O-Sulfated Bacterial Polysaccharides with Low Anticoagulant Activity Inhibit Metastasis

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ABSTRACT

Heparin-like polysaccharides possess the capacity to inhibit cancer cell proliferation, angiogenesis, heparanase-mediated cancer cell invasion, and cancer cell adhesion to vascular endothelia via adhesion receptors, such as selectins. The clinical applicability of the antitumor effect of such polysaccharides, however, is compromised by their anticoagulant activity. We have compared the potential of chemically O-sulfated and N,O-sulfated bacterial polysaccharide (capsular polysaccharide from E. coli K5 [K5PS]) species to inhibit metastasis of mouse B16-BL6 melanoma cells and human MDA-MB-231 breast cancer cells in two in vivo models. We demonstrate that in both settings, O-sulfated K5PS was a potent inhibitor of metastasis. Reducing the molecular weight of the polysaccharide, however, resulted in lower antimetastatic capacity. Furthermore, we show that O-sulfated K5PS efficiently inhibited the invasion of B16-BL6 cells through Matrigel and also inhibited the in vitro activity of heparanase. Moreover, treatment with O-sulfated K5PS lowered the ability of B16-BL6 cells to adhere to endothelial cells, intercellular adhesion molecule-1, and P-selectin, but not to E-selectin. Importantly, O-sulfated K5PSs were largely devoid of anticoagulant activity. These findings indicate that O-sulfated K5PS polysaccharide should be considered as a potential antimetastatic agent.

KEYWORDS: K5 polysaccharide, heparin, cancer, metastasis, anticoagulation

Clinical data have indicated that anticoagulant doses of heparin may retard cancer progression and prolong disease-free survival of cancer patients (for reviews, see Zacharski and Ornstein¹ and Hejna et al²). Furthermore, tumor metastasis in various experimental cancer models can be inhibited by heparin (reviewed by Smörenburg et al³). There are multiple mechanisms by which heparin-like polysaccharides may inhibit cancer growth and metastasis. Such mechanisms include (1) inhibition of heparanase and possibly proteases that are intimately involved in tumor cell invasion; (2) effects on tumor cell adhesion to vascular endothelia via adhesion...
receptors, such as selectins; and (3) inhibition of growth factors that stimulate cancer cell proliferation or tumor neovascularization.3

In the clinic, the usefulness of heparin as an anticancer drug is limited by its modest therapeutic efficacy and the narrow therapeutic window due to its anticoagulant activity. Multiple polyanionic heparin mimetics, including chemically modified heparins,4–6 chemically sulfated natural polymers,7,8 and synthetic polymers,9,10 have therefore been tested as anticancer agents to identify compounds with better efficacy and safety profile. Some compounds, such as phosphomannopentaose sulfate (PI-88) have entered clinical trials that aim to evaluate its therapeutic potential as an anticancer agent.11,12

The capsular polysaccharide of Escherichia coli K5 bacteria (K5PS) has been used as starting material to synthesize heparin-like compounds with various biological activities. K5PS consists of alternating D-glucuronic acid and D-N-acetylgalactosamine units (GlcA\(\alpha\)1,4GlcNAc\(\beta\)1,4)\(n\) and thus has the same structure as the primary polymerization product of mammalian heparin/heparan sulfate (HS) biosynthesis. Depending on their extent and pattern of sulfation, chemically sulfated K5PSs have been shown to possess antiproliferative,14 antiangiogenic,15,16 antimetastatic,17 and/or antiviral activities.18 We have previously described N-acetylated, O-sulfated K5PSs that are potent inhibitors of fibroblast growth factor (FGF) signaling and FGF-dependent cancer cell proliferation.14 This article reports that same N-acetylated, O-sulfated polysaccharides also inhibit cancer cell invasion and metastasis in mice, but are virtually devoid of anticoagulant activity.

