Identification of Sulfated Oligosaccharide-based Inhibitors of Tumor Growth and Metastasis Using Novel in Vitro Assays for Angiogenesis and Heparanase Activity

Christopher R. Parish, Craig Freeman, Kathryn J. Brown, Douglas J. Francis, and William B. Cowden

Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra 2601, Australia

ABSTRACT

Inhibitors of tumor angiogenesis and metastasis are rapidly emerging as important new drug candidates for cancer therapy. To facilitate the identification of such drugs, we recently developed novel and rapid in vitro assays for human angiogenesis and for the extracellular matrix-degrading enzyme heparanase, which has been implicated in tumor metastasis. In this study, sulfated oligosaccharides, which are structural mimics of heparan sulfate, were investigated as drug candidates because these compounds may interfere with heparan sulfate recognition by many angiogenic growth factors and may inhibit cleavage of heparan sulfate by heparanase. In the preliminary screening studies, it was found that inhibitory activity in both assay systems was critically dependent on chain length and degree of sulfation, highly sulfated linear oligosaccharides of five or more monosaccharides in length being the most active. However, two sulfated oligosaccharides stood out as potential antitumor drugs, phosphomannopentaose sulfate (PI-88) and maltohexaose sulfate, both of these compounds having the important property of simultaneously being potent inhibitors of in vitro angiogenesis and heparanase activity. Due to the ease of manufacture of the starting material, phosphomannopentaose sulfate (PI-88) was studied in more detail. PI-88 was shown to inhibit the primary tumor growth of the highly invasive rat mammary adenocarcinoma 13762 MAT by $\geq 50\%$, inhibit metastasis to the draining popliteal lymph node by $\geq 40\%$, and reduce the vascularity of tumors by $\geq 30\%$, all of these effects being highly significant. Acute hematogenous metastasis assays also demonstrated that PI-88 was a potent (>$90\%$) inhibitor of blood-borne metastasis. Thus, by the use of novel in vitro screening procedures, we have identified a promising antitumor agent.

INTRODUCTION

It is now well established that solid tumor growth is critically dependent on the growth of new vessels from preexisting blood vessels surrounding the tumor, a process called angiogenesis (1–4). On the basis of this finding, the development of drugs that inhibit angiogenesis has become an attractive approach to cancer therapy (2–6). Furthermore, such drugs have the advantage of selective toxicity, ready access to target tissue (i.e., tumor blood vessels), and, because the drugs target the tumor-associated vasculature rather than the tumor cells, little chance of drug resistance developing during therapy. One of the major difficulties in identifying antiangiogenic drugs, however, is the availability of simple and physiologically relevant in vitro assays for human angiogenesis. Current screening procedures usually involve highly artificial, expensive, time consuming, and technically complex in vivo models of angiogenesis. Recently, our laboratory developed a novel in vitro assay for human angiogenesis that is ideally suited to identifying substances that inhibit human angiogenesis (7). The procedure measures the spontaneous angiogenic response of human placental blood vessel fragments embedded in a fibrin gel, can be performed in microcultures, and can be readily quantified by digital image analysis.

Inhibition of metastasis represents another attractive approach for the treatment of highly malignant tumors. In many patients, it is the tumor metastases and not the primary tumor that are life-threatening. Although antiangiogenic drugs would be expected to restrict the growth of secondary tumors (2, 4, 6), substances that directly interfere with tumor cell invasion and the subsequent spread of tumor cells to distant sites would also be of considerable clinical benefit. A popular approach in this area has been the development of compounds that inhibit degradative enzymes involved in tumor cell invasion. Such enzymes facilitate tumor cell spread by degrading the ECM surrounding tumors and by solubilizing the vascular basement membrane, thus, enabling tumor cells to both enter into and escape from blood vessels and lymphatics. ECM and basement membrane consist of a complex network of molecules, the predominant molecular components being collagen, fibronectin, laminin, vitronectin, and HSPG (8). In the past, most drug development programs have concentrated on the identification of compounds that inhibit proteases involved in ECM solubilization, recently the most notable of these being inhibitors of matrix metalloproteinases (9). In contrast, the endoglycosidase heparanase, which degrades the heparan sulfate sidechains of the HSPGs in the ECM, has not often been a target for metastasis inhibition despite there being considerable evidence implicating the enzyme in tumor cell invasion (10, 11), with heparanase seeming to act synergistically with proteases in degrading the ECM (11). A major reason for the lack of studies of heparanase inhibition has been due to the absence of a simple and rapid assay for heparanase activity. In fact, heparanase activity has been known for over 20 years and yet there is still considerable controversy about the molecular properties and identity of the enzyme (reviewed in Ref. 12). Recently, we reported a highly quantitative and rapid heparanase assay (13) that has enabled us to purify human platelet heparanase to homogeneity (12). Subsequent studies have led us to propose that the heparanase expressed by metastatic tumor cells and other cell types is identical to the platelet enzyme (14). Therefore, the platelet enzyme has been used in all our subsequent screening studies for inhibitors of tumor heparanase. Furthermore, these data suggest that, unlike the large number of proteases that can solubilize polypeptides in the ECM, there is only one heparanase used by cells to degrade ECM heparan sulfate. Thus, the heparanase enzyme represents an extremely attractive target for the development of new antmitastatic drugs.

With the availability in our laboratory of novel in vitro assays for angiogenesis and heparanase enzyme activity, a comprehensive screening program was undertaken to identify new angiogenesis and heparanase inhibitors. Structural mimics of heparan sulfate were con-

Received 1/25/99; accepted 5/14/99.

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 Funded by Progen Industries Limited, Brisbane, Australia.

2 To whom requests for reprints should be addressed, at Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra, ACT 0200, Australia. Phone: 61-2-6249-2604; Fax: 61-2-6249-2595; E-mail: Christopher.Parish@anu.edu.au.

3 The abbreviations used are: ECM, extracellular matrix; BM, basement membrane; HSPG, heparan sulfate proteoglycan; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; VEGF, vascular endothelial growth factor; PI-88, phosphomannopentaose sulfate; HRG, histidine-rich glycoprotein; mAb, monoclonal antibody.

