Metastasis is a multistep cascade involving the migration of tumor cells from their site of origin, evasion of host defense systems, subsequent seeding of distant organs, and growth of secondary tumors. During metastatic dissemination, cells shed from the primary tumor enter the blood circulation and interact with platelets and leukocytes forming neoplastic emboli that can arrest in the microvasculature and adhere to the endothelium. Tumor cell adhesion is mediated in part through selectins, a family of cell surface carbohydrate-binding proteins (1–3). P-selectin on platelets facilitates platelet attachment to tumor cells forming a protective cloak and preventing tumor cell lysis by elements of the innate immune system (4–6). L-selectin-mediated adhesion of leukocytes results in formation of leukocyte-tumor cell emboli as well as local secretion of cytokines and growth factors thought to aid in secondary tumor growth (7–9). P-selectin and E-selectin on the endothelium facilitate tumor cells and emboli to anchor in the microvasculature (1, 2, 10, 11). The combined action of the selectins provides a mechanism that enables tumor cells to survive and to populate distant organs.

Sialylated, fucosylated tetrasaccharides, such as sLe\(^X\) (Sia\(\alpha\)2,3 Gal\(\beta\)1,4(Fuc\(\alpha\)1,3)GlcNAc\(\beta\)-) or sLe\(^a\) (Sia\(\alpha\)2,3Gal\(\beta\)1,3(Fuc\(\alpha\)1,4) GlcNAc\(\beta\)-), are two major carbohydrate determinants common to many selectin ligands (12, 13). A number of clinical studies show that expression of sLe\(^X\) and sLe\(^a\) on tumor cell mucins correlates directly with metastasis, tumor progression, and poor prognosis (see ref. 3 and references therein). Thus, therapeutic agents that target these carbohydrate ligands would offer a promising avenue for intervention (reviewed in refs. 14, 15). Towards this goal, we have developed carbohydrate-based inhibitors of sLe\(^X\) expression (6, 16–18). These agents consist of a disaccharide conjugated to a hydrophobic moiety, the most potent being acetylated per-O-acetylated GlcNAc\(\beta\)1,3Gal\(\beta\)-O-naphthalenemethan (AcGnG-NM; Fig. 1). Peracetylation facilitates passive diffusion of the disaccharide across cell membranes; inhibitory activity is lost when the disaccharide is deacetylated.

<table>
<thead>
<tr>
<th>Authors’ Affiliations:</th>
<th>1Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, 2Department of Medicine, Division of Pulmonary and Critical Care, and 3Department of Pathology, University of California, San Diego, La Jolla, California</th>
</tr>
</thead>
<tbody>
<tr>
<td>Received</td>
<td>12/15/05; revised 2/8/06; accepted 2/27/06.</td>
</tr>
<tr>
<td>Grant support:</td>
<td>NIH grants CA46462 and CA112278 (J.D. Esko), University Biotechnology Research and Training Grant (J.R. Brown), Tobacco-Related Disease Research Program fellowship grant TRDRP #BFT-0118 (M.M. Fuster), and U.S. Department of Veterans Affairs Research Career Development Award (M.M. Fuster).</td>
</tr>
<tr>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>Note:</td>
<td>C.A. Glass is currently at Zacharon Pharmaceuticals, 505 Coast Boulevard South, La Jolla, CA 92037. R. Li is currently at the Department of Ophthalmology, University of California, San Diego, La Jolla, CA 92039-0946.</td>
</tr>
<tr>
<td>Requests for reprints:</td>
<td>Jeffrey D. Esko, Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92039-0687. Phone: 858-822-1100; Fax: 568-534-5616; E-mail: <a href="mailto:jesko@ucsd.edu">jesko@ucsd.edu</a>.</td>
</tr>
<tr>
<td>©2006 American Association for Cancer Research.</td>
<td></td>
</tr>
<tr>
<td>doi:10.1158/1078-0432.CCR-05-2745</td>
<td></td>
</tr>
</tbody>
</table>

**Abstract**

**Purpose:** The binding of hematogenously borne malignant cells that express the carbohydrate sialyl Lewis X (sLe\(^X\)) to selectin adhesion receptors on leukocytes, platelets, and endothelial cells facilitates metastasis. The glycosylation inhibitor, per-O-acetylated GlcNAc\(\beta\)1,3Gal\(\beta\)-O-naphthalenemethan (AcGnG-NM), inhibits the biosynthesis of sLe\(^X\) in tumor cells. To evaluate the efficacy of AcGnG-NM as an antimetastatic agent, we examined its effect on experimental metastasis and on spontaneous hematogenous dissemination of murine Lewis lung carcinoma and B16BL6 melanoma cells.

