[CANCER RESEARCH 56, 5319–5324, December 1, 1996]

Advances in Brief

Inhibition of Human Breast Cancer Metastasis in Nude Mice by Synthetic Glycoamines[1]

Gennadi V. Glinsky,[2] Janet E. Price, Vladislav V. Glinsky, Valery V. Mossine, Galina Kiriakova, and John B. Metcalf

Cancer Research Center [G. V. G., V. V. G.] and Department of Biochemistry [G. V. G., V. V. G., V. V. M.], University of Missouri, Columbia, Missouri 65201; Metastat, Inc., Portland-Eugene, Oregon, and Columbia, Missouri 65201 [G. V. G., J. B. M.]; Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77054 [J. E. P., G. K.]

Abstract

We have examined the effect of synthetic low molecular weight glycoamine analogues on the metastasis of MDA-MB-435 human breast carcinoma xenografts growing in the mammary fat pads of nude mice. Initial *in vitro* screening of a panel of synthetic glycoamines was performed using a clonogenic growth assay in 0.9% agarose. Eight of nine compounds manifested a significant dose-dependent inhibition of colony formation by MDA-MB-435 cells in 0.9% agarose. The relative activity ranks of the compounds, based on ID_{50}s independently determined for each synthetic glycoamine analogue, identified *N*-(1-deoxy-D-lactulos-1-yl)-L-leucine (Lac-L-Leu), *N*-(1-deoxy-D-fructos-1-yl)-D-leucine (Fru-D-Leu), *N*-(1-deoxy-D-fructos-1-yl)-L-phenylalanine, and *N*-(1-deoxy-D-fructos-1-yl)-L-leucine as the most effective inhibitors of colony formation. Two separate experimental treatment protocols were used to examine the effect of selected synthetic glycoamines on human breast cancer growth and metastasis in athymic nude mice. Group A mice were treated intraperitoneally daily from day 2 after injection of the breast cancer cells until the end of the experiment (17 weeks). In group B, the mice were untreated until the mean tumor diameter was 10 mm, at which time daily i.p. treatment began. After 7 days, the primary tumors were resected, and the mice were treated for an additional 4 weeks (a total of 5 weeks of treatment). The synthetic glycoamines did not have significant antitumor effects, and there was no difference in the tumor incidence or tumor growth rates in mice treated continuously with synthetic glycoamines or PBS. The significant antimetastatic activity of synthetic glycoamines was detected in both experimental treatment protocols. In mice continuously treated with synthetic glycoamines according to protocol A, the incidence of metastasis was decreased 4.6-fold ($P = 0.014$) and 2.7-fold ($P = 0.031$) in mice treated with Fru-D-Leu and Lac-L-Leu, respectively. In mice in protocol B, the incidence of pulmonary metastasis was decreased 1.9-fold ($P = 0.069$) and 2.5-fold ($P = 0.042$) in mice treated with Fru-D-Leu and Lac-L-Leu, respectively. Correspondingly, the average number of spontaneous pulmonary metastases was reduced from 37 in control mice to 0.2 ($P = 0.005$) and 0.9 ($P < 0.02$) in mice treated according to the protocol A with Fru-D-Leu and Lac-L-Leu, respectively. Treatment of mice with *N*-(1-deoxy-D-fructos-1-yl)-L-leucine did not have significant antimetastatic effects, and no reduction in metastasis incidence or number was noted in mice treated with this synthetic glycoamine analogue. The treated animals had no apparent toxicity from chronic daily injection (up to 17 weeks of treatment) of synthetic glycoamines, and no obvious pathology was noted in the histological slides of the livers, kidneys, or spleens of the treated mice. Therefore, we have identified two synthetic glycoamines (Fru-D-Leu and Lac-L-Leu) that were the effective inhibitors of spontaneous human breast cancer metastasis in nude mice. Potential mechanisms for antimetastatic activity of synthetic glycoamines may include the inhibition of β-galectin-mediated homotypic cancer cell aggregation and induction of apoptosis in target cells.

Introduction

Breast cancer is the most common cancer among women in North America and western Europe and is the second leading cause of female cancer deaths in the United States (1, 2). In the United States, age-adjusted breast cancer incidence rates have increased considerably during this century (3). Approximately one in every nine American women will develop breast cancer during her lifetime. Breast cancer incidence rates in the United States have increased about 3% a year since 1980. An estimated 180,000 new breast cancer cases were diagnosed in the United States in 1995, and more than 46,000 deaths from breast cancer were reported (2). Approximately 40% of patients diagnosed with breast cancer have disease that has regional or distant metastases and, at present, there is no efficient curative therapy for breast cancer patients with advanced metastatic disease.

The anchorage-independent growth of primary breast cancer cells in a semisolid medium was found to be an independent variable associated with shorter disease-free survival and overall survival after mastectomy (4, 5). The clonogenic growth in agar cultures was also reported to correlate with the progression of breast neoplasms from benign to malignant (4, 5). In several experimental systems using rodent cells, colony-forming efficiency in agarose was shown to be correlated with metastatic potential (6, 7), and a particularly discriminating assay was to test colony formation in higher densities of agar medium (8, 9). It has also been reported that for human tumor cell lines, including breast cancer cells, the ability to grow in dense agarose cultures (>0.6% agarose) corresponds well with metastatic potential tested in nude mice (9–12). Thus, the colony formation assay in agarose cultures of >0.6% density distinguishes cancer cells with high metastatic potential (10–12). Therefore, the clonogenic growth assay could be used for *in vitro* screening of potential antimetastatic compounds.