4 C. Freeman, A. M. Browne, and C. R., Parish. Evidence that platelet and tumor heparanases are similar, if not identical, enzymes, submitted for publication.
sidered as an attractive class of compounds to investigate because there is now clear evidence that many angiogenic growth factors, such as bFGF and VEGF, are heparan sulfate binding with recognition of cell surface heparan sulfate being required for growth factor action (15–16). Thus, an objective of the present study was to synthesize sulfated oligosaccharides as heparan sulfate mimics, which block heparan sulfate recognition by growth factors and inhibit cleavage of heparan sulfate by heparanase. In the case of inhibiting the binding of growth factors to cell surface HS PGs, it was reasoned that low molecular weight mimics of heparan sulfate should be particularly effective because it is now believed that cell surface heparan sulfates aid dimerization of growth factor receptors by growth factors (15). Furthermore, sulfated oligosaccharides should be effective heparanase inhibitors by acting as noncleavable substrates of this enzyme. In addition, a particular emphasis of the drug screening program was to identify sulfated oligosaccharides that simultaneously inhibited angiogenesis, by blocking angiogenic growth factor action, and reduced tumor metastasis by inhibiting heparanase activity. This study describes the successful use of this in vitro approach to identify PL-88 as a drug candidate, subsequent in vivo studies demonstrating that PL-88 significantly inhibits tumor growth, metastasis, and angiogenesis.

**MATERIALS AND METHODS**

**Preparation of Sulfated Oligosaccharides.** Maltose, raffinose, stachyose, chondroitin-6-sulfate, bovine lung heparin, and the cyclohexa-, hepta-, and octa-amyloses were purchased from Sigma Chemical Co. (St. Louis, MO). Maltootriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were obtained from Seikagaku (Tokyo, Japan) and represent oligosaccharides purified from limited amylase digests of the exopolysaccharide produced by the yeast *Pichia holstii* (strain NRRL Y-2448, formerly *Hansenula holstii*). The method for the growth of *P. holstii* and isolation of phosphomannopentaose was based on that described previously (18). Briefly, the crude exopolysaccharide was isolated from aerobically grown yeast culture supernatants as a potassium salt by ethanol precipitation. Acid hydrolysis was then used to liberate the phosphomannopentaose from the phosphomannan monoester core of the exopolysaccharide. The phosphomannan monoester core and the phosphomannopentaose were then separated from each other as barium monoester core of the exopolysaccharide. The phosphomannopentaose was then separated from each other as barium monoester core of the exopolysaccharide. The phosphomannopentaose was prepared from the exopolysaccharide produced by the diploid yeast *Pichia holstii* (strain NRRL Y-2448, formerly *Hansenula holstii*). The method for the growth of *P. holstii* and isolation of phosphomannopentaose was based on that described previously (18). Briefly, the crude exopolysaccharide was isolated from aerobically grown yeast culture supernatants as a potassium salt by ethanol precipitation. Acid hydrolysis was then used to liberate the phosphomannopentaose from the phosphomannan monoester core of the exopolysaccharide. The phosphomannan monoester core and the phosphomannopentaose were then separated from each other as barium salts by differential ethanol precipitation and, subsequently, by gel filtration. The oligosaccharide that inhibited cell adhesion by 50% (IC50). One volume of a solution of sulfur trioxide-pyridine complex (Aldrich, Castle Hill, NSW, Australia) in dimethyl formamide was added to a suspension of oligosaccharide (–50 mg/ml) in dimethyl formamide and pyridine (2 vol:3 vol). Routinely, the sulfur trioxide-pyridine complex was used at a 2-fold molar excess over the number of free hydroxyl groups in the oligosaccharide. In some cases, undersulfated oligosaccharides were prepared by using lower concentrations of sulfur trioxide-pyridine complex. The mixture was heated at 80°C for 2 h. The supernatant was decanted while still warm, and the sticky residue was washed thoroughly with methanol three times. After decanting the residual methanol, the product was dissolved in water and neutralized (to pH 6) with barium acetate (–0.7 g in 5 ml of water) with vigorous stirring. After centrifugation (3000 × g), the overlying liquid was decanted and the precipitated barium sulfate pellet was washed thoroughly with water. The overlying liquid and washings were combined and applied to a column (2.5 × 14 cm) of DOWEX 50W-X8–400 cation exchange resin (H+ form; Bio-Rad Laboratories, Hercules, CA). The column was eluted with water until the eluate was neutral. The eluate was stirred and neutralized (to pH 7) with sodium acetate. The solution was diluted with acetone and centrifuged (3000 × g) to separate the product. The pellet was finely pulverized by crushing under methanol, stirred while still under methanol, and then the solid was filtered and washed several times with methanol to give the sulfated oligosaccharide. The resultant sulfated oligosaccharides were not contaminated with barium ion (determined by microanalysis and flame ionization) nor nitrogen (microanalysis). To assess purity and degree of sulfation the C, H, S, Na, and P content of each sulfated oligosaccharide preparation was determined by microanalysis. The homogeneity of sulfated oligosaccharide preparations was also assessed by electrophoresis of samples in 30% polyacrylamide gels using the discontinuous buffer system of Laemmli (19) in the absence of SDS. Sulfated oligosaccharides were visualized in the polyacrylamide gels by toluidine blue staining (20).