**Experimental Design:** Tumor cells were treated *in vitro* with AcGnG-NM, and the degree of selectin ligand inhibition and experimental metastasis was analyzed in wild-type and P-selectin-deficient mice. Conditions were developed for systemic administration of AcGnG-NM, and the presence of tumor cells in the lungs was assessed using bromodeoxyuridine labeling *in vivo*. The effect of AcGnG-NM on inflammation was examined using an acute peritonitis model.

**Results:** *In vitro* treatment of Lewis lung carcinoma cells with AcGnG-NM reduced expression of sLe\(^X\) and P-selectin-dependent cell adhesion to plates coated with P-selectin. Treatment also reduced formation of lung foci when cells were injected into syngeneic mice. Systemic administration of the disaccharide significantly inhibited spontaneous dissemination of the cells to the lungs from a primary s.c. tumor, whereas an acetylated disaccharide not related to sLe\(^X\) in structure had no effect. AcGnG-NM did not alter the level of circulating leukocytes or platelets, the expression of P-selectin ligands on neutrophils, or sLe\(^X\)-dependent inflammation.

**Conclusion:** Taken together, these data show that AcGnG-NM provides a targeted glycoside-based therapy for the treatment of hematogenous dissemination of tumor cells.
membranes, allowing entry into the Golgi, where \( \text{SLe}^X \) assembly takes place. The acetylated disaccharide undergoes deacetylation by endogenous esterases, which allows it to act as an intermediate in the biosynthesis of \( \text{SLe}^X \). Assembly of oligosaccharides on the disaccharide “decoys” the assembly of \( \text{SLe}^X \) from cellular glycoconjugates; thus, AcGnG-NM acts as an inhibitor of selectin-ligand formation. We have previously shown that AcGnG-NM diminishes tumor seeding in an experimental metastasis model in which human tumor cells pretreated with disaccharide were injected i.v. into immunocompromised mice (6). Although these data showed the antimitastatic potential of AcGnG-NM, the experimental conditions did not mimic the relevant clinical setting of spontaneous metastasis in which tumor cells seed and colonize sites distant from the primary tumor.

As a prelude to clinical trials, we have developed a model for systemic administration of the acetylated disaccharide in mice using syngeneic tumor cell lines to study spontaneous metastasis. Herein, we show that systemic treatment with AcGnG-NM attenuates spontaneous pulmonary metastatic seeding. The effect was remarkably selective because the inhibitor had no effect on leukocyte infiltration in response to an experimental inflammatory stimulus, which also depends on selectin-carbohydrate interactions. These findings suggest that treatment with acetylated disaccharide uniquely targets the metastatic seeding behavior of tumors cells, setting the stage to explore the use of disaccharides as adjuvant therapy for blocking hematogenous tumor metastasis.

**Materials and Methods**

**Disaccharides and cell lines.** AcGnG-NM and per-O-acetylated Gal[3GlcNAc]-O-naphthalenaldehyde were prepared as described (19). Murine Lewis lung carcinoma (LLC) cells were purchased from the American Type Culture Collection (LLC1, CRL-1642; Manassas, VA). Murine B16BL6 melanoma cells were a kind gift from J.I. Fidler (University of Texas, M.D. Anderson Cancer Center). Cells were grown in DMEM (4.5 g glucose/L) supplemented with sodium bicarbonate (1.59 g/L for LLC and 3.7 g/L for B16BL6), 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT), glutamine (0.3 g/L), streptomycin sulfate (100 \( \mu \)g/mL), and penicillin (100 units/mL). Cells were maintained at 37°C in a humidified incubator containing 5% CO\(_2\) and 95% air and passed every 4 days using ATV solution (Invitrogen, Carlsbad, CA).