The glycoamines are a naturally occurring low molecular weight component of mammalian blood serum that were studied as potential humoral cancer markers (13–15). The prototype compounds were originally isolated from human and rodent blood serum using a combination of conventional chromatography, analytical ultrafiltration, and size-exclusion and reverse-phase high pressure liquid chromatography (13–18). Subsequently, the compounds were structurally characterized as glycoconjugates of amino acids with covalent structures of Schiff base, ester, and Amadori bonding (13, 15–18). The inhibitory activity of high-pressure liquid chromatography-purified natural and synthetic glycoamines on rodent cancer cell aggregation *in vitro* and experimental metastasis *in vivo* were reported (13, 14, 19–22). Recently, we described a method of synthesis, purification, and full structural characterization of a series of synthetic glycoamine analogues using nuclear magnetic resonance, mass spectometry, and X-ray analysis (23–25). The availability of synthetic glycoamines in

Received 8/12/96; accepted 10/16/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by NIH/National Cancer Institute Grant 1R43 CA61402-01 and Metastat, Inc.

[2] To whom requests for reprints should be addressed, at Cancer Research Center, 3501 Berrywood Drive, Columbia, MO 65201. Phone: (573) 875-2255; Fax: (573) 443-1202.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/23/5319/2462122/cr0560235319.pdf by guest on 24 August 2022

pure form in multigram quantities allowed us to demonstrate a high inhibitory effect of the synthetic glycoamines on clonogenic growth in agarose of metastatic human cancer cells (25). The main objective of this study was to select the most effective synthetic glycoamines acting as inhibitors of clonogenic growth in hard agarose of metastatic breast carcinoma cells and to evaluate the antimetastatic activity of selected glycoamine analogues using the nude mouse model of human breast cancer metastasis (11, 26).

In this paper, we report the significant inhibition (>70%) of the incidence of spontaneous human breast cancer metastasis in the nude mouse model by synthetic glycoamine analogues. We also report that antimetastatic synthetic glycoamines inhibit homotypic aggregation of human MDA-MB-435 breast carcinoma cells and induce apoptosis in metastatic cancer cells.

Materials and Methods

Human Breast Carcinoma Cells

The MDA-MB-435 human breast carcinoma cell line was isolated originally from the pleural effusion of a patient with breast carcinoma and was found to be highly metastatic from the mfp[3] tumors (11, 26). The metastatic and tumorigenic properties of human breast carcinoma cell lines in nude mice were found to correspond well with their colony-forming efficiency in dense agarose (11).

Mouse Melanoma Cell Lines

B16 melanoma cell lines exhibiting high (B16-F10 and B16-BL6) and low (B16-F1) metastatic potential (27–30) were kindly provided by Dr. I. Fidler (M. D. Anderson Cancer Center, Houston, TX). The cells were maintained as monolayer cultures as described below.

Cell Culture

The tumor cells were maintained in tissue culture in MEM supplemented with 5 or 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold vitamin solution (Life Technologies, Grand Island, NY). The cultures were maintained on plastic and incubated in 5% CO_2/95% air at 37°C in a humidified incubator. All cultures were free of *Mycoplasma* and the following murine viruses: *Reovirus* type 3; pneumonia virus; K virus; Theiler's encephalitis virus; Sendai virus; minute virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; lactate dehydrogenase virus (all assayed by MA Bioproducts, Walkersville, MD).

Tumor cells were harvested from subconfluent cultures (50–70% confluence) by rinsing the monolayer with a 0.25% trypsin/0.02% EDTA solution. After 1 min, the flask was tapped to dislodge the cells, serum-supplemented medium was added, and the suspension pipetted to produce a single-cell suspension. Only single-cell suspensions of >90% viability (determined by trypan blue dye exclusion) were used for *in vivo* studies.

The inhibition of colony formation by MDA-MB-435 cells was performed as described previously (25). Briefly, synthetic glycoamines (see below) were suspended in culture medium to achieve a final concentration, after mixing with agarose, of 0.375–6.0 mM and filtered. Single-cell suspensions of MDA-MB-435 cells were mixed with the glycoamines and incubated for 1 h at 37°C, then mixed with agarose in a final concentration of 0.9%. No toxicity was measured at this step, using a trypan blue dye exclusion assay. Aliquots of 1.5 ml containing 10^3 cells were plated in triplicate 35-mm culture wells over a base layer of 0.6% agarose and allowed to gel. Colonies of >50 μm diameter were counted after 21 days of incubation. Percentage inhibition was calculated by comparison with the average colony numbers in triplicate control cultures (cells treated with medium alone).

In Vivo Studies Using MDA-MB-435 Metastasis Model

A model of spontaneous human breast cancer metastasis to the lungs after orthotopic implantation of MDA-MB-435 cells in nude mouse mfps (11, 26) was used for evaluation of antimetastatic activity of synthetic glycoamines.

Mice. Four-to-five-week-old athymic NCr *nu/nu* female mice were purchased from the Animal Production Area, National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The care and use of the animals were in accordance with institutional guidelines.

Tumor Growth in the mfp. Mice were anesthetized with Metofane (Pitman Moore, Inc., Washington, NJ), and a 5-mm incision was made in the skin over the lateral thorax. The mfp was exposed, and 10^6 cells in a volume of 0.1 ml of PBS was injected into the fatpad. The incision was closed with wound clips. The growth of the tumors was monitored by weekly examination, and growth rates were determined as the increase in mean tumor diameter, calculated from caliper measurements of two orthogonal diameters.

The Metastasis Therapy Protocol. For each synthetic glycoamine, the mice were divided into two experimental groups. For protocol A, mice were injected daily intraperitoneally, starting two days after tumor inoculation with either 0.2 ml of PBS or 0.2 ml of glycoamines in PBS (a total of 17 weeks of treatment). In group B, the treatment was started only when the mean tumor diameter was 10 mm. After 1 week, the mice were anesthetized, the tumors were resected, and the skin incisions were closed with wound clips. The mice were treated for an additional 4 weeks (a total of 5 weeks of treatment). Mice were killed at the end of treatment schedule, or when moribund, and examined for presence of lung metastases both macroscopically and microscopically. The lungs and tumors were fixed in 10% buffered formalin, and paraffin-embedded sections stained with H&E were examined.