**Human Angiogenesis Assay.** The assay method used is based on a previously described procedure (7). Blood vessels, –1–2 mm in diameter and 2–5 cm in length, were excised from the surface of human placenta obtained from the Canberra Hospital within 24 h of an elective cesarean birth. Approval to use human placentas was granted by the Australian Capital Territory Department of Health and Community Care Ethics Committee. The vessels were placed in HBSS containing 2.5 μg/ml fungizone and cut into 1–2 mm length fragments. Similar angiogenic responses were obtained from blood vessels of venular and arterial origin, but, for each assay, vessel fragments from only one vessel were used. Angiogenesis assays were performed in 24- or 48-well culture plates (Costar, Cambridge, MA). In the 24-well format, 30 μl of bovine thrombin (0.15 M NaCl; Sigma Chemical Co.) were added to each well, followed by 1.0 ml/well of 3 mg/ml bovine fibrinogen (Sigma Chemical Co.) in Medium 199. The thrombin and fibrinogen were mixed rapidly, and one vessel fragment was quickly placed in the center of the well before clot formation. Usually, fibrin gel formation occurred in 30 s, and the vessel fragment was left suspended in the gel. After gel formation, 1.0 ml/well of Medium 199 supplemented with 20% FCS, 0.1% α-arginine caproic acid, L-glutamine, and antibiotics (genticin and fungizone), and with or without inhibitors was added. In the 48-well format, all reagent volumes were halved. Vessels were cultured at 37°C in a humidified environment for 14–21 days, with the medium being changed twice weekly. Angiogenesis was quantified by computer-based image analysis, using NIH Image software of digital images of the cultures obtained with a Dycam 3.04 digital camera (Dycam Inc., Chatsworth, CA), mounted on an inverted microscope (Olympus, Tokyo, Japan).

**Heparanase Assay.** The heparanase assay has been described in detail elsewhere (13). The assay is based on the observation that the serum protein HRG binds to heparan sulfate chains, masking the heparanase cleavage site, and that heparanase-cleaved heparan sulfate fails to bind to immobilized HRG. Briefly, human platelet heparanase (10 mg of protein), purified to homogeneity as described previously (12), was added to an incubation mixture consisting of 90 pmol of radiolabeled [*3H*] heparan sulfate in 0.05 M-sodium acetate buffer (pH 5.1) containing 5 mM-N-acetylmannosamine, 0.1 mg/ml BSA, and differing concentrations of the inhibitor to be tested in a total volume of 20 μl. After incubation for 30 min at 37°C, the products were separated from the substrate by passage through a mini-column containing HRG-Sepharose beads. Enzyme activity was expressed as pmol product formed/hour/mg protein.

**Assessment of the Effect of Sulfated Oligosaccharides on the FGF-Heparan Sulfate Interaction.** The FGF-heparan sulfate interaction was assessed, as reported earlier (21), by measuring the binding of BALB/c 3T3 fibroblasts to plastic immobilized FGFs, cell binding being detected by Rose Bengal staining of adherent cells. Sulfated oligosaccharides were examined for their ability to inhibit this cell adhesion process, which is totally dependent on heparan sulfate structures on the surface of BALB/c 3T3 cells, as previously described (21). Data were expressed as the concentration of sulfated oligosaccharide that inhibited cell adhesion by 50% (IC50).

**Metastasis and Tumor Growth Assays.** The antitumor activity of the different sulfated oligosaccharides was assessed using the highly metastatic rat mammary adenocarcinoma 13762 MAT (22). The tumor cells were maintained in vitro as previously reported (22). In the acute hematogenous metastasis assay, female Fischer 344 rats (10–13 weeks of age) were given injections of 2 × 106 13762 MAT cells in 0.6 ml of RPMI 1640 (Life Technologies, Inc., Grand Island, NY) medium containing 10% FCS in a lateral tail vein. Usually, at the time of tumor cell injection, animals were also injected with different doses of sulfated oligosaccharide, similar results being obtained if the oligosaccharide was injected i.v., i.p., or s.c. However, in some experiments, the sulfated oligosaccharides were injected up to 6 h before or 6 h after the tumor cells or were administered for 3 days before tumor cell injection by i.p.-inserted 7-day Alzet mini-osmotic pumps (model 2 ML1; Alza Corp., Palo Alto, CA).
Lungs were removed from the rats 13 days after tumor cell injection, placed in Bouin’s solution for at least 24 h, and lung metastases were then assessed under a dissecting microscope. The number of lung metastases in sulfated oligosaccharide-treated rats was compared with that observed in control animals, with a minimum of four animals being included in each group.

In the primary tumor growth and lymph node metastasis experiments, rats were given s.c. injections of 10^6 13762 MAT tumor cells in the hind footpad. The sulfated oligosaccharide PI-88 was continually administered to the animals by i.p.-inserted 14-day Alzet mini-osmotic pumps (model 2 ML2; Alza Corp.), which were usually inserted 7 days after tumor cell injection and continually delivered drug until the animals were sacrificed. On days 18–21, the animals were sacrificed and primary tumor diameters were measured, with tumor volume being calculated according to the formula:

\[
\text{Tumor volume} = \frac{\text{length} \times \text{width}}{2}
\]

The draining popliteal lymph nodes were removed and weighed, and lymph node cell suspensions were prepared in PBS containing 0.1% BSA, with viable cells being separated from dead cells by centrifugation of the cell suspension on a cushion of Isopaque/Ficoll, as described earlier (23). Lymph node cell suspensions were reacted, using previously published methods (24), with a leukocyte-specific mAb against rat CD45 [clone OX-1; kindly provided by Dr. J. Sedgwick (Centenary Institute, Sydney, Australia)] and a myeloid-specific mAb against rat Mac-1 [clone OX-42; Serotec Ltd., Blackthorn, Bicester, United Kingdom], mAb binding being detected by phycoerythrin-coupled rabbit F(ab')₂, antimouse immunoglobulin (DAKO Corp., Carpinteria, CA), and fluorescence flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). Rat 13762 MAT tumor cells were detected by their ability to bind fluorescein-labeled hyaluronic acid, after incubation with 25 μg/ml fluorescein-labeled hyaluronic acid for 30 min on ice, and their failure to bind the leukocyte-specific mAb CD45. Hyaluronic acid (human umbilical cord; Sigma Chemical Co.) was coupled with fluorescein isothiocyanate after CNBr activation, as described previously (25).

In some experiments, tumors were grown in s.c. air pouches to aid removal and assessment of vascularization by hemoglobin content. Air pouches were created on the dorsum of female Fischer 344 rats (16–18 weeks of age) by the leukocyte-specific mAb against rat CD45. Hyaluronic acid (human umbilical cord; Sigma Chemical Co.) was coupled with fluorescein isothiocyanate after CNBr activation, as described previously (25).