**ELISA,** **flow cytometry,** **cell adhesion,** and **enzyme assays.** To measure the presence of selectin ligands, cells were grown to confluency for 4 to 7 days in six-well plates with and without AcGnG-NM. Binding of monoclonal antibody (mAb) CSLEX-1 was measured by ELISA as described (18). Sialylated and fucosylated cell surface glycans were measured by reacting cells with biotinylated \( \text{Vibrio cholerae} \) haemaglutinin (10 \( \mu \)g/mL) or biotinylated \( \text{Aleuria aurantia} \) lectin (2 \( \mu \)g/mL; Vector Laboratories, Burlingame, CA), respectively. After incubation with streptavidin-R-phycocerythrin (1.85 \( \mu \)g/mL; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), the cells were analyzed by flow cytometry (FACScan; BD Biosciences, San Jose, CA).

Cell adhesion to immobilized P-selectin was done in 96-well plates coated overnight at 4°C with recombinant P-selectin (2 \( \mu \)g/mL; R&D Systems, Minneapolis, MN) and blocked with 1% bovine serum albumin in PBS as described (6, 18).

Total fucosyltransferase and sialyltransferase activities were assayed in cell lysates prepared from LLC cells as described (18).

In some experiments, cells or whole blood were incubated with mAbs to the following antigens: CA19-9 (anti-\( \text{SLe}^X \), 120 \( \mu \)g/mL; Chemicon, Temecula, CA), Gr-1 (myeloid differentiation antigen, 12 \( \mu \)g/mL; CD45 (leukocyte common antigen, 26 \( \mu \)g/mL), CD41 (integrin \( \alpha_{IIb}\beta_{3} \) chain expressed on platelets, 10 \( \mu \)g/mL), CD62P (P-selectin, 10 \( \mu \)g/mL; BD Biosciences). Murine selectin/IgM chimeras were used to probe the tumor cells for selectin ligands (Ps-IgM, Ls-IgM, and Es-IgM, a kind gift from J.B. Lowe, University of Michigan). The cells were washed and incubated with goat antibodies against mouse or human IgM or IgG (30 \( \mu \)g/mL, Sigma, St. Louis, MO) conjugated to FITC and analyzed by flow cytometry.

**Experimental metastasis using AcGnG-NM-pretreated cells.** LLC cells were grown for 4 to 5 days in the presence or absence of 50 \( \mu \)mol/L AcGnG-NM. The cells were then harvested with EDTA, resuspended in 0.9% saline, and injected (2 \( \times 10^4 \) per 150 \( \mu \)L) into the lateral tail vein of anesthetized (inhaled isoflurane, Janssen Pharmaceuticals, Titusville, NJ) 8- to 12-week-old Es1(e) mice (bred in a C57Bl/6 background, a kind gift from P. Potter, University of North Carolina via Charles River Laboratories; ref. 20). These mice carry a mutant allele of the esterase locus Es-1 designated Es1(e) (21) that results in reduced plasma esterase activity (22). After 10 days, lung metastases were detected by visible nodules on the surface of the lungs and quantitated under a dissecting microscope. Representative pictures were taken.

**Spontaneous metastatic seeding of LLC cells after systemic AcGnG-NM treatment.** To measure the effect of systemic administration of AcGnG-NM on metastasis, Alzet osmotic minipumps (Durect Corp., Cupertino, CA) containing vehicle (DMSO/propylene glycol, 1:1, v/v) or AcGnG-NM (150 mg/mL) were surgically implanted in a dorsal skin fold of LLC cells (5 \( \times 10^4 \) per 150 \( \mu \)L) were injected s.c. in the hindquarter of P-selectin-deficient mice (bred in a C57Bl/6 background, a kind gift from P. Potter, University of North Carolina) and stained with H&E. Nuclei were counterstained with methyl green. Representative pictures were taken.

To measure the extent of BrdUrd labeling, lungs were incubated with collagenase (10 mg/mL, 1 h, 37°C), dispersed by repeated passage through an 18-gauge needle, and filtered through a 40-\( \mu \)m pore nylon filter. The cells were fixed (70% ethanol, 1 \( \times 10^4 \) cells/mL), and the relative number of BrdUrd-labeled cells was determined by reacting the cells with mouse FITC-labeled anti-BrdUrd antibody followed by flow cytometry. Each lung dispersion was incubated with FITC-conjugated isotype control antibody to set the lower threshold for BrdUrd-positive cells, and we gated on labeled cells that were positive for propidium iodide staining. In some experiments, the animals received a control disaccharide, per-O-acetylated Gal[3GlcNAc]-O-naphthalenaldehyde. To determine how metastatic seeding depended on P-selectin, LLC cells (6 \( \times 10^4 \) ) were implanted s.c. in the hindquarter of P-selectin-deficient mice (P-/-) bred on a C57Bl/6 background (The Jackson Laboratory, Bar Harbor, ME).

