and other cells. Excess administered galectin-3C, in which the N-terminal carbohydrate binding part of the protein has been removed, will occupy binding sites of endogenous galectin-3 and prevent its cross-linking activities. Galectin-3C itself will not have cross-linking activity since it lacks the N-terminal part of galectin-3, and should act like a dominant-negative inhibitor of galectin-3. The hypothesis to be tested is that therapy with recombinant galectin-3C will be efficacious for inhibition of tumor invasion and metastasis in breast cancer. The overall purpose of the research is to determine the efficacy, safety and mechanism of action of galectin-3C in treatment of metastatic breast cancer using a nude mouse model of metastasis.

(6) BODY

Task 1: Characterization of biological activity of galectin-3C in vitro.

A: Months 1-4: Determine if exogenous radiolabeled galectin-3C is uptaken in breast epithelial cells using Transwell chambers.

Our plan was to use $^{125}$I-labeled galectin-3C for this portion of the study. We decided to postpone this portion of the work until we could obtain $^{35}$S-labeled galectin-3C from Dr. Leffler, since he is producing this for the pharmacokinetic analysis (see Task 2 below). It will be easier to perform this experiment technically with the $^{35}$S-labeled protein since $^{35}$S is a beta emitter, and $^{125}$I is a gamma emitter. We plan to perform these experiments by the end of month 14 (September).

B: Months 5-17: Assay efficacy of galectin-3C in prevention of invasion of various breast cancer cell lines in vitro using Matrigel invasion assay.

We have obtained a human breast cancer cell line labeled internally with green fluorescent protein (ONCOBRITE™; GFP MDA-MB435) from AntiCancer, Inc. that metastasizes to bone in nude mice. This is a stable transductant that expresses green fluorescent protein similar to other transductants which they have reported (Yang et al. 1998; Yang et al. 1999). Detection of this cell line in the Matrigel invasion assay will be greatly simplified as it will be easy to distinguish extracellular debris and other noncellular material from the cells themselves without staining the cells. These cells are easily cultured and have been passed a number of times (>10) in our laboratory.

In the invasion assay, metastatic cancer cells loaded into the top of Transwell chambers squeeze through Matrigel-coated pores in response to conditioned media from fibroblasts that is placed in the bottom chamber. We have harvested and stored conditioned media from cultured rat 3T3-like fibroblasts for the invasion assays (Le Marer and Hughes, 1996). Transwell chambers with Matrigel-occluded membranes containing small pores (8 mm) are normally available from Becton-Dickinson Labware (Bedford, MA). However, these chambers have been unavailable from the manufacturer for more than 6 months. Therefore, we have purchased Matrigel and chambers separately and are in the process of learning how to make and preparing our own Matrigel-coated Transwell chambers for the invasion assays. We expect these experiments to be completed by end of month 17 (December).
Task 2: Prepare for animal studies.

A: Months 1-2: Begin and continue the production of recombinant galectin-3C until there is sufficient quantity and purity for the proposed animal work.

The carbohydrate recognition domain of galectin-3 (galectin-3C) was produced as described previously (Massa et al., 1993). First, intact recombinant galectin-3 was produced in *Escherichia coli* BL21/DE3 containing the pET3c plasmid with the human galectin-3 coding DNA (pET3cGal3). The organisms were lysed by sonication and the galectin-3 protein purified by affinity chromatography on lactosyl-Sepharose (Leffler et al., 1989). The purified galectin-3 was dialyzed to remove lactose and cleaved with *Clostridium perfringens* collagenase type VII (Sigma), and the resulting galectin-3C purified again by affinity chromatography on lactosyl-Sepharose. For storage and shipment we developed a new procedure involving dialysis against water followed by lyophilization. The dry galectin-3C powder was stored at -20 °C for various amounts of time up to 3 months and the retention of the carbohydrate binding activity of an aliquot was ascertained by testing on a small lactosyl-Sepharose column. Other batches (with or without enrichment in 15N) were sent to a collaborator (not associated with this project) in the USA for analysis by NMR-spectroscopy. This analysis confirmed that the protein had retained its proper folding. Hence, we feel confident that galectin-3C can be stored and shipped as a lyophilized powder without losing activity.