Quantification of Tumor Vascularization. Tumors from the s.c. air pouches were weighed, individually frozen in test tubes and, usually 24 h later, thawed. Approximately 20 ml of distilled water were then added/gram of tumor tissue, and the tumor homogenized with a blade homogenizer until it had fully disintegrated. The debris was then pelleted by centrifugation (3000 g, 5 min), and the supernatant, which contained hemoglobin, was collected. The concentration of hemoglobin in the supernatant was determined by the catalytic action of hemoglobin on the oxidation of 3,3′, 5,5′-tetramethylbenzidine by hydrogen peroxide, as outlined by the manufacturers (Plasma Hemoglobin Kit; Sigma Chemical Co.).

**Statistical Analysis.** Data are shown as mean ± SE. The values were analyzed by a two-tailed unpaired t test between the drug-treated group and the untreated control group. A P < 0.05 was considered statistically significant.

**Animal Ethics.** All animal experimental protocols were approved by the Australian National University Animal Experimentation Ethics Committee and were carried out according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

### RESULTS

**Preparation and Characterization of Sulfated Oligosaccharides.** To produce a range of sulfated oligosaccharides for *in vitro* testing, advantage was taken of two classes of naturally occurring oligosaccharides of defined structure. The first class consisted of oligosaccharides that required no further degradation and fractionation, examples of this class being maltose [Glc-(1→4)-Glc], raffinose [Gal-α-(1→6)-Glc-α-(1→2)-Frc], stachyose [Gal-α-(1→6)-Gal-α-(1→6)-Glc-α-(1→2)-Frc], and the cyclodextrans [cyclic Glc-α-(1→6)-Glc containing oligosaccharides]. The second class consisted of oligosaccharides obtained from naturally occurring polysaccharides that were partially degraded enzymatically or chemically and size fractionated. Examples of this class are the amylose (source of maltose series of oligosaccharides) and chondroitin-derivated oligosaccharides and phosphomannopentaose from the yeast *P. holstii*. Initially, all oligosaccharides were sulfated under conditions that resulted in maximal sulfation although, in later studies, variably sulfated preparations of phosphomannopentaose and maltohexaose were synthesized. Due to steric crowding effects, it was usually extremely difficult to achieve 100% substitution of all free hydroxyl groups of an oligosaccharide. The extent of sulfation of oligosaccharides was analyzed by electrophoresis of the preparations on high-density polyacrylamide gels. Differently sulfated forms of the oligosaccharides were then visualized by toluidine blue staining. This technique could readily distinguish oligosaccharides that differed by as little as a single sulfate moiety. Usually, each sulfated oligosaccharide preparation was found to contain two to three dominant species of sulfated material on toluidine blue-stained gels. With oligosaccharides that were more extensively studied, the sulfate content was accurately determined by microanalysis.

#### Table 1 Inhibition of human angiogenesis, heparanase activity, and tumor metastasis by sulfated forms of naturally occurring oligosaccharides

<table>
<thead>
<tr>
<th>Compound</th>
<th>Number of saccharide units</th>
<th>50% inhibitory concn (μg/ml)</th>
<th>Angiogenesis</th>
<th>Heparanase</th>
<th>Metastasis (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>60</td>
<td>&gt;2000</td>
<td>1</td>
<td>20 ± 3^a</td>
<td>20 ± 3^a</td>
</tr>
<tr>
<td>Phosphomannopentaose SO₄ (PT-88)a</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>31 ± 3^b</td>
<td></td>
</tr>
<tr>
<td>Raffinose SO₄</td>
<td>3</td>
<td>200</td>
<td>50</td>
<td>73 ± 4</td>
<td></td>
</tr>
<tr>
<td>Stachyose SO₄</td>
<td>4</td>
<td>2000</td>
<td>12</td>
<td>48 ± 9^c</td>
<td></td>
</tr>
<tr>
<td>Maltose SO₄</td>
<td>2</td>
<td>2000</td>
<td>&gt;1000</td>
<td>99 ± 9</td>
<td></td>
</tr>
<tr>
<td>Maltotetraose SO₄</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>72 ± 9</td>
<td></td>
</tr>
<tr>
<td>Maltohexaose SO₄</td>
<td>6</td>
<td>2</td>
<td>1.5</td>
<td>24 ± 7b</td>
<td></td>
</tr>
<tr>
<td>Cyclohexaamytlose SO₄</td>
<td>6</td>
<td>200</td>
<td>8</td>
<td>67 ± 17</td>
<td></td>
</tr>
<tr>
<td>Cycloheptaamyllose SO₄</td>
<td>7</td>
<td>200</td>
<td>7</td>
<td>53 ± 25</td>
<td></td>
</tr>
<tr>
<td>Cyclooctaamyllose SO₄</td>
<td>8</td>
<td>200</td>
<td>5</td>
<td>36 ± 6^d</td>
<td></td>
</tr>
<tr>
<td>Chondroitin tetra SO₄</td>
<td>4</td>
<td>2000</td>
<td>&gt;30</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Chondroitin hexa SO₄</td>
<td>6</td>
<td>2000</td>
<td>45</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Chondroitin octa SO₄</td>
<td>8</td>
<td>1000</td>
<td>ND</td>
<td>ND</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>Suramin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Percentage control metastasis ± SE (n = 4) in lungs of rats receiving 2 × 10^3 13762 MAT cells i.v. and 2 mg/rat of each compound i.v. at the time of tumor cell injection.

^b Highly significant (P < 0.001) inhibition of metastasis.

^c Phosphomannopentaose isolated from the yeast *P. holstii*.

^d Significant (P < 0.05) inhibition of metastasis.

^e ND, not determined.
Effect of Different Sulfated Oligosaccharides on Angiogenesis, Heparanase Activity, and Metastasis. After synthesis of a range of sulfated oligosaccharides, they were examined for their ability to inhibit human angiogenesis and human heparanase activity. Initially, they were tested in our in vitro assays for these biological activities, and then selected sulfated oligosaccharides were tested for antimetastatic activity in vivo using the highly metastatic rat mammary adenocarcinoma 13762 MAT in an acute hematogenous metastasis assay (22). Table 1 summarizes the results obtained with 12 representative sulfated oligosaccharides. The biological activities of suramin, a modest antiangiogenic compound and heparanase inhibitor (26, 27), and heparin are also included in Table 1 for comparison.