The effect of systemic administration of AcGnG-NM on experimental metastasis was measured in animals 2 weeks after implantation of
pumps. LLC cells (2 × 10^5 cells per 150 μL) were injected into the lateral tail vein, and 2 weeks later, each animal was injected i.p. with BrdUrd and processed as described above.

**Thioglycollate inflammation model.** Acute peritoneal inflammation (peritonitis) was induced by injection of 3% thioglycollate broth (2 mL), with sterile pyrogen-free saline as control (25). Groups of five animals per experimental condition were injected and sacrificed after 3 hours. The peritoneal cavities were lavaged with ice-cold saline containing 3 mmol/L EDTA (8 mL) to prevent aggregation, and cells were counted in the lavage fluid using a particle counter. The cells were also stained with FITC-conjugated rat anti-mouse Gr-1 mAb and counted by flow cytometry.

**Statistics.** All statistical analyses were done using Prism software. Statistics were calculated by either one-way ANOVA test comparing three groups of four to seven animals or by Student’s t test comparing two groups of four to seven animals as indicated in the individual experiments.

**Results**

**Sensitivity of LLC to AcGnG-NM.** LLC was derived from a C57BL/6 mouse and spontaneously metastasizes from primary s.c. tumors to the lung, mimicking the aggressiveness of clinical pulmonary metastatic tumors (26–28). LLC cells also reacted strongly in vitro with a chimeric selectin composed of the carbohydrate recognition domain of murine P-selectin and the Fc region of human IgM (ref. 29; Fig. 2A). In contrast, the cells only weakly reacted with an L-selectin-IgM chimera and not at all with an E-selectin-IgM chimera. The cells also bound mAbs that recognize sLe^X (CSLEX-1) and sLe^A (CA19-9; data not shown).

Treatment of LLC cells with AcGnG-NM decreased binding of CSLEX-1 in a dose-dependent manner, with partial inhibition occurring as low as ~ 10 μmol/L (Fig. 2B). In contrast, the compound had no effect on sLe^A expression, which only differs from sLe^X in the linkage arrangement of the sugars [Siaα2,3Galβ1,3(Fucα1,4)GlcNAc-] versus Siaα2,3Galβ1,4(Fucα1,3)GlcNAc-]. Treatment of cells with low concentrations of AcGnG-NM also significantly reduced cell adhesion to plates coated with recombinant P-selectin (Fig. 2B). Control experiments using sialidase, which removes the crucial sialic acid determinant for selectin binding, decreased adhesion to the same extent as AcGnG-NM. Addition of a P-selectin antibody and EDTA blocked adhesion confirming the specificity of the interaction. These agents decreased adhesion to a greater extent than AcGnG-NM, suggesting that a second ligand insensitive to AcGnG-NM might be present, such as sulfatide (3-sulfoGalβ1,4Glcβ1-O-ceramide; ref. 30). Disaccharide inhibition of adhesion also depended on the structure of the ligand.

**Fig. 2.** Selectin ligands on LLC cells. A. LLC cells were incubated for 1 h with mouse P-selectin, L-selectin, and E-selectin IgM chimeras and analyzed by flow cytometry (solid lines; Materials and Methods). The shaded peaks represent samples incubated without chimeras. Incubation with 5 mmol/L EDTA prevented binding of P-selectin chimera to the cells (dashed line). B. Treatment of LLC cells with AcGnG-NM showed a concentration-dependent decrease in binding of mAb CSLEX-1 as measured by ELISA (Materials and Methods). Adhesion to immobilized P-selectin was also altered. The extent of inhibition was comparable with that achieved by sialidase treatment. Adhesion was blocked by anti-P-selectin mAb (20 μg/mL) or by the inclusion of 5 mmol/L EDTA in the incubation. Columns, average of triplicate measurements, which varied by <10%.