Glycoamine solutions were prepared daily, by suspension of preweighed aliquots in sterile PBS to a final concentration of 10 μM/0.1 ml. The solutions were filtered through a 0.2-μm syringe filter before use.

Statistical analysis of the incidence of lung metastasis was performed using Fisher's exact test. The significance of differences in the numbers of lung metastasis was evaluated using the Mann-Whitney ranked sum test (31).

Multicell Aggregate Formation Assay

The cell aggregation assay was performed as described previously (25, 32, 33). Briefly, tumor cells were obtained from tumor tissue or harvested from subconfluent cultures using the standard trypsinization procedures. Aliquots containing 0.5–1.0 ml of cell suspension (0.5–1.0 × 10^6 cells/ml in serum-free RPMI 1640 or CMF-PBS) were placed in siliconized glass tubes or plastic cell culture tubes and agitated at 80 rpm for 1 h at 37°C. The aggregation was then terminated by fixing the cells with 1% formaldehyde in CMF-PBS [Ca^{2+}- and Mg^{2+}-free PBS (pH 7.2)]. Three samples from each tube were used for counting the number of single cells using a hemacytometer, and aggregation was calculated according to the equation: $(1 - N_t/N_c) \times 100$, where N_t and N_c represent the number of single cells in the presence of the test compounds and the number of single cells in the control medium (RPMI 1640 or CMF-PBS), respectively. Alternatively, three 20-μl aliquots from each sample were used for preparing total cytological preparations. In each preparation, the numbers of single cells and aggregated cells were counted in at least 10 random high-power microscopic fields, and the average number was calculated for individual samples. All cultures retained >90% viability throughout the duration of the experiments. Each determination was performed in triplicate, and the typical results of at least two separate experiments are presented.

Competition of Synthetic Glycoamine Analogues for Binding with β-Galactoside-specific PNA Lectin

Three synthetic glycoamines (Lac-L-Leu, Fru-D-Leu, and Fru-Gly) were analyzed for their abilities to compete for binding with β-galactoside-specific PNA lectin to the MDA-MB-435 cells as well as to the reference glycoprotein ASF (Sigma Chemical Co., St. Louis, MO), which displays β-Gal as a terminal sugar residue. The ASF was dissolved in transfer buffer [20 mmol/liter

[3] The abbreviations used are: mfp, mammary fat pad; ASF, asialofetuin; Fru-D-Leu, *N*-(1-deoxy-D-fructos-1-yl)-D-leucine; Fru-L-Leu, *N*-(1-deoxy-D-fructos-1-yl)-L-leucine; Lac-L-Leu, *N*-(1-deoxy-D-lactulos-1-yl)-L-leucine; Fru-Gly, *N*-(1-deoxy-D-fructos-1-yl)-glycine; Fru-L-Phe, *N*-(1-deoxy-D-fructos-1-yl)-L-phenylalanine; CMF, Ca^{2+}- and Mg^{2+}-free; PNA, peanut agglutinin; TBS, Tris-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/23/5319/2462122/cr0560235319.pdf by guest on 24 August 2022

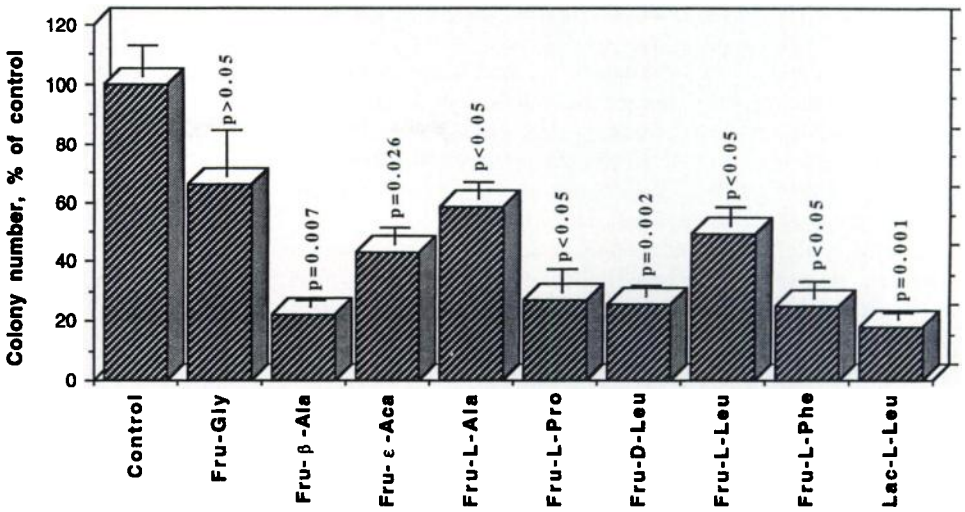

Fig. 1. Effect of synthetic glycoamines on MDA-MB-435 colony formation in 0.9% agarose. Clonogenic growth assay in agarose was performed as described in "Materials and Methods." The presence of synthetic glycoamines significantly inhibited the colony formation ability in hard agarose of MDA-MB-435 cells compared with control PBS-treated cells. The results are presented as the mean of triplicate experiments. *Bars*, SD.

Tris-base and 150 mmol/liter glycine (pH 7.5)], immobilized for 30 min at room temperature in humidified chamber (1 γ of glycoprotein per dot) on the nitrocellulose membranes (Gene Screen Hybridization Transfer Membrane; DuPont New England Nuclear), and blocked with 5% BSA-TBS [0.01 mol/liter Tris-HCl and 0.15 mol/liter NaCl (pH 7.4)] for 1 h at room temperature. The membranes were washed twice for 10 min each in TBS and once with buffer 1 and incubated for 1 h at room temperature in buffer 1 [0.05 mol/liter Tris-HCl, 0.15 mol/liter NaCl (pH 7.5), 1 mmol/liter $MgCl_2$, 1 mmol/liter $MnCl_2$, and 1 mmol/liter $CaCl_2$ (pH 7.5)], containing 6.25 γ/ml of horseradish peroxidase-PNA (Sigma) alone or with addition of 3 mM (final concentration) synthetic glycoamines. The membranes were subjected to 2 × 10 min washes in TBS [0.05 mol/liter Tris-HCl and 0.15 mol/liter NaCl (pH 7.5)] and developed with 3,3′-diaminobenzidine. Each determination was performed in duplicate, and the typical results of the three separate experiments are presented.