Three of the sulfated oligosaccharides were quite potent inhibitors of in vitro human angiogenesis, namely PI-88 (P. holstii derived), maltotetraose sulfate, and maltohexaose sulfate (Table 1). PI-88 and maltohexaose sulfate were the most potent of these compounds with a 50% inhibitory concentration of 2 μg/ml, whereas maltotetraose sulfate gave 50% inhibition at 10 μg/ml. An example of the pronounced inhibition of angiogenesis induced by 20 μg/ml maltohexaose sulfate is depicted in Fig. 1. Representative titrations of angiogenesis inhibition by the maltose series of sulfated oligosaccharides and by PI-88 are depicted in Fig. 2. It can be seen that with the maltose series, maltose sulfate had little inhibitory activity, whereas maltotetraose and maltohexaose sulfate were quite potent inhibitors (Fig. 2). These data indicate that chain length is a critical factor in determining the antiangiogenic activity of a sulfated oligosaccharide, with an oligosaccharide of more than four monosaccharides in length being the most effective. However, the nature of the oligosaccharide backbone also seems to be important because sulfated chondroitin tetra-, hexa-, and octa-oligosaccharides lacked inhibitory activity. Similarly, sulfated cyclohexa-, hepta-, and octa-amylases displayed only low antiangiogenic activity. Furthermore, it is interesting to note that in this angiogenesis assay system the polysulfonated compound, suramin, an antiangiogenic compound that has entered clinical trials (28), was 25-fold less inhibitory than the most potent sulfated oligosaccharides, whereas the sulfated glycosaminoglycan heparin exhibited no antiangiogenic activity (Table 1).

All of the angiogenesis experiments presented in Table 1 involved the addition of sulfated oligosaccharides to the culture medium at the commencement of the angiogenesis assays. Although less effective, PI-88 or maltohexaose sulfate, when added after commencement of the angiogenesis response, also inhibited further vessel outgrowth (data not shown).
The sulfated oligosaccharides also differed markedly in their heparanase inhibitory activity, the most potent inhibitors being PI-88 and maltohexaose sulfate, the activity of these two compounds resembling that of heparin (i.e., 50% heparanase inhibitory concentrations of 2, 1.5, and 1 μg/ml, respectively (Table 1). Interestingly, these two compounds were also the most effective antiangiogenic compounds detected. However, angiogenesis inhibition did not correlate with the heparanase inhibitory activity of many compounds. The most striking example of this is heparin, which was a potent heparanase inhibitor, but did not inhibit angiogenesis. Similarly, the sulfated cycloamyloses were relatively effective heparanase inhibitors, but poor angiogenesis inhibitors. The maltose series was also very informative regarding chain length and heparanase inhibition, with the disaccharide (maltose sulfate) being noninhibitory, maltotetraose sulfate exhibiting modest inhibitory activity, and maltohexaose sulfate exhibiting high inhibitory activity (Table 1). Additional experiments (data not shown) revealed that the maltopenta-, hexa-, and hepta-saccharide sulfates were comparable heparanase inhibitors. Thus, a sulfated pentasaccharide or greater is required for optimal heparanase inhibition although, as with angiogenesis inhibition, the nature of the oligosaccharide backbone is also an important factor.

The sulfated oligosaccharides were also tested in vivo for their antimetastatic activity (Table 1), the compounds being administered at the same time as the i.v. injection of 13762 MAT tumor cells, and their effect on subsequent lung metastases was determined. With the experimental data presented in Table 1, the compounds were injected i.v., but similar results were obtained when the compounds were injected s.c. or i.p. (data not shown). In general, there was a correlation between in vitro heparanase inhibition and the ability of a compound to inhibit in vivo metastasis. Thus, PI-88 and maltohexaose sulfate, the two sulfated oligosaccharides with the highest heparanase inhibitory activity, exhibited the greatest antimetastatic activity, and, in fact, they closely resembled heparin in their ability to prevent metastasis (Table 1). Two other compounds, cyclooctaamylose sulfate and stachyose sulfate, also significantly inhibited tumor metastasis, a property consistent with their modest heparanase inhibitory activity. A number of other modest heparanase inhibitors (e.g., cyclohexa- and cyclohepta-amyllose sulfate) caused some reduction in lung metastases, but these effects were not statistically significant. Similarly, suramin was a modest heparanase inhibitor, but only produced a slight reduction in tumor metastases, which was not statistically significant.

Although heparanase is involved in angiogenesis, the fact that the antiangiogenic activity of the compounds did not always directly correlate with their heparanase-inhibitory activity indicated that the sulfated oligosaccharides were inhibiting angiogenesis by some other mechanism. Sulfated oligosaccharides have been shown to perturb the action of angiogenic growth factors by disrupting growth factor-heparan sulfate interactions (29–31). Also, we have previously shown (7) that the human angiogenesis assay used in this study is largely dependent on endogenous bFGF and, to a lesser extent, on aFGF and VEGF action, all these growth factors being heparan sulfate binding. Thus, the various sulfated oligosaccharides were examined for their ability to act as competitors of the interaction of bFGF and aFGF with cell surface heparan sulfate. The competition assay involved measuring the ability of the sulfated oligosaccharides to inhibit the binding of murine fibroblasts to immobilized bFGF or aFGF, bound cells being quantified by Rose Bengal staining. We have previously successfully used this assay to study the binding of FGF to cell surface heparan sulfate and to identify inhibitors of this interaction (21). It was found that, with increasing chain length, the maltose series of sulfated oligosaccharides became more effective inhibitors of the interaction of bFGF and aFGF with cell surface heparan sulfates (i.e., maltose was weakly inhibitory), whereas the penta-, hexa-, and hepta-saccharides were the most active (Table 2). PI-88 also exhibited considerable inhibitory activity in this system (Table 2). The complete inhibition curves for the inhibition of the aFGF-heparan sulfate interaction by the maltose series of sulfated oligosaccharides are presented in Fig. 3.