C. Treatment of LLC cells with 50 μmol/L AcGnG-NM reduced binding of *A. aurantia* lectin (*AAL*), which reacts with α1,3/6-linked fucose, but had no effect on binding of *M. amurensis* hemaglutinin (*MAH*), which reacts with α2,3-linked sialic acid. Cells were analyzed by flow cytometry, and the average fluorescence value was normalized to that obtained for a sample of cells that had not been treated with AcGnG-NM. D. Total sialyltransferase (*SiaT*) and fucosyltransferase (*FucT*) activity was measured in LLC cells by assaying the transfer of radiolabeled sialic acid to Galα1,4GlcNAcβ1-O-NM (SiaT) and radiolabeled fucose to Galα1,4GlcNAcβ1-O-NM (FucTb). Columns, average of duplicate measurements, which varied by <10%.
compound because per-O-acetylated GaLβ1,3Galβ-O-naphthalenemethanol, which is not an intermediate in sLeX biosynthesis, did not affect binding to CSLEX-1 or adhesion to P-selectin-coated plates (data not shown).

To determine how AcGnG-NM inhibited P-selectin ligand formation in LLC cells, cells were reacted with FITC-conjugated M. amurensis hemagglutinin, which recognizes α2,3-linked sialic acid and 3-O-sulfated galactose containing glycans (31) and A. aurantia lectin, which recognizes α1,3/6-linked fucose (32). Binding of M. amurensis hemagglutinin did not change after treatment with AcGnG-NM, whereas binding of A. aurantia lectin decreased by ~40% (Fig. 2C), suggesting that fucosylation was selectively altered. In previous studies of human LS180 colon carcinoma cells, we showed that the disaccharide can also affect sialylation. The different modes of inhibition of sLeX formation in different cell types (i.e., fucosylation versus sialylation) seems to correlate inversely with the relative activities of fucosyltransferases and sialyltransferases involved in sLeX formation (18). In LLC cells, fucosyltransferase activity using Galβ1,4GlcNAcβ-O-NM (FucTa; 33.4 pmol/mg/h) or NeuAcα2,3Galβ1,4GlcNAc (FucTb; 13.2 pmol/mg/h) as the acceptor was lower than the sialyltransferase activity (65.6 pmol/mg/h) measured with Galβ1,4GlcNAcβ-O-NM as the acceptor (Fig. 2D), consistent with idea that the compound inhibits P-selectin ligand formation by blocking fucosylation.

AcGnG-NM inhibits experimental pulmonary metastasis of LLC cells. To test whether inhibition of sLeX by AcGnG-NM diminished experimental pulmonary metastasis, mice were injected i.v. with AcGnG-NM-treated or vehicle-treated LLC cells. After 10 days, the animals were sacrificed, and the lungs were analyzed for surface tumor foci. Disaccharide treatment dramatically reduced the incidence of macroscopic tumor foci [16 ± 6 (n = 7) versus 77 ± 12 (n = 5); P = 0.003; Fig. 3]. Some animals injected with disaccharide-treated cells had only one to two nodules, showing the strong antimetastatic effect of the compound in this model.

Prior studies have shown that genetic deletion of P-selectin can reduce experimental metastasis of tumor cells by blocking platelet-tumor cell aggregation and tumor cell-endothelial cell interactions (33, 34). To determine if LLC cell metastasis also depended on P-selectin, cells were i.v. injected in wild-type and P-selectin-deficient mice (Fig. 3C). The number of tumor foci was dramatically reduced in P-selectin deficient mice (12 ± 6 versus 73 ± 27, P < 0.001). When disaccharide-treated cells were injected into P-selectin-deficient mice, no further reduction in lung metastases was observed. The lack of additive or synergistic effects of P-selectin deficiency and AcGnG-NM suggested that the disaccharide inhibited experimental pulmonary metastasis through a pathway involving P-selectin.