To produce 35S labeled galectin-3C for pharmacokinetic studies, the plasmid pET3cGal3 was transfected into *E.coli* B834 (Novagen), which is a methionine auxotroph derived from BL21/DE3. The *E.coli* were adapted for growth on M9 minimal medium supplemented with ampicillin (50 mg/ml) and methionine (40 mg/ml)(M9-Met) by passage on M9-Met plates three times. To produce 35S galectin-3, a colony from the last plate was inoculated into 0.5 liters of M9-Met supplemented with 1.0 mCi 35S-Met. The bacteria were cultured, induced with IPTG and harvested as described previously (Massa et al., 1993). To lyse the radioactive *E.coli*, sonication was avoided because of aerosol formation. Various alternative methods were tested and the following method was determined to be most efficient. To the bacterial pellet was added 5 ml sucrose (25%) in 50 mM TrisHCl, pH 8.0 with 50 mM NaCl, 20 mM EDTA, and 8 mg lysozyme. After 10 minutes on ice, 16 ml water was added and the sample kept on ice another 30 minutes. The sample was centrifuged at 12000 rpm for 30 minutes and the supernatant applied to lactosyl-Sepharose. The galectin-3 was eluted, dialyzed and treated with collagenase to generate galectin-3C as described above.

The determination of the Maximum Tolerated Dose (toxicity) and approximately half of the pharmacokinetic analysis has been performed (see Task 3 below). Only 5.5 mg (150 µg/animal) are required to complete the pharmacokinetic analysis, including up to 2 more time points with 5 animals per group for intravenous testing and 6 time points with 5 animals each. Considerably more galectin-3C protein will be required to complete the efficacy study. We foresee no problem with this requirement as Dr. Leffler is in the process of and committed to producing all of the protein required. However, until the pharmacokinetic analyses and toxicity studies are complete, the quantities required remain to be determined.

B: Months 2-7: Immunization of four chickens with purified, N-terminally truncated galectin-3 and purification of polyclonal Ig from chicken eggs.

Dr. Leffler immunized two chickens with purified galectin-3C and the polyclonal Ig was purified. In theory, the Ig should bind to both galectin-3 and galectin-3C. When tested, the chicken polyclonal anti-galectin-3C was of very low affinity as determined by repeated nitrocellulose dot blots of galectin-3 and galectin-3C following lactose elution of each protein from a lactosyl-Sepharose column. The presence of galectin-3 and galectin-3C protein in specific fractions from the column was confirmed by the measurement of UV absorbance at 280 nm. Detection of anti-galectin-3 antibody (rat IgG) binding to galectin-3 was used as a positive control on a separate dot blot using anti-rat IgG labeled with alkaline phosphatase (AP). For the chicken polyclonal anti-galectin-3C antibody, an anti-chicken Ig antibody (Zymed, South San Francisco) labeled with biotin was used followed by AP-conjugated streptavidin and AP substrate. The results of these
studies provided no evidence that immunization of chickens would produce a high affinity antibody specific for galectin-3C. We therefore decided to explore the alternative strategy of generating $^{35}$S-labeled galectin-3C (see Task 2 above).

C: Months 8-10: Development of an ELISA (or alternative) assay for galectin-3C protein. Determine the sensitivity and reproducibility of the assay and plot standard curves.

For the reasons outlined above, we generated $^{35}$S-labeled galectin-3C for use as a detection system in the pharmacokinetic studies.

Task 3: Pharmacokinetic analysis & determination of Maximum Tolerated Dose.

A: Months 10-12: Determine the Maximum Tolerated Dose or MTD of a single injection of 4 different doses and a control group of nu/nu mice for galectin-3C. Observation of mice over 48 hours (total 25 mice).

A dose determination study was carried out in non-tumor bearing female athymic nude mice in order to determine the MTD of galectin-3C using a single bolus dose. The dose finding study comprised 4 dose groups with each group consisting of 5 mice. The subcutaneous doses administered were 1 mg/kg, 5 mg/kg, 25 mg/kg, and 125 mg/kg. In addition, a vehicle treated control group consisting of 5 mice was evaluated. No abnormal signs were observed within 48 hours of injection. Animals were observed for 5 days total after injection at which time body weight and viability were determined (Figure 1; Appendix A). We conclude from these results that galectin-3C can be injected into nude mice at a dose as high as 125 mg/kg without adverse effects.