Apoptosis Induction Experiments

Induction of apoptosis in MDA-MB-435 human breast carcinoma cells by synthetic glycoamine analogues was performed as described previously (32). Cell viability in RPMI 1640 containing 0.1% FCS was assayed by acridine orange staining and/or trypan blue exclusion as reported earlier (32). Identification of apoptotic cells using the TUNEL method was performed exactly as described (32). The *in situ* cell death detection kit POD (Boehringer Mannheim Co., Indianapolis, IN) was used; the kit uses fluorescein-dUTP and an anti-fluorescein antibody conjugated to a peroxidase reporter molecule as detection reagent. DNA fragmentation analysis was performed at day 2 in each culture as described previously, using the same culture conditions of serum deprivation and saturation density (32), except that the induction of apoptosis in MDA-MB-435 cells was performed in the RPMI 1640 containing 0.1% FCS. The results shown are representative of at least three separate experiments.

Data Calculation and Presentation

Results of colony formation, cell aggregation, and apoptosis induction experiments are presented as means + 1 SD and *n* (the number of separate experiments). Statistical analysis was made using a two-tailed paired Student's *t* test to evaluate the statistical significance of the difference between the means. The concentration of the synthetic glycoamine analogues causing 50% inhibition of colony formation in agarose was obtained by plotting the percentage of inhibition *versus* the concentration of the compounds.

Synthesis, Purification, and Structural Characterization of Synthetic Glycoamine Analogues

Synthesis, purification, and full structural characterization of compounds tested in this study were described previously (23–25). Compound 1 was designated as Fru-Gly; compound 2 was designated as *N*-(1-deoxy-D-fructos-1-yl)-β-alanine; compound 3 was designated as *N*-(1-deoxy-D-fructos-1-yl)-γ-aminobutyric acid; compound 4 was designated as *N*-(1-deoxy-D-fructos-1-yl)-ε-aminocaproic acid; compound 5 was designated as *N*-(1-deoxy-D-fructos-1-yl)-L-alanine; compound 6 was designated as Fru-D-Leu; compound 7 was designated as Fru-L-Leu; compound 8 was designated as Fru-L-Phe; compound 9 was designated as *N*-(1-deoxy-D-fructos-1-yl)-L-proline); compound 10 was designated as Lac-L-Leu.

Results

Selection of Inhibitors of Colony Formation by MDA-MB-435 Cells in Dense Agarose

We have reported previously the inhibition of colony formation by MDA-MB-435 cells following exposure to a panel of nine synthetic glycoamines (25). Fig. 1 summarizes the results that led to the identification of four compounds that were most effective in the *in vitro* assay: Lac-L-Leu, Fru-D-Leu, Fru-L-Phe, and Fru-L-Leu. The IC_{50} doses of these compounds in the agarose assay were 2.3, 2.3, 2.7, and 3.0 mM, respectively. The three Leu analogues were selected for the *in vivo* study.

Nude Mouse-Human Breast Cancer Xenograft Experiments

Two experimental treatment protocols were used to determine the *in vivo* activity of the three selected synthetic glycoamines. In protocol A, the mice were treated daily from 2 days after tumor cell injection until the end of the experiment (17 weeks). In group B, daily treatment started when the mean tumor diameter was 10 mm. After 7 days of treatment, the primary tumors were removed, and the mice were treated for an additional 4 weeks (a total of 5 weeks of treatment).

Effect of Synthetic Glycoamines on Incidence and Growth of Primary Tumors. The growth curves of MDA-MB-435 mfp tumors in mice treated continuously from day 2 after cancer cell injection with Fru-D-Leu or Fru-L-Leu did not differ from the growth curves of the PBS-treated mice (data not shown). The weights of tumors on the control and treated mice at time of autopsy were similar, and the mean weights showed no statistical difference. The incidence of tumors in these mice did not differ, with 82% incidence in the PBS-treated group and 75% incidence in mice treated with either Fru-D-Leu or Fru-L-Leu. In mice that were scored as not having tumor at the end of the experiment, either no tumor was found at the site of injection or a mass of 2–3 mm was found that had shown no progressive growth. Histological evaluation of the small masses showed residual tumor

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/23/5319/2462122/cr0560235319.pdf by guest on 24 August 2022

cells, surrounded by fibrous connective tissues. The growth curves of MDA-MB-435 mfp tumors in mice treated continuously with Lac-L-Leu showed a trend toward inhibition of the local tumor, although a comparison of the weights of tumors removed from these mice at the time of autopsy [control mean weight = 0.69 + 0.6 g (mean + SD), test mean weight = 0.44 + 0.54 g] showed no statistically significant difference (Student's t test). A 100% incidence of tumors was recorded in group A mice treated with Lac-L-Leu.

Thus, the synthetic glycoamines did not have significant antitumor effects, and there was no difference in the tumor incidence or tumor growth rates of MDA-MB-435 tumors in mice treated continuously with synthetic glycoamines or with PBS.

Effect of Synthetic Glycoamines on Tumor Host. The treated animals had no apparent toxicity from the daily injection (up to 17 weeks of treatment) of synthetic glycoamines. Animal weights, skin texture, and overall behavior were unchanged between the control and the treated groups. No obvious pathology was noted in the histological slides of the livers, kidneys, or spleens of the treated mice. Thus, chronic treatment with synthetic glycoamines did not apparently lead to toxicity.