The influence of degree of sulfation on the biological activity of maltohexaose sulfate, one of the most active antiangiogenic and antimetastatic compounds, was examined in some detail (Table 3). Sulfation was found to be essential for biological activity because unsulfated maltohexaose and phosphomannopentaose were inactive in all assays. With increasing sulfation, there was a steady increase in the ability of maltohexaose to inhibit heparanase activity and FGF binding to heparan sulfate. However, inhibitory activity plateaued in both systems when sulfation was 85% or greater. Metastasis inhibition studies (Table 3) also demonstrated that with an increasing degree of sulfation, maltohexaose sulfate became a more effective antiangiogenic drug, with the extensively sulfated variants being highly significant inhibitors of tumor metastasis. Angiogenesis inhibition experiments also showed that only the highly sulfated preparations exhibited antiangiogenic activity (data not shown). In related studies, it was shown that two undersulfated phosphomannopentaose preparations, which were 21% and 56% sulfated, respectively, exhibited little or no angiogenesis, heparanase, or metastasis inhibitory activity.

<table>
<thead>
<tr>
<th>Sulfated oligosaccharide</th>
<th>Sulfation</th>
<th>% sulfation</th>
<th>IC$_{50}$ (μg/ml)$^b$</th>
<th>bFGF</th>
<th>aFGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose SO$_4$</td>
<td>6/9</td>
<td>75</td>
<td>≥200</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>Maltoolitrose SO$_4$</td>
<td>10/11</td>
<td>91</td>
<td>145</td>
<td>58.7</td>
<td></td>
</tr>
<tr>
<td>Maltotetraose SO$_4$</td>
<td>11/14</td>
<td>79</td>
<td>65</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>Maltopentaose SO$_4$</td>
<td>15/17</td>
<td>88</td>
<td>37.5</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Maltohexaose SO$_4$</td>
<td>18/20</td>
<td>90</td>
<td>31.3</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Maltoheptaose SO$_4$</td>
<td>18/23</td>
<td>78</td>
<td>10</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Phosphomannopentaose SO$_4$</td>
<td>12.5/16</td>
<td>78</td>
<td>25</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Average number of sulfate groups attached/theoretical maximum number of sulfate groups that can be coupled to each molecule.

$^b$ Concentration of compound required to inhibit by 50% binding of mouse 3T3 cells to immobilized aFGF/BFGF.

$^c$ Phosphomannopentaose isolated from the yeast P. holstii.

Table 2. Inhibition of FGF binding to cell surface heparan sulfates by different sulfated oligosaccharides

![Fig. 3. Assessment of the ability of sulfated maltose oligosaccharides of different chain length to inhibit the binding of plastic immobilized aFGF to cell surface heparan sulfates on BALB/c 3T3 cells. Bound 3T3 cells were quantified by Rose Bengal staining and by measuring dye absorbance at 540 nm. The degree of sulfation of the different maltose oligosaccharides is listed in Table 2: ■, maltose SO$_4$; ▲, maltoolitrose SO$_4$; ○, maltotetraose SO$_4$; ●, maltopentaose SO$_4$; △, maltohexaose SO$_4$; □, maltoheptaose SO$_4$.](image-url)
Maltohexaose sulfate and PI-88 were also examined for their ability to directly inhibit the growth of 13762 MAT tumor cells in vitro, and no significant effect on tumor growth rate was observed. However, the maltotetra-, penta-, and hexa-saccharide sulfates were found to inhibit the proliferation of human umbilical vein endothelial cells grown in the presence of exogenous bFGF (data not shown), a result consistent with the bFGF binding and angiogenesis inhibitory activity of these sulfated oligosaccharides.

Antitumor Activity of PI-88. In the preliminary screening studies described above, two sulfated oligosaccharides stood out as potential antitumor drugs, PI-88 and maltohexaose sulfate. Both of these compounds have the important property of simultaneously being potent antitumor drugs, PI-88 and maltohexaose sulfate. One of the compounds, PI-88, was selected for more detailed study, this compound being chosen because of the ease of preparation of this compound being chosen because of the ease of preparation of PI-88 and maltohexaose sulfate. Both of these compounds have the important property of simultaneously being potent antitumor drugs, PI-88 and maltohexaose sulfate. One of the compounds, PI-88, was selected for more detailed study, this compound being chosen because of the ease of preparation of large quantities of the starting oligosaccharide, phosphomannopentaose, from the polysaccharide secreted by the yeast P. holstii. The structure of PI-88 is depicted in Fig. 4.

Initial studies further examined the activity of PI-88 in the acute metastatic rat mammary adenocarcinoma 13762 MAT. When different doses of PI-88 were injected s.c. at the same time as tumor cells, a clear cut dose-response curve emerged with high doses (16–32 mg/kg), resulting in >90% inhibition of tumor metastasis and 50% inhibition of metastasis still being observed with the lowest dose tested of 2 mg/kg (Fig. 5A). Using a single s.c. dose of 16 mg/kg, similar inhibition of tumor metastasis was observed when PI-88 was injected at the same time, 1 h before or 1 h after the tumor cells (Fig. 5B). Considerable inhibition of metastasis was still seen when PI-88 was injected 3 h after the tumor cells, but this effect was lost if PI-88 administration was delayed 6 h after tumor cell injection. An earlier study noted that the antimitotic activity of heparin followed a similar time course (22). In contrast, there was only slight metastasis inhibition if PI-88 was given 3 h before tumor cell injection, a result presumably due to the anticipated short half-life of PI-88 in plasma.

In preparation for experiments involving continuous administration of PI-88 over long periods in tumor-bearing animals, different doses of PI-88 were delivered to animals by mini-osmotic pumps to establish a continuously infused dose that inhibited tumor metastasis. In these experiments, mini-osmotic pumps delivered the drug for 7 days, the pumps being inserted 3 days before the i.v. injection of the tumor cells. This ensured that a stable plasma level of PI-88 was achieved before the animals were exposed to tumor cells. It was found that PI-88 administered at 20 and 50 mg/kg/day gave a similar, and very substantial (~90%), inhibition of metastasis with a lower dose of 5 mg/kg/day, still inhibiting metastasis by 55% (Fig. 5C). In subsequent experiments, PI-88 was usually administered by mini-osmotic pumps to tumor-bearing animals at a dose of 20 mg/kg/day.