MATERIALS AND METHODS

Synthesis of K5PSs

The K5PS preparations used in this study were synthesized and characterized as described elsewhere.17,19,20 Briefly, a series of sulfated K5PSs were generated by chemical N-deacetylation/N-sulfation and/or direct O-sulfation of the unmodified K5PS structure (GlcAGlcNAc)\(n\), followed by molecular weight fractionation. Low molecular weight (LMW) derivatives were prepared in different ways for N-acetylated and N-sulfated precursors. Whereas the N-sulfated LMW derivative was obtained by classical nitrous acid depolymerization (at pH 12, followed by borohydride reduction) to avoid side reactions usually associated with the N-deacetylation step, the N-acetylated LMW derivative was obtained by radical depolymerization with Fenton reagent (2 hours at 50°C; to be published elsewhere). The synthesis resulted in four different compounds including high and low molecular weight species (designated as HMW (~11 to 15 kDa) and LMW (~2 to 3 kDa), respectively) of O-sulfated and N,O-sulfated saccharides (OS and NSOS, respectively). The compounds had an overall degree of sulfation (sulfate groups/disaccharide unit) of ~3.0 to 4.0. The O-sulfation of the NSOS species was somewhat lower than that of the OS species, mainly due to resistance to sulfation of 3-OH groups of GlcN residues of N-sulfated K5PSs. The NSOS-LMW derivative was also sulfated incompletely in the GlcA residues. However, in both N-sulfated and N-acetylated preparations the degree of GlcN 6-O-sulfation was 100%.

Cell Cultures

Human MDA-MB-231 breast cancer cells (a kind gift from Dr. Theresa Guise, University of Virginia, Charlottesville, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 1% penicillin/streptomycin, and nonessential amino acids (GIBCO BRL). Mouse B16-BL6 melanoma cells (National Institutes of Health, Frederick, MD) were cultured in DMEM (Sigma) supplemented with 5% FCS, 1 mM sodium pyruvate, 1% MEM nonessential amino acid solution, and 1% MEM vitamin solution. Rat endothelial Ax cells were cultured in RPMI 1640 (Sigma) supplemented with 20% FCS, 10 μM β-mercaptoethanol, and 1% nonessential amino acids.

Model of Melanoma Metastasis to Lung

The potential of various K5PSs to inhibit metastasis of B16-BL6 cells to lung was studied in accordance with the protocols approved by the Ethical Animal Care and Use Committee of University of Turku using a previously described method.4,5 Briefly, B16-BL6 cells (1 × 10\(^5\)) suspended in 100 μL serum-free medium and the test compounds dissolved in 100 μL phosphate-buffered saline (PBS) at various concentrations were mixed prior to intravenous injection (total injected volume 200 μL) into the lateral tail vein of approximately 7-week-old female C57BL6 mice (Bomholtgaard, Denmark, or Harlan, The Netherlands). K5PSs and heparin from porcine intestinal mucosa (Sigma) were administered in concentrations of 10 or 25 mg/kg. The number of animals per group varied between five and nine. On day 14, the mice were sacrificed with CO₂, and the lungs were excised and photographed in situ; thereafter, they were fixed with formalin. The number of metastasized colonies on the lung surface was counted from the digital photographs by using Microsoft Photo Editor with a grid overlay. The number of colonies was evaluated and counted independently by three scientists. The differences between animal groups were tested using Kruskal–Wallis test followed by paired comparisons.
based on Mann-Whitney U test with Bonferroni correction.