Effect of Synthetic Glycoamines on Metastasis. As shown in Table 1, daily treatment with synthetic glycoamines caused a significant reduction in both the incidence and number of spontaneous pulmonary metastases of MDA-MB-435 human breast carcinoma. The antimetastatic activity of synthetic glycoamines was detected in both experimental treatment protocols. Only 18–22% of control mice (4 of 18 control mice in group A and 3 of 17 control mice in group B) failed to develop pulmonary metastases compared with 83% to 57% of mice treated with Fru-D-Leu (5 of 6 treated mice in group A and 4 of 7 treated mice in group B) or 71% to 67% of mice treated with Lac-L-Leu (5 of 7 treated mice in group A and 4 of 6 treated mice in group B). In mice continuously treated with synthetic glycoamines according to the protocol A, the incidence of metastasis was decreased 4.6-fold ($P = 0.014$) and 2.7-fold ($P = 0.031$) in mice treated with Fru-D-Leu and Lac-L-Leu, respectively (Table 1). The antimetastatic activity of synthetic glycoamines was less profound in mice treated according to the protocol B, probably due to significantly shorter treatment period (a total of 17 and 5 weeks of treatment in protocols A and B, respectively). In mice treated daily with synthetic glycoamines according to the protocol B, the incidence of pulmonary metastasis was decreased 1.9-fold ($P = 0.069$) and 2.5-fold ($P = 0.042$) in mice treated with Fru-D-Leu and Lac-L-Leu, respectively (Table 1). Correspondingly, the average number of spontaneous pulmonary metastases was reduced from 37 in control mice to 0.2 ($P = 0.005$) and 0.9 ($P < 0.02$) in mice treated according to the protocol A with Fru-D-Leu and Lac-L-Leu, respectively (Table 1). A similar trend toward inhibition of the number of pulmonary metastasis in mice treated according to the protocol B was noticed in mice treated with Fru-D-Leu and Lac-L-Leu (Table 1). Treatment of mice with Fru-L-Leu did not have significant antimetastatic effect, and no reduction in metastasis incidence or number was noted in mice treated with this synthetic glycoamine analogue.

Therefore, we have identified two synthetic glycoamines (Fru-D-Leu and Lac-L-Leu) that were the effective inhibitor of spontaneous human breast cancer metastasis in nude mice.

Induction of Apoptosis in MDA-MB-435 Human Breast Carcinoma Cells by Synthetic Glycoamines

It had been demonstrated previously that synthetic glycoamines could inhibit homotypic aggregation of murine B16 melanoma cells by interfering with β-galactoside-specific lectin-mediated cell-cell interactions (25). Blocking of cell surface galectin-mediated cell-cell interactions resulted in induction of apoptosis in highly metastatic B16-F10 murine melanoma cell variants (32). We investigated whether these mechanisms are relevant to the bioactivity of synthetic glycoamines on MDA-MB-435 human breast carcinoma cells as well. Similar to the murine B16 melanoma model, synthetic glycoamines significantly inhibited homotypic aggregation of MDA-MB-435 cells and blocked binding of β-galactoside-specific PNA lectin to the MDA-MB-435 cells (Fig. 2). More than 50% reduction in homotypic aggregation of human breast carcinoma cells treated with 5 mM of Fru-D-Leu and Lac-L-Leu was observed. Treatment of MDA-MB-435 cells with synthetic glycoamines caused a 4–6-fold increase in the apoptosis level as determined by the TUNEL assay (Fig. 3*A*). Induction of apoptosis in glycoamine-treated highly metastatic MDA-MB-435 human breast carcinoma cells was confirmed using DNA fragmentation analysis, which clearly demonstrated an accumulation of low molecular weight apoptotic DNA in MDA-MB-435 cells at 48 h after treatment with antimetastatic synthetic glycoamines (Fig. 3*B*).

Discussion

We have identified two synthetic glycoamines that were effective inhibitors of spontaneous human breast cancer metastasis in nude mice. Continuous i.p. injection of Lac-L-Leu or Fru-D-Leu, beginning

Table 1 *Spontaneous lung metastasis of MDA-MB-435 human breast carcinoma in nude mice treated with synthetic glycoamines*

Protocol A: The mice were treated intraperitoneally daily from day 2 after injection of the MDA-MB-435 breast cancer cells, until the end of the experiment (17 weeks).
Protocol B: The mice were untreated until the mean tumor diameter was 10 mm, at which time daily i.p. treatment began. After 7 days, the tumors were resected, and the mice were treated for an additional 4 weeks (total of 5 weeks of treatment).

	Lung metastasis number[a]			
Treatment	Protocol A	*P*	Protocol B	*P*
Control (PBS)	37 (0–150)		32 (0–150)	
Fru-D-Leu (FDL)	0.2 (0–1)	0.005	22 (0–100)	>0.1
Lac-L-Leu (LL)	0.9 (0–5)	<0.02	8 (0–40)	>0.1
Fru-L-Leu (FLL)	39 (0–150)	>0.1	37 (0–150)	>0.1
	Incidence of lung metastasis[b]			
	Protocol A	*P*	Protocol B	*P*
Control (PBS)	78% (14/18)		82% (14/17)	
Fru-D-Leu (FDL)	17% (1/6)	0.014	43% (3/7)	0.069
Lac-L-Leu (LL)	29% (2/7)	0.031	33% (2/6)	0.042
Fru-L-Leu (FLL)	67% (4/6)	0.341	78% (7/9)	0.372

[a] Average number (and range) of lung metastases. Metastasis scored both macroscopically and microscopically. The mice that did not develop a primary tumor have been eliminated from the count. *P*s were calculated using the Mann-Whitney ranked sum test.