### Table 3 Effect of degree of sulfation of maltohexaose on inhibition of heparanase activity, growth factor binding to cell surface heparan sulfates, and tumor metastasis

<table>
<thead>
<tr>
<th>Sulfated oligosaccharide</th>
<th>Sulfation</th>
<th>% sulfation</th>
<th>IC₅₀ (µg/ml)</th>
<th>Heparanase</th>
<th>bFGF</th>
<th>aFGF</th>
<th>Metastasis (%) control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltohexaose SO₄</td>
<td>3/20</td>
<td>15</td>
<td>&gt;100</td>
<td>187</td>
<td>180</td>
<td>200</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>Maltohexaose SO₄</td>
<td>9/20</td>
<td>45</td>
<td>50</td>
<td>45.6</td>
<td>79</td>
<td>ND</td>
<td>62 ± 11</td>
</tr>
<tr>
<td>Maltohexaose SO₄</td>
<td>14/20</td>
<td>70</td>
<td>20</td>
<td>12.5</td>
<td>12.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Maltohexaose SO₄</td>
<td>17/20</td>
<td>85</td>
<td>6</td>
<td>5.4</td>
<td>10.4</td>
<td>ND</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>Maltohexaose SO₄</td>
<td>18/20</td>
<td>90</td>
<td>6</td>
<td>5.4</td>
<td>18.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Maltohexaose SO₄</td>
<td>20/20</td>
<td>100</td>
<td>5</td>
<td>5.4</td>
<td>19.7</td>
<td>ND</td>
<td>21 ± 6</td>
</tr>
</tbody>
</table>

a Actual number of sulfate groups attached/theoretical maximum number of sulfate groups that can be coupled to each molecule.

b Concentration of compound required to inhibit by 50% human platelet heparanase activity or binding of mouse 3T3 cells to immobilized aFGF/bFGF. In the case of the heparanase assay, the IC₅₀ for heparin was 2 µg/ml.

c Percentage control metastasis ± SE in the lungs of rats receiving 2 × 10⁶ 13762 MAT cells i.v. and 8 mg/kg of each sulfated oligosaccharide i.v. at the time of tumor cell injection.

d ND, not determined.

e Highly significant (P ~ 0.001) inhibition of metastasis.
and, interestingly, the vast majority of leukocytes in the lymph nodes at this time point were found to be myeloid (i.e., Mac-1+), rather than lymphoid, in nature.

Additional experiments showed that commencement of PI-88 treatment at the time of tumor cell injection, rather than delaying treatment until 7 days after tumor implantation, also gave similar results (data not shown). There was no evidence of macroscopic tumor metastases in the lungs of tumor-bearing animals, although histological examination of the lungs revealed some microscopic lung tumors. However, the appearance of lung micrometastases was highly variable, with many untreated animals containing no lung metastases and others containing very high numbers. Unfortunately, this extreme variability made it very difficult to measure the effect of PI-88 treatment on the incidence of lung metastases in this tumor model.

Because our earlier experiments demonstrated that PI-88 inhibited an in vitro model of angiogenesis (Table 1 and Fig. 2), it was important to determine whether PI-88 also inhibited tumor-associated angiogenesis. Rather than using tedious and subjective immunohistochemical measurements of tumor vessel density, it was decided to quantify the vascularization of the tumors by measuring their hemoglobin content. This procedure has been validated previously by a number of groups as a simple and objective measure of tumor vascularity and has been used to assess the activity of antiangiogenic substances (32–36). To facilitate tumor excision for weighing and assessment of hemoglobin content, the 13762 MAT tumor cells were grown in s.c. air pouches rather than in hind footpads. PI-88 was administered at 20 mg/kg/day by mini-osmotic pumps throughout the experiment. It was found that tumors from PI-88-treated animals, based on hemoglobin content of tumor tissue (µM/g), were significantly (P < 0.05) less vascularized than tumors from untreated controls, the hemoglobin content of PI-88-treated tumors being 69 ± 11% that of controls [i.e., 1.59 ± 0.26 µM (n = 8) versus 2.32 ± 0.15 µM (n = 10) in untreated controls].
examined for total, CD45<sup>+</sup> hind footpads and, 21 days later, the draining popliteal lymph nodes were collected and
oligosaccharides between 6 and 10 monosaccharides in length inhibit
interpretation is supported by studies showing that heparin-derived
and receptors for heparan sulfate binding angiogenic factors. Such an
sulfated oligosaccharides inhibit angiogenesis by interfering with the
inhibitor. This finding is consistent with the hypothesis that the
inhibitory activity, whereas this molecule was a potent heparanase
ever, when considering angiogenesis, heparin was found to lack
optimum inhibitory activity was achieved in both systems with linear
be discussed further here. In the case of oligosaccharide chain length,
three-dimensional space before interpretation and, therefore, will not
affected structural investigations of the positioning of sulfate groups in
Clearly oligosaccharide chain length is critical, with a high degree of
ment can be made about the structural requirements for a sulfated
oligosaccharide to inhibit angiogenesis and heparanase activity. Also, the drug can be prepared
in large quantities from a readily available starting material, the
exopolysaccharide of the yeast P. holstii, which can be conveniently
depolymerized by acid hydrolysis of phosphodiester bonds to obtain,
in high yield, the starting oligosaccharide, phosphomannopentaose.