Model of Breast Cancer Metastasis to Bone

The potential of chemically modified K5PSs to inhibit metastasis of human MDA-MB-231 cells to bone was studied in accordance with the protocols approved by the Ethical Animal Care and Use Committee of University of Turku using a well-established animal model described elsewhere. Briefly, cell suspension (100 μL of 1 × 10^5 cells/mL in PBS) was inoculated into the left cardiac ventricle of 5-week-old female athymic nude mice (Harlan) on day 0. Subcutaneous administration of PBS or intravenous administration of the test compounds, OS-HMW or NSOS-HMW (5 mg/kg), was started on day 1 and continued daily for 21 days. Prior to sacrifice on day 22, the animals were anesthetized and x-rayed in a prone position using the Faxitron Specimen Radiographic System MX-20 DC-2 (34 kV, 3 seconds; Faxitron Corp.). The number of osteolytic lesions and area in the hind and fore limbs per mouse were determined from the radiographs by MetaMorph image analysis software (Universal Imaging, Downington, PA). In addition, tissue samples from each animal were collected at sacrifice. One hind limb (including both tibia and femur) from each animal was randomly selected, decalcified, and embedded in paraffin. The paraffin-embedded samples were then cut into 4-μm-thick sagittal mid-level sections and stained with hematoxylin-eosin (H&E). In the subsequent histomorphometric analysis, tumor area per hind limb was measured using MetaMorph image analysis software. Eventually, 13, 12, and 11 animals receiving PBS, OS-HMW, or NSOS-HMW, respectively, were analyzed. The differences between animal groups were tested using Kruskal-Wallis test followed by paired comparisons based on Mann-Whitney U test with Bonferroni correction.

Cell Invasion Assay

B16-BL6 cells (5 × 10^4) were suspended in 500 μL serum-free medium, with or without K5PSs (5 μg/mL), and plated into cell culture inserts layered with Matrigel (BD Biocoat Matrigel Invasion Chambers; BD Biosciences), a reconstituted basement membrane–like matrix. Two parallel inserts were plated per sample. The inserts were then set on the 24-well plates containing medium and 3% FCS as a chemoattractant. After incubation for 72 hours, cells on the lower surface of the insert were fixed with 4% formalin and stained using hematoxylin. Next, cells on the upper surface of the insert were removed with a cotton swab. The number of invaded cells per insert was scored under a microscope by counting the cells within 16 1-mm² areas in a minimum of three fields, on the lower surface of the insert.

Heparanase Inhibition Assay

The ability of various K5PSs to inhibit the activity of heparanase was assessed as described elsewhere. Briefly, recombinant human heparanase (40 ng/mL) with or without heparin or K5PS preparations (1 μg/mL) were added into 35-mm cell culture plates coated with sulfate-labeled extracellular matrix, and incubated for 4 hours at 37°C at pH 6.0. The incubation medium (1 mL) containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 mL) were eluted with PBS and their radioactivity counted in a β-scintillation counter. Degradation fragments of HS side chains are eluted at 0.5 < Kav < 0.8 and represent heparanase degradation products.

Adhesion Assays

For studying adhesion to endothelial cells, rat endothelial Ax cells were plated onto 96-well plates (5000 to 10,000 cells/well) and cultured until confluency. Prior to use, the wells were washed with PBS. B16-BL6 cells were trypsinized, stained with 200 μg/mL Rhodamine (Fluka) in PBS for 15 minutes at room temperature, washed with PBS, and suspended in serum-free medium.
with or without test compounds (0.25 mg/mL). Heparin from porcine intestinal mucosa (LEO Pharma Q15) was tested as a control. Fluorescent B16-BL6 cells (150,000 cells/well) were then seeded over endothelial cells, and allowed to adhere for 30 minutes at 37°C. Next, the nonadherent B16-BL6 cells were removed, the wells were washed with serum-free medium, and fluorescence was measured by Victor Q16 (Wallac Q17).

To study adhesion of B16-BL6 cells to various adhesion receptors, 2 µg/well of P-selectin, E-selectin, or intercellular adhesion molecule-1 (ICAM-1) -Fc chimeras (R&D Systems Q18) were incubated for 1 hour at room temperature in Reacti-Bind Protein A–coated wells (Pierce Q19), and washed with PBS. B16-BL6 cells were trypsinized, washed twice with PBS, and suspended in serum-free medium with or without K5PSs or heparin (0.2 mg/mL). Next, the cells were plated onto the precoated wells (150,000 cells/well) and allowed to adhere for 30 minutes at 37°C. After incubation, the nonadherent cells were removed and fresh serum-free medium together with WST-1 reagent (Roche Q20) was added into the wells. After an incubation for 1 hour at 37°C, the plates were measured spectrophotometrically at 450 nm with a plate reader.