[b] Mice with lung metastases/mice with mfp tumors. Metastasis scored both macroscopically and microscopically. The mice that did not develop a primary tumor have been eliminated from the count. *P*s were calculated using Fisher's exact test.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/23/5319/2462122/cr0560235319.pdf by guest on 24 August 2022

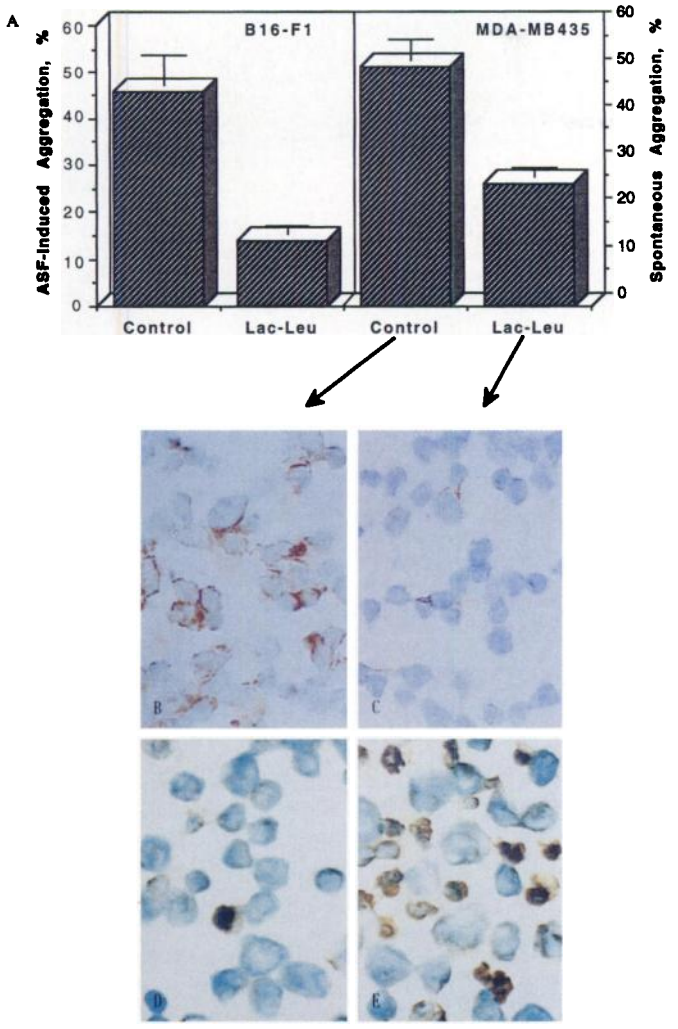

Fig. 2. Inhibition of ASF-induced B16-F1 murine melanoma and spontaneous MDA-MB-435 human breast carcinoma cell aggregation (*A*) and binding of β-galactoside-specific PNA lectin to the MDA-MB-435 cells (*B* and *C*) is accompanied by induction of apoptosis in target cells (*D* and *E*). Cell aggregation (*A*), lectin binding (*B* and *C*), and apoptosis induction (*D* and *E*) experiments were performed as described in "Materials and Methods." The evaluation of both cell aggregation and PNA lectin-binding activities was performed after 1 h of incubation of MDA-MB-435 cells. The apoptosis level was determined using the TUNEL assay after 2 days of cultivation of MDA-MB-435 cells in RPMI 1640 containing 0.1% FCS without (*Control*) and with addition of 2 mM (final concentration) synthetic glycoamines. Representative results of one of two similar experiments are presented. *Bars*, SD.

2 days after the inoculation of MDA-MB-435 human breast carcinoma cells in mfps of athymic nude mice, decreased the incidence of pulmonary metastasis by 2.7-fold and 4.6-fold, respectively. The average number of spontaneous pulmonary metastases was reduced from 37 in PBS-treated mice to 0.9 in Lac-L-Leu-treated mice and 0.2 in Fru-D-Leu-treated mice. The relative number of metastasis-free mice (without either macroscopic and microscopic metastases) was increased 3.8-fold in Fru-D-Leu-treated mice and 3.2-fold in Lac-L-Leu-treated mice. Chronic treatment (daily i.p. injection during 5 or 17 weeks of treatment) with synthetic glycoamines did not lead to any apparent toxicity.

In vitro screening of potential antimetastatic compounds using colony formation assay in dense agarose could be useful for selecting promising compounds for subsequent *in vivo* studies. According to our results, the most promising compounds were those that produced more than 70–80% inhibition of the clonogenic growth of the MDA-MB-435 cells in dense agarose. The effective *in vivo* antimetastatic compounds Fru-D-Leu and Lac-L-Leu caused 74 and 82% inhibition, respectively, of the colony formation in 0.9% agarose. Fru-L-Leu showed a 51% inhibition of colony formation in 0.9% agarose by MDA-MB-435 cells, yet did not produce any significant antimetastatic effect *in vivo*.

The synthetic glycoamines did not have a significant effect on tumor growth, and there was no difference in the primary tumor incidence of MDA-MB-435 mfp tumors in treated or control group mice. This observation is not surprising because synthetic glycoamines were described as inhibitors of cancer cell aggregation and adhesion mediated by cell surface β-galactoside-specific lectins (19, 25). Carbohydrate-mediated homotypic and heterotypic cancer cell aggregation and adhesion is thought to be one of the key events in the metastatic process (19–22, 23), which apparently does not play a significant role in growth of primary neoplasms. A similar selective antimetastatic effect without noticeable antitumor activity has been reported recently for another carbohydrate-based metastasis inhibitor. A modified citrus pectin was reported to act by interfering with β-galectin-mediated adhesion of metastatic