AcGnG-NM inhibits spontaneous metastatic seeding of the lung. The blockade of experimental metastasis by AcGnG-NM encouraged us to examine the effect of the disaccharide on spontaneous pulmonary metastasis. Because these studies would require systemic administration of the compound to mice, we first examined the stability of AcGnG-NM. Incubation of radiolabeled disaccharide with mouse serum for 3 hours resulted in extensive removal of the acetyl groups due to serum esterases but no hydrolysis of the glycosidic linkages. Because uptake of the compound depends on the presence of the acetyl groups to facilitate passive diffusion (16), we examined the stability of the compound in serum obtained from an esterase-reduced Es1(e) mouse, which has a hypomorphic allele of serum Es-1 (21). Acetylated disaccharide in serum from Es1(e) mice was stable for several hours, which approximated conditions in human serum (22). Thus, all subsequent studies were done in Es1(e) mice.

We first attempted to administer the compound by tail vein injection. However, this method of delivery led to the collapse of the vein and necrosis of the tail after repeated injection of the vehicle. To circumvent this problem, the compound was dissolved in DMSO/propanol glycol (1:1, v/v) and placed in small Alzet osmotic pumps, which were surgically implanted under a dorsal skin fold. Based on the theoretical delivery rate of 0.25 μL/h, the animals received an effective dose of ~0.9 mg/d/mouse, which translates to a maximum dose of ~45 mg/kg/d. Detailed pharmacokinetic and pharmacodynamic studies to determine the actual steady-state concentration achieved by continuous infusion have not been done. Thus, the calculated concentration should be considered the maximum that could be achieved under these conditions.

To measure spontaneous pulmonary metastasis, animals were injected s.c. with LLC cells in the hindquarter. After 4 weeks, large tumors arose at the s.c. site, but secondary metastatic nodules were rare in the lungs and other tissues. In histologic sections stained with H&E, we found numerous cells with characteristics of malignant tumor cells embedded in the
lung parenchyma (Fig. 4A; ref. 28). These cells exhibited nuclear pleomorphism, increased nuclear/cytoplasmic ratios, irregular nuclear membranes, and in some cases prominent nucleoli. Many of the metastatic cells were within capillaries, and some had already entered the alveolar spaces. Because humane treatment of the animals required euthanasia when the primary tumors reached about 20% of the animal mass, we could not extend the duration of the experiments to determine if micrometastatic foci formed in the lungs. Attempts to surgically resect the primary tumor to extend the experiments were unsuccessful because the primary tumors were invasive and grew back rapidly before nodule formation in other organs.

To determine the effect of AcGnG-NM on tumor cell seeding of the lungs, we injected BrdUrd and stained tissue sections with anti-BrdUrd mAb. Numerous brown-red–stained cells were present in samples obtained from animals with a tumor (Fig. 4A, arrows). The brown-red stain left by the substrate highlights the sharp but irregularly shaped nuclear membranes of the malignant cells. Examination of lung sections from BrdUrd-labeled mice without a tumor did not show any malignant cell infiltrates (Fig. 4B). Sections were prepared from the primary tumor as well (Fig. 4C). H&E staining of the tumor mass showed malignant cells with pleomorphic nuclei, altered nuclear/cytoplasmic ratios, and many mitotic figures. Immunohistochemical staining for incorporated BrdUrd showed mitotically active nuclei scattered within the tumor but did not label all of the tumor cells presumably because they were at different stages of the cell cycle.

To determine whether BrdUrd labeling was suitable for quantitative measurement of tumor cells, the lungs were removed and treated with collagenase. The resulting cell suspension was then analyzed by flow cytometry after permeabilizing the cells and staining them with anti-BrdUrd mAb. The percentage of BrdUrd-positive cells in the lungs from animals bearing tumors was $3.1 \pm 0.8\%$, whereas animals that had no tumor contained $0.7 \pm 0.2\%$ BrdUrd-positive cells ($n = 4, P = 0.0018$). Because not all tumor cells took up BrdUrd, this measurement actually underestimates the tumor cell burden in the lungs (Fig. 4C). Nevertheless, the labeling method provides a way to estimate metastatic seeding of the lungs and the effect of potentially antimetastatic agents (28, 35).