On the basis of the initial screening studies, some general com-
ments can be made about the structural requirements for a sulfated
oligosaccharide to inhibit angiogenesis and heparanase activity. Clearly
oligosaccharide chain length is critical, with a high degree of
sulfation and the nature of the backbone oligosaccharide also being
important. The latter two factors are highly complex, requiring de-
tailed structural investigations of the positioning of sulfate groups in
three-dimensional space before interpretation and, therefore, will not
be discussed further here. In the case of oligosaccharide chain length,
optimum inhibitory activity was achieved in both systems with linear
oligosaccharides of five or more monosaccharides in length. How-
ever, when considering angiogenesis, heparin was found to lack
inhibitory activity, whereas this molecule was a potent heparanase
inhibitor. This finding is consistent with the hypothesis that the
sulfated oligosaccharides inhibit angiogenesis by interfering with the
formation of a ternary complex between cell surface heparan sulfates
and receptors for heparan sulfate binding angiogenic factors. Such an
interpretation is supported by studies showing that heparin-derived
oligosaccharides between 6 and 10 monosaccharides in length inhibit
bFGF action, whereas longer oligosaccharides and intact heparin are
either inactive or, in some cases, potentiate the activity of heparan sulfate
binding growth factors (30, 31). It has been suggested that, due to its large
size, heparin is unable to disrupt the heparan sulfate-growth factor com-
plex and, in fact, can substitute for cell surface heparan sulfates in
stabilizing the growth factor/growth factor receptor interaction (30–31).
There are conflicting data in this field, however, because very small
fragments of heparin have been reported to enhance bFGF action under
certain circumstances (37). It should be emphasized, however, that intact
heparin and some heparin fragments, which are inactive when adminis-
tered alone, can exhibit antiangiogenic activity when combined with
certain angiostatic steroids (38, 39). For this initial screen, we have
concentrated on identifying sulfated oligosaccharides that are antiangi-
genic in their own right rather than requiring coadministration of certain
steroids for activity. On the other hand, in the case of the heparanase
enzyme, presumably both heparin and the sulfated oligosaccharides oc-
cupy the active site of the enzyme as noncleavable substrates (27), a
sulfated pentasaccharide or greater being highly active.

More extensive studies with our lead compound PI-88 revealed that
it can inhibit the primary tumor growth of the highly invasive rat
mammary adenocarcinoma 13762 MAT by ~50%, inhibit metastasis
to the draining popliteal lymph node by ~40%, and reduce the
vascularity of tumors by ~30%, all of these effects being highly
significant. Acute hematogenous metastasis assays also demonstrated
that PI-88 was a potent (>90%) inhibitor of blood-borne metastasis.
In fact, the ability of the sulfated oligosaccharides, including PI-88, to
inhibit tumor metastasis in this acute assay correlated well with their
heparanase inhibitory activity.

The demonstration that PI-88 can reduce tumor vascularity was a
critical finding because it supports the in vitro angiogenesis inhibition
data obtained with PI-88. Previous studies have shown that the in vitro
angiogenesis response used to screen the sulfated oligosaccharides is
dependent on endogenous bFGF and to a lesser extent aFGF and
VEGF (7), all these growth factors being heparan sulfate binding.
Furthermore, the ability of the sulfated oligosaccharides to block
FGF-heparan sulfate binding tended to correlate with their capacity to
inhibit in vitro angiogenesis. Recent biosensor studies also suggest
that PI-88 can interfere with the VEGF-heparin interaction. Nevertheless,
with the availability of multiple angiogenic growth factors in vivo,
there was always the possibility that PI-88 would be unable to
inhibit tumor associated angiogenesis. Of course there is the addi-
tional possibility that PI-88 is inhibiting in vivo angiogenesis not only by
blocking angiogenic growth factor action, but also via heparanase
inhibition. Heparanase activity has been implicated in several aspects
of neovascularization, such as degradation of the endothelial ECM
during endothelial cell migration and the release of heparan sulfate-
bound angiogenic factors associated with the ECM (15).

An earlier study has also shown that sulfated malto-oligosacca-
rides of four to seven monosaccharides in length can block the
bFGF-heparan sulfate interaction (29). This study also revealed that
such sulfated malto-oligosaccharides can inhibit endothelial cell pro-
iferation in vitro and interfere with the ability of endothelial cells to
form tubes on Matrigel. Furthermore, the sulfated malto-oligosacca-
rides exhibiting in vitro activity can inhibit tumor growth and metas-
tasis in vivo. (40) In contrast, sulfated maltohexaose and maltohep-
taose have been shown to enhance, rather than inhibit, the activity of
the heparan sulfate binding growth factor hepatocyte growth factor
(41), suggesting that the effects of sulfated oligosaccharides on
growth factor action may be growth factor-specific.

There have been a number of earlier studies by us and other
investigators demonstrating that heparin, chemically modified hep-
rins, and related sulfated polysaccharides are effective antimitastatic

---

5 K. J. Brown, unpublished observation.
compounds, there being a reasonably good correlation between the antmitotic activity of these compounds and their heparanase inhibitory activity (10, 27, 42–45). Although heparin and the sulfated polysaccharides are anticoagulants, several studies have shown that heparin, drastically depleted of anticoagulant activity by antithrombin III column fractionation (45), depolymerization (43, 44), or chemical treatment (10, 42–44), still retains its antimitic and heparanase inhibitory activity. On the basis of the earlier observations, it is not surprising that some sulfated oligosaccharides were found to be potent heparanase and tumor metastasis inhibitors. Additional studies have revealed that PI-88 and related sulfated oligosaccharides have ~6–20-fold less anticoagulant activity than heparin. Furthermore, the residual anticoagulant activity of the sulfated oligosaccharides is mediated by their interaction with heparin cofactor II, and not antithrombin III.6 Whether the low, but significant, anticoagulant activity of PI-88 contributes to its antitumor properties remains to be determined. However, compared with sulfated polysaccharides, sulfated oligosaccharides such as PI-88 have the advantage that they are structurally more homogeneous, exhibit less toxicity due to reduced anticoagulant activity and likely ease of excretion, and are of sufficiently low molecular weight that oral delivery may be feasible.

ACKNOWLEDGMENTS

We thank Gavin Bartell, Anna Bezos, Anna Brown, Jorge Gepella, Ros Henderson, Karen Jakobsen, Susan Maynes, and Tom Teitel for excellent technical assistance.

REFERENCES


Identification of Sulfated Oligosaccharide-based Inhibitors of Tumor Growth and Metastasis Using Novel in Vitro Assays for Angiogenesis and Heparanase Activity

Christopher R. Parish, Craig Freeman, Kathryn J. Brown, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/14/3433

Cited articles
This article cites 42 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/14/3433.full#ref-list-1

Citing articles
This article has been cited by 39 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/14/3433.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, contact the AACR Publications Department at permissions@aacr.org.