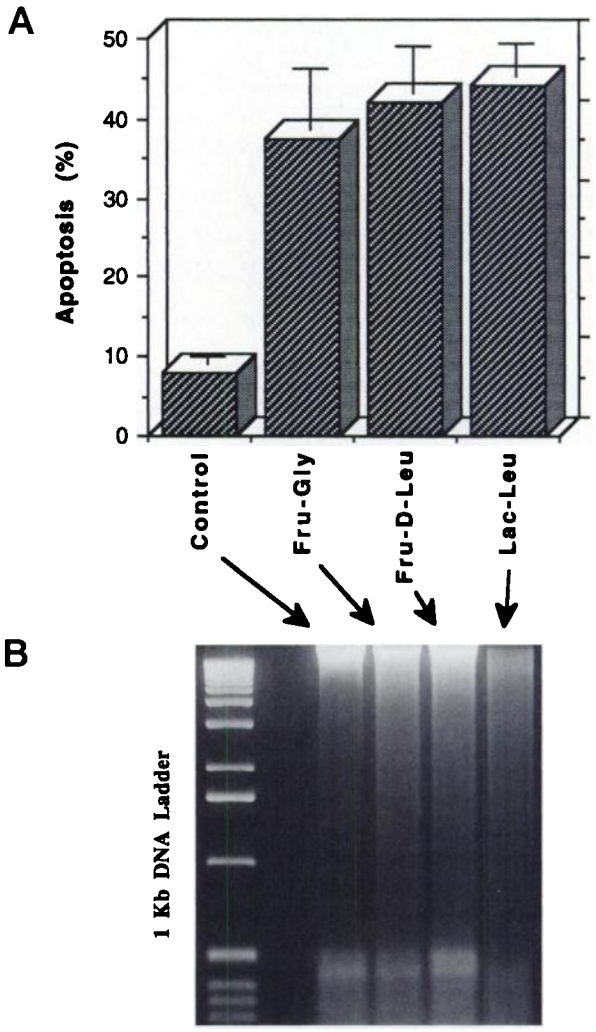

Fig. 3. Induction of apoptosis in MDA-MB-435 human breast carcinoma cells by antimetastatic synthetic glycoamines. MDA-MB-435 cells were incubated in RPMI 1640 containing 0.1% FCS without (*Control*) and with addition of 2 mM (final concentration) synthetic glycoamines. After 2 days of cultivation, the apoptosis level was determined using the TUNEL assay (*A*) and DNA fragmentation analysis by electrophoresis in 1.2% agarose gel and ethidium bromide staining (*B*) as described in "Materials and Methods." The counts of apoptotic cells were made on a minimum of 200 cells per culture. Representative results from one of two similar experiments are presented as a mean of triplicate determinations. *Bars*, SD.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/23/5319/2462122/cr0560235319.pdf by guest on 24 August 2022

cancer cells in a rat prostate carcinoma spontaneous metastasis model (34).

Two apparently unrelated chemical modifications of Fru-L-Leu molecule (substitution of L-Leu for D-Leu in Fru-D-Leu and addition of β-Gal residue in Lac-L-Leu) resulted in a dramatic increase in biological activities, as determined in both the clonogenic growth assay and the nude mouse experiments. It will be interesting to examine whether a combination of these two chemical modifications leads to the synthesis of even more effective antimetastatic compounds. The observation that synthetic glycoamines induce apoptosis *in vitro* in human metastatic breast cancer cells certainly may add a new dimension to our understanding of the molecular mechanisms of the biological activity of this class of molecules. However, it is not yet clear how the *in vitro* apoptosis induction activity of synthetic glycoamines is related to their *in vivo* antimetastatic activity and, obviously, further experiments are needed in this area.

This study used a single metastatic human tumor; experiments using additional cancer cell lines are necessary to fully establish the effectiveness of the synthetic glycoamines as antimetastatic agents. However, our data add further support to the concept of anticell adhesion therapy for cancer, particularly for treatment of a cancer metastasis of breast, ovarian, prostate, and colon tumors, and malignant melanoma (19–22, 25, 33, 34). Interference with the carbohydrate-mediated cell-cell recognition and adhesion may lead to reduced metastatic dissemination and may be useful as an adjuvant treatment along with surgery, radiation, and chemotherapy.