To determine the antimetastatic effect of AcGnG-NM, pumps containing vehicle or disaccharide were surgically implanted, and 1 day later, LLC cells were injected s.c. in the hindquarter. After 4 weeks, the animals were labeled with BrdUrd, and the lungs were processed for flow cytometry. As shown in Fig. 5A, continuous systemic administration of disaccharide resulted in a decreased proportion of BrdUrd-labeled cells compared with animals receiving only vehicle. The experiment was repeated with three sets of mice on different days, with consistently the same result (average values: $1.4 \pm 0.2\%$ BrdUrd-labeled cells in disaccharide-treated animals versus $2.9 \pm 0.3\%$ in animals treated with vehicle; $P < 0.001$). Subtracting the background of BrdUrd-labeled cells in naive animals from the data ($0.7 \pm 0.1\%$) showed that the disaccharide decreased seeding by $\approx 3$-fold. Averaging the data in this way underestimates the efficacy of AcGnG-NM because several of the
A potential approach to block hematogenous metastasis involves targeting selectins and their ligands, which facilitate the aggregation of tumor cells with platelets and leukocytes and tumor cell adhesion to the endothelium. These cell-cell interactions enhance tumor cell resistance to cytolytic leukocytes.

### Discussion

The majority of therapeutic strategies for treating cancer focus on cytotoxic agents designed to alter growth and/or differentiation of the primary tumor (37). These agents have helped reduce the death rate from many types of cancer, but the overall cancer mortality rate has not changed significantly since the 1950s due to changes in cancer demographics (38). An open area for therapeutic development includes agents that target aspects of tumor progression required for metastasis, such as invasion, angiogenesis, and survival of metastatic cells in the microvasculature. These steps constitute the major cause of morbidity and mortality in patients with epithelial tumors, suggesting that antimitastatic agents in conjunction with cytotoxic agents could greatly improve survival. One recently successful approach involves inhibition of tumor angiogenesis. Avastin (bevacizumab) targets endothelial growth factors and their receptors and has proven effective in combination with cytostatic agents for increasing progression-free survival and decreasing overall mortality (39). However, at present, the ability to intervene in other steps of metastasis is extremely limited.

A potential approach to block hematogenous metastasis involves targeting selectins and their ligands, which facilitate the aggregation of tumor cells with platelets and leukocytes and tumor cell adhesion to the endothelium. These cell-cell interactions enhance tumor cell resistance to cytolytic leukocytes.
present in the circulation (4–6) and provide growth factors that enhance tumor growth (7–9), suggesting that inhibitors of selectins would decrease tumor cell survival and metastasis. In this report, we showed that systemic administration of an acetylated synthetic disaccharide will inhibit spontaneous metastatic seeding of the lungs from cells shed from a primary s.c. tumor. To our knowledge, this is the first report showing that pharmacologic inhibition of tumor glycosylation in this way can reduce spontaneous metastatic seeding of the lungs.

In addition to AcGnG-NM, other metabolic inhibitors of the carbohydrate ligands for selectins have been described. For example, 4-fluoro-N-acetylglucosamine and N-acetylgalactosaminides inhibit sLeX synthesis (40–42). Treating tumor cells with N-acetylgalactosaminides also prevents tumor cell adhesion (43), but to our knowledge, this compound has not been tested in models of spontaneous metastasis. Very high concentrations (usually 1–10 mmol/L) of both 4-fluoro-N-acetylglucosamine and N-acetylgalactosaminides are needed to alter selectin ligand expression. In contrast, AcGnG-NM acts in the 10 to 50 μmol/L range in vitro, making it less likely to have toxic side effects in vivo. Indeed, systemic administration of the compound for 28 days at ~45 mg/kg/d did not cause acute toxicity, weight loss, obvious changes in behavior, or alterations of standard blood chemistry and inflammatory responses.

Other strategies for blocking selectin-carbohydrate interactions may prove useful for treating tumor cell dissemination and metastasis as well, including (a) competition by soluble recombinant forms of selectins or their glycopeptide and glycolipid ligands; (b) competition by peptides derived from the primary sequence of the carbohydrate binding site in selectins; (c) anti-selectin antibodies, (d) oligosaccharides related to sLeX and sLeα; (e) inositol polyanions and sulfated sugars; (f) molecular mimics of sLeX, including oligonucleotides; and (g) unfraccionated and low molecular weight heparins (for a review, see refs. 44–47). Heparin also blocks tumor metastasis probably through multiple mechanisms (e.g., blockade of P- and L-selectin and inhibition of chemokine/cytokine interactions with cell surface proteoglycans expressed on tumor cells or the vasculature reviewed in refs. 47, 48). Unlike heparin, AcGnG-NM has a specific mechanism of action, blocking selectin ligand formation without causing any change in hemostasis.