B: Months 10-12: Perform pharmacokinetic analysis using standard methodology. Analyze the concentration of galectin-3C in plasma of nu/nu mice after tail vein injection using the ELISA assay previously developed. Determinations will be made at 6 points over 48 hours with 5 mice per time point, and 5 controls. Analysis will be repeated with s.c. injections of the two proteins. The concentration of protein in each sample will be determined by the ELISA assay previously developed (total 65 mice).

The pharmacokinetic analysis of the intravenous administration of galectin-3C is nearly complete. Groups of five mice (approximately 0.03 kg/mouse) were each injected with 150 μg/mouse (1 μg per μl; 5 mg/kg = dose) of a mixture of $^{35}$S-labeled galectin-3C and unlabeled galectin-3C in a weight ratio of 1:9 (labeled:unlabeled). The animals were sacrificed and serum samples were obtained by terminal cardiac puncture at five time points: 15 min, 1 h, 2 h, 4 h, and 24 h after injection. In addition, serum samples were obtained from one control group of five animals 1 h after injection of vehicle only (1 mg/ml lactose in PBS). Each 200 μl serum sample was analyzed for radioactivity in triplicate. The vehicle only control and the 15 min groups of animals were injected on June 8, 1999, whereas the 1, 2, 4, and 24 h groups were injected on June 24, 1999. The results are shown in Figure 2, Appendix B.

The total dose of galectin-3C was 150 μg containing 15 μg $^{35}$S-labeled galectin-3C. The radioactivity of 15 μg $^{35}$S-labeled galectin-3C was 7,500 cpm (counts per minute) on May 20, 1999. The half-life ($T_{1/2}$) of $^{35}$S is 87.2 days. The disintegration constant (λ) per day is equal to 0.693/$T_{1/2}$ or 0.00975 day$^{-1}$ (Martin et al., 1966). Thus, on June 24, 1999 the sample had decayed by 72.2% to 5,415 cpm (361 cpm per μg protein). On June 9, 1999, the 7,500 cpm had declined by 84.1% to 6,308 cpm. The background level of $^{35}$S detected in the vehicle only animals had a mean value of 48.93 cpm. The serum samples collected 24 h after injection had a mean CPM of 98.85, approximately two times background. The mean and standard deviations of the data are presented graphically in Figure 2, Appendix B. We plan to analyze one more group of five animals, as planned, but at 12 h after intravenous injection rather than after 48 h.
During the distribution phase after an intravenous dose, changes in the concentration of drug are primarily due to movement of drug within the body. The distribution phase primarily determines the early rapid decline in plasma concentration of a drug after an intravenous dose. With time, equilibrium is reached in the distribution of the drug between the plasma and the tissues, and changes in plasma reflect proportional changes in all the other tissues. During the elimination phase after the rapid decline of the distribution phase, the decline in plasma concentration is due only to elimination of the drug from the body and is characterized by the elimination half-life \( T_{1/2} \) and the apparent volume of distribution \( V \) (Martin et al., 1966; Rowland and Tozer, 1995). The elimination half-life is the time it takes for the concentration of the drug in the plasma (and body) to be reduced by one-half. The apparent volume of distribution is the apparent volume of distribution of the drug in the body at equilibrium. The volume of distribution is equal to the amount of drug in the body at \( T_0 \) divided by the plasma drug concentration at \( T_0 \) (Rowland and Tozer, 1995).

As seen in Figure 2, the distribution phase for galectin-3C apparently lasted until approximately 4 h. The peak level in the serum was detected at 2 h, with a rapid decline to the 4 h time point, and a less rapid decline during the elimination phase between 4 and 24 h. In a first-order elimination process the half-life is independent of the concentration of the drug in the body and the following equations apply (Rowland and Tozer, 1995).

\[ T_{1/2} = \frac{0.693}{k} \]  
\[ k = \frac{2.303}{\log_{10} \frac{\text{conc}_{\text{Time}(2)}}{\text{conc}_{\text{Time}(1)}}} \]

The elimination half-life for galectin-3C can be calculated from the means of the concentrations at 4 and 24 h. After subtracting the mean of the background cpm (48.9), the mean at 4 h was 259.4 cpm and the mean at 24 h was 49.9 cpm. Thus \( k = 0.0824 \text{ h}^{-1} \) (fractional rate of drug removal) and \( T_{1/2} = 8.41 \text{ h} \).