References

1. Howe, G. R., Hirohata, T., Hislop, T. G., Iscovich, J. M., Yuan, J-M., Katsouyami, K., Lubin, F., Marubini, E., Modan, B., Rohan, T., Toniolo, P., and Shunzhang, Y. Dietary factors and risk of breast cancer: combined analysis of 12 case-control studies. J. Natl. Cancer Inst., *82:* 561–569, 1990.
2. Wingo, P. A., Tong, T., and Bolden, S. Cancer statistics, 1995. CA Cancer J. Clin., *45:* 8–30, 1995.
3. Harris, J. R., Lippman, M. E., Veronesi, U., and Willett, W. Breast cancer (part 1). N. Engl. J. Med., *327:* 319–328, 1992.
4. Nomura, Y., Tashiro, H., and Hisamatsu, K. *In vitro* clonogenic growth and metastatic potential of human operable breast cancer. Cancer Res., *49:* 5288–5293, 1989.
5. Hug, V., Rashid, R., Johnston, D., and Hortobagyi, G. Clonogenic growth and hormone sensitivity of benign and malignant breast tissues. Br. J. Cancer, *56:* 619–621, 1987.
6. Cifone, M. A., and Fidler, I. J. Correlation of patterns of anchorage-independent growth with *in vivo* behavior of cells from a murine fibrosarcoma. Proc. Natl. Acad. Sci USA, *77:* 1039–1043, 1980.
7. Price, J. E. Clonogenicity and experimental metastatic potential of spontaneous mouse mammary neoplasms. J. Natl. Cancer Inst., *77:* 529–535, 1986.
8. Saiga, T., Kashu, I., Tabuchi, K., *et al.* Increased contraenvironmental pressure division capability: a decisive force in metastasis and invasion of mouse lung adenocarcinoma cell lines. Invasion & Metastasis, *9:* 254–268, 1989.
9. Fidler, I. J., Li, L., Ananthaswamy, H., Esumi, N., Radinsky, R., and Price, J. E. Correlation of growth capacity in hard agarose with successful transfection by the activated c-Ha-*ras* oncogene and *in vivo* proliferative capacity at metastatic sites. Anticancer Res., *11:* 17–24, 1991.
10. Li, L., Price, J. E., Fan, D., Zhang, R. D., Bucana, C. D., and Fidler, I. J. Correlation of growth capacity of human tumor cells in hard agarose with their *in vivo* proliferative capacity at specific metastatic sites. J. Natl. Cancer Inst., *81:* 1406–1412, 1989.
11. Zhang, R. D., Fidler, I. J., and Price, J. E. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. Invasion & Metastasis, *11:* 204–215, 1991.
12. Zhang, R. D., Price, J. E., Schackert, G., Itoh, K., and Fidler, I. J. Malignant potential of cells isolated from lymph node or brain metastases of melanoma patients and implications for prognosis. Cancer Res., *51:* 2029–2035, 1991.
13. Glinsky, G. V. Glycoamines. Biochemistry of a new class of humoral tumor markers. J. Tumor Markers Oncology, *4:* 193–221, 1989.
14. Glinsky, G. V. Glycoamines. Structural-functional characterization of a new class of humoral tumor markers *In:* S. Sell (ed.), Serological Cancer Markers, pp. 233–260. Totowa, NJ: The Humana Press, 1992.
15. Kuo, K. C., Gehrke, J. C., Allen, W. C., Holsbeke, M., Li, Z., Glinsky, G. V., Zumwalt, R. W., and Gehrke, C. W. High-performance liquid chromatographic analysis of glycoamines in serum. J. Chromatogr., *656:* 295–302, 1994.
16. Glinsky, G. V., Linetsky, M. D., Shemyakin, V. V., Markin, V. V., Liventsov, V. V., and Ivanova, A. B. Structural analysis of a new class of humoral tumor markers: characterization of 48 aminoglycoconjugates (glycoamines) isolated from rat and human blood plasma. J. Tumor Markers Oncology, *5:* 107–118, 1990.
17. Glinsky, G. V., and Linetsky, M. D. Structural analysis of the carbohydrate core of amino-glycoconjugates (glycoamines) from blood plasma of oncological patients. J. Tumor Markers Oncology, *5:* 137–160, 1990.
18. Glinsky, G. V., Linetsky, M. D., Liventsov, V. V., Surmilo, N. I., Ivanova, A. B., Sidorenko, M. V., and Vinnitsky, V. B. Glycoamine-synthesizing reaction: molecular mechanism of the incorporation of monomers into aminoglycoconjugates. J. Tumor Markers Oncology, *5:* 119–136, 1990.
19. Glinsky, G. V. Cell adhesion and metastasis: is the site specificity of cancer metastasis determined by leukocyte- endothelial cell recognition and adhesion? Crit. Rev. Oncol. Hematol., *14:* 229–278, 1993.
20. Glinsky, G. V. Glycodeterminants of melanoma cell adhesion. A model for antimetastatic drug design. Crit. Rev. Oncol. Hematol., *14:* 1–13, 1993.
21. Glinsky, G. V. The blood group antigen-related glycoepitopes: biological functions and implications in immunogenesis, cancer, and AIDS pathogenesis. The Medical Intelligence Units. Austin, TX: RG Landes Co., 1992.
22. Glinsky, G. V. The blood group antigens (BGA)-related glycoepitopes. A key structural determinant in immunogenesis and cancer pathogenesis. Crit. Rev. Oncol. Hematol., *12:* 151–166, 1992.
23. Mossine, V. V., Glinsky, G. V., and Feather, M. S. The preparation and characterization of some Amadori compounds (1-amino-1-deoxy-D-fructose derivatives) derived from a series of aliphatic ω-amino acids. Carbohydr. Res., *262:* 257–270, 1994.
24. Mossine, V. V., Glinsky, G. V., Barnes, C. L., and Feather, M. S. Crystal structure of an Amadori compound. *N*-(1-Deoxy-β-D-fructopyranos-1-yl)-glycine ("D-fructose-glycine"). Carbohydr. Res., *266:* 5–14, 1995.
25. Glinsky, G. V., Mossine, V. V., Price, J. E., Bielenberg, D., Glinsky, V. V., Ananthaswamy, H. N., and Feather, M. S. Inhibition of colony formation inagarose of metastatic human breast carcinoma and melanoma cells by synthetic glycoamine analogs. Clin. & Exp. Metastasis, *14:* 253–267, 1996.
26. Price, J. E., Polyzos, A., Zhang, R. D., and Daniels, L. M. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res., *50:* 717–721, 1990.
27. Fidler, I. J. The relationship of embolic homogeneity, number, size, and viability to the incidence of experimental metastasis. Eur. J. Cancer, *9:* 223–227, 1973.
28. Fidler, I. J. Selection of successive tumor lines for metastasis. Nat. New Biol., *242:* 148–149, 1973.
29. Fidler, I. J. Biological behavior of malignant melanoma cells correlated to their survival *in vivo*. Cancer Res., *35:* 218–224, 1975.
30. Hart, I. R. The selection and characterization of an invasive variant of the B16 melanoma. Am. J. Pathol., *97:* 587–592, 1979.
31. Zar, J. H. Biostatistical analysis, 2nd Ed. Englewood Cliffs, NJ: Prentice-Hall, Inc., 1984.
32. Glinsky, G. V., and Glinsky, V. V. Apoptosis and metastasis: a superior resistance of metastatic cancer cells to programmed cell death. Cancer Lett., *101:* 43–51, 1996.
33. Meromsky, L., Lotan, R., and Raz, A. Implications of endogenous tumor cell surface lectins as mediators of cellular interactions and lung colonization. Cancer Res., *46:* 5270–5275, 1986.
34. Pienta, K. J., Naik, H., Akhtar, A., Yamazaki, K., Replogle, T. S., Lehr, J., Donat, T. L., Tait, L., Hogan, V., and Raz, A. Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. J. Natl. Cancer Inst., *87:* 348–353, 1995.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/23/5319/2462122/cr0560235319.pdf by guest on 24 August 2022