Because AcGnG-NM inhibits the formation of selectin ligands on a number of tumor cell lines in vitro (6, 16–18), it may also be applicable to blocking metastatic seeding of organs from a variety of carcinomas in vivo. Several human tumor lines (e.g., LS180 colon carcinoma, PC-3 prostatic carcinoma, and A549 and A427 lung adenocarcinomas) possess selectin ligands sensitive to AcGnG-NM (6, 18). However, measuring spontaneous metastatic tumor formation using these models has been difficult due to the low efficiency of tumor growth from spontaneously shed cells. To circumvent this problem, indirect methods have been used to measure disseminated tumor cells in tissues [e.g., lacZ expression (28), green fluorescent protein fluorescence of tagged cells (49), and PCR to detect species specific sequence tags (50)]. The advantages of BrdUrd labeling include its low cost, the absence of any prior manipulation of the tumor cell or immune response (e.g., by transfection of green fluorescent protein or lacZ; ref. 51), and its effectiveness for measuring mitotic cells independent of the type of tumor. Its primary disadvantage is that it underestimates the extent of seeding because only mitotic cells take up the nucleoside.

### Table 1. Blood cell counts

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Untreated (n = 375)</th>
<th>Vehicle treated (n = 15)</th>
<th>AcGnG-NM treated (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6.5 ± 2.8</td>
<td>7.9 ± 1.6</td>
<td>7.8 ± 2.6</td>
</tr>
<tr>
<td>Neutrophils (10⁴/μL)</td>
<td>1.1 ± 0.7</td>
<td>1.9 ± 1.0</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Lymphocytes (10³/μL)</td>
<td>5.0 ± 2.5</td>
<td>5.6 ± 0.7</td>
<td>6.1 ± 2.1</td>
</tr>
<tr>
<td>Platelets (10³/μL)</td>
<td>981 ± 267</td>
<td>1146 ± 251</td>
<td>1031 ± 174</td>
</tr>
<tr>
<td>Red blood cells (10⁹/μL)</td>
<td>8.64 ± 1</td>
<td>10.8 ± 1.8</td>
<td>10.5 ± 0.9</td>
</tr>
</tbody>
</table>

Fig. 6. AcGnG-NM has no effect on leukocytes. Blood samples were collected from Es1(e) mice 4 weeks after surgical implantation of osmotic pumps containing vehicle or AcGnG-NM. Gr-1-positive neutrophils (A) and CD45-positive lymphocytes (B) were stained for P-selectin ligands using P-selectin IgM chimera. CD41-positive platelets were stained for P-selectin using CD62P mAb. D, acute peritoneal inflammation (peritonitis) was induced by injection of 3% thioglycollate (ref. 25; Materials and Methods). Cells in the peritoneal lavage fluid were stained with FITC-conjugated rat anti-mouse Gr-1 mAb and counted by flow cytometry. Statistics were calculated by a Student’s t test comparing two groups (n = 4–5 animals).
The data presented here provide crucial preclinical animal data needed to begin phased trials in cancer patients. Because AcGN-G-NM is “first in class,” further studies are needed to determine the optimal formulation, bioavailability, and maximum tolerable dose. Modification of the disaccharide by deoxygenation, fluorination, or by addition of reactive groups may enhance its pharmacologic properties. Because the formation of selectin ligands depends on a complex biosynthetic pathway involving several enzymes and oligosaccharide intermediates, other acetylated disaccharide intermediates could provide additional candidates for drug development (19). AcGN-G-NM may be just the first of a new class of disaccharide-based agents that function as specific inhibitors of cancer metastasis.

Acknowledgments

We thank David Ditto of the University of California, San Diego, Hematology Core for blood counts and Drs. Ajit Varki and Jennifer Stevenson for many helpful discussions.

References

A Disaccharide-Based Inhibitor of Glycosylation Attenuates Metastatic Tumor Cell Dissemination

Jillian R. Brown, Mark M. Fuster, Ruixia Li, et al.


Updated version Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/12/9/2894

Cited articles This article cites 49 articles, 15 of which you can access for free at: http://clincancerres.aacrjournals.org/content/12/9/2894.full#ref-list-1

Citing articles This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/12/9/2894.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/12/9/2894. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.