Calculation of the volume of distribution requires that distribution equilibrium be achieved between the drug in the tissues and the plasma (Rowland and Tozer, 1995). After an intravenous bolus, the amount of the drug in the body is the dose administered, but the distribution equilibrium has not yet been achieved. To estimate the plasma concentration that would have resulted if the drug immediately distributed into its final volume of distribution, use is made of the linear decline during the elimination phase in the semilogarithmic plot (Figure 2) (Rowland and Tozer, 1995). By regression analysis of the linear portion of the curve between the 4 and 24 h (Figure 2), the initial concentration can be estimated as 301 cpm (per 200 ul; 1,505 cpm per ml). The total dose (in radioactivity) per animal was 5,415 cpm. Therefore the volume of distribution can be determined:

\[ V = \frac{5,415 \text{ cpm}}{1,505 \text{ cpm/ml}} = 3.60 \text{ ml} \]

Total clearance (Cl) relates concentration to the rate of elimination, and is equal to the elimination rate constant times the volume of distribution.

\[ \text{Cl} = k \times V \]

\[ \text{Cl} = 0.0824 \text{ h}^{-1} \times 3.60 \text{ ml} = 0.297 \text{ ml h}^{-1} \]
Dr. Leffler is currently producing additional quantities of radiolabeled galectin-3C so that we can complete the intravenous pharmacokinetic analysis with at least one more time point. We will then generate the data with subcutaneous dosing.

We expected that the highest concentrations would be detected in serum from the first time point at 15 min. However, both the samples from the 15 min and 1 h time points were significantly less than the maximum concentration detected at 2 h. This is the type of curve usually produced from some type of extravascular dose rather than an intravenous dose. However, there is at least one likely explanation for the data. Galectin-3C may bind to β-galactosides on the vascular walls, capillary matrix, or red blood cells initially and be slowly released as the plasma without galectin-3C reaches the site. Secondly, galectin-3C might exist in associated form in concentrated solution and act like a particulate upon injection leading to a delay in the even distribution in the blood. Analysis of whole blood instead of serum samples would allow us to address these possibilities.

Task 4: Compare in the MetaMouse\textsuperscript{R} model of metastatic breast cancer, the efficacy of treatment with galectin-3C to control animals (vehicle only).

A: Months 13-16: Prepare the 80 mice by surgical orthotopic implantation of human breast cancer tissues. 20 mice would form the control group. The remaining 60 mice would be put into 3 groups, with 3 different s.c. dosing regimens of galectin-3C. Start treatment 1 week after implantation and continue for 60 days or longer (up to 6 months) depending on the survival of the mice. Weigh mice and observe (total 80 mice).

B: Months 14-18: Post-mortem analysis of mice as they die, for up to 6 months at which time surviving mice would be sacrificed. Parameters to be determined are as follows:

1. weight of primary tumor—calculated as \( \text{width}^2 \times \text{length}/4 \)
2. local/regional invasion by tumor
3. number of metastases
4. survival
5. tumor histology (fixed in 10\% formalin followed by paraffin embedding and sectioning, and then hematoxylin and eosin stained).
6. animal weight

B: Months 19-24: Analysis of all of the data from the efficacy study and preparation of report/manuscript describing results.

Work on Task 4 will begin following completion of the pharmacokinetic analyses and production of additional quantities of galectin-3C by Dr. Leffler.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Development of system for large-scale production of recombinant galectin-3C
- Development of system for detection of galectin-3C in nude mice using \(^{35}\text{S}-\text{labeled galectin-3C}
- Maximum tolerated dose of galectin-3C in nude mice determined to be a minimum of 125 mg/kg
- Intravenous half-life of galectin-3C in nude mice acceptable
- Volume of distribution of galectin-3C moderate; will consider using higher dose for efficacy studies
- Use of breast cancer cell line labeled internally with green fluorescent protein for \textit{in vitro} invasion assays
(8) REPORTABLE OUTCOMES

None at this time

(9) CONCLUSIONS

Large-scale quantities of recombinant galectin-3 were produced from which galectin-3C was successfully derived by collagenase enzyme digestion and affinity chromatography. This amount of production scale-up was essential for generating sufficient quantities of galectin-3C for dosage and efficacy studies, as well as for the proposed in vitro invasion studies. For ELISA detection of injected galectin-3C in nude mice, chickens were immunized with galectin-3C but the response yielded only low affinity antibody. We then developed an alternative detection strategy by metabolically labeling galectin-3 with $^{35}$S methionine prior to collagenase cleavage. This allowed us to directly detect galectin-3C in vivo by measuring serum radioactivity levels. The maximum tolerated dose of galectin-3C in nude mice at 5 days was determined to be a minimum of 125 mg/kg without adverse effects. This suggests that the safety threshold for the use of galectin-3C in vivo is high. The pharmacokinetic analysis of the intravenous administration of $^{35}$S-labeled galectin-3C into nude mice, although not complete, indicated an elimination half-life of galectin-3C of 8.4 hours. In conclusion, the data indicate that galectin-3C is well-tolerated in mice and that a sufficient concentration of galectin-3C is sustained in serum to warrant subsequent efficacy testing.

SO WHAT SECTION

1. The development of a system for detection of galectin-3C in nude mice using $^{35}$S-labeled galectin-3C allowed us to accurately determine the in vivo pharmacokinetics of intravenously injected galectin-3C.
2. Knowing the half-life of a drug allows one to determine whether the drug is accumulating in the body with multiple doses, and to determine the maximum and minimum amounts in the body with multiple doses. Thus, one can more rationally optimize a dosing interval. In general, drugs with half-lives between 30 min and 8 h need only be administered every 1 to 3 half-lives if the drug has a high therapeutic index (i.e. there is a large difference between the toxic and the therapeutic dose). Drugs with half-lives between 8 and 12 h often can be given every half-life (Rowland and Tozer, 1995). To reach a steady state more quickly, a loading dose of twice the maintenance dose can be administered. Thus, if the remainder of the intravenous and subcutaneous pharmacokinetic data indicate that galectin-3C has a half-life of 8 h or more, we would plan to use twice a day dosing. Since there was no toxicity observed, we can use as large a dose as is practical.
3. The concentration of a drug in the plasma after distribution reflects the dose and the extent of tissue distribution. A small volume of distribution implies that there is little distribution of the drug into the tissue. In humans the average mass is 70 kg, plasma volume is 3 L, extracellular space 15 L, and total body water is 42 L. The apparent volume of distribution can be larger than the total body water for a drug that is highly distributed. The apparent volume of distribution that we calculated for our average 30 g mouse is 3.6 ml. This would be greater than the plasma volume of the mouse. However, adequate distribution of galectin-3C could be of concern for therapeutic efficacy. We are planning to perform some histological analyses of the mice treated with the radiolabeled protein to address the tissue distribution of the drug.
4. The use of GFP MDA-MB-435 cells will be excellent for detection of metastases and for invasion assays.


Figure 1. Four groups of 5 mice were injected subcutaneously with the indicated dose of galectin-3C. A control group of 5 mice was injected with the vehicle only. Animals were observed for 5 days at which time body weight was determined. The mean body weights for each group were statistically identical (p>0.10) at 5 days indicating that all doses of galectin-3C were well-tolerated by the mice and that galectin-3C at the doses tested did not effect the normal physiological growth of the mice.
Intravenous Pharmacokinetic Study

Figure 2. Pharmacokinetic analysis of the intravenous administration of galectin-3C. Mice were injected with $^{35}$S-labeled galectin-3C and at the indicated time points the animals were sacrificed and serum levels of radioactivity were determined. Data is presented as the mean and standard deviation of the radioactive counts detected in 5 mice at each time point. From this data, the elimination half-life of galectin-3C was calculated to be 8.41 hours.