**In Vitro Coagulation Tests**

Anti–factor (F) IIa Q21 and anti-FXa activities of the test compounds were determined utilizing Chromogenic IL Test Heparin assay kit (Instrumentation Laboratory Q22 Co.) according to the manufacturer’s protocol and ACL 7000 automated coagulation analyzer (Instrumentation Laboratory Co.). In the assays, the residual FIIa and FXa were quantified with a synthetic chromogenic substrate. The buffer solution 0.9% NaCl was used in the anti-FXa activity measurements, whereas buffer solution 0.05 M Tris, 0.15 M NaCl, 1% bovine serum albumin Q23, pH 7.4 was used in the anti-FIIa activity measurements. All of the test compounds were measured against international low molecular weight heparin standard (“low molecular mass heparin,” European Pharmacopoeia Commission).

The effects of the test compounds on activated partial thromboplastin time (aPTT Q24) in citrated plasma were measured using IL Test aPTT lyophilized silica kit (ILS Laboratories Q25) according to the manufacturer’s recommendations. The low molecular weight heparin dalteparin (Fragmin; Upjohn and Pharmacia Q26) was used as a reference compound. Dose–response curves as a clotting time versus saccharide concentration were prepared for the test and

**Figure 2** The effect of disaccharide units of capsular polysaccharide from *E. coli* K5 (K5PSs) on metastasis of melanoma cells to lung. (A) Representative lung photographs of mice inoculated with B16-BL6 mouse melanoma cells (1 × 10⁵) with or without O-sulfated saccharide high molecular weight (OS-HMW) or N,O-sulfated saccharide (NSOS) -HMW (10 mg/kg) into the lateral tail vein. The lungs were photographed and the number of metastatic foci on the lung surface was counted on day 14. (B) The number of lesions in mice treated with the test compounds as indicated. Each dot represents the number of metastatic foci in the lungs of one animal; the columns represent the median of the number of metastatic foci in a treatment group. *p < 0.05 and **p < 0.01 according to Mann-Whitney U test with Bonferroni correction. PBS, phosphate-buffered saline.
reference compounds, and the doses causing a coagulation time of 100 seconds were estimated from the trend lines.

RESULTS

Synthesis and Structure of K5PSs

The K5PS compounds were synthesized as described in Materials and Methods, and characterized by heteronuclear two-dimensional nuclear magnetic resonance spectroscopy\(^1\)\(^2\)\(^3\)\(^4\) and molecular weight determination. The preparations included four compounds distinguished from each other by their sulfate substitution (OS and NSOS; Fig. 1), and by their molecular weight (HMW, ~11 to 15 kd) versus LMW, ~2 to 3 kd). The synthesis thus yielded four compounds, hereafter designated as OS-HMW, OS-LMW, NSOS-HMW, and NSOS-LMW. The O-sulfation reactions led to similar degrees of total sulfation for the OS and NSOS compounds; the NSOS compounds have a somewhat lower degree of O-sulfation.

O-Sulfated K5PS Inhibits Metastasis of Melanoma Cell to Lung

We have reported previously that NSOS species of K5PS are efficient inhibitors of FGF signaling.\(^1\)\(^4\) To evaluate their antimetastatic potential in vivo, B16-BL6 mouse melanoma cells (\(1 \times 10^5\)) were injected into the lateral tail vein of C57BL6 mice with or without various K5PS preparations. After 14 days, the mice were sacrificed and the metastatic lesions macroscopically visible on the lung surface (Fig. 2A) were counted. Both OS-HMW and NSOS-HMW inhibited the metastasis of B16-BL6 cell to lungs in a concentration-dependent manner (data not shown). However, when compared with the control group, a

Figure 3  The effect of disaccharide units of capsular polysaccharide from *E. coli* K5 (K5PSs) on metastasis of breast cancer cells to bone as measured by radiographic analysis. (A) Representative radiographs of female athymic mice inoculated with MDA-MB-231 human breast cancer cells and treated with O-sulfated saccharide high molecular weight (OS-HMW) or N,O-sulfated saccharide (NSOS) -HMW (5 mg/kg) for 21 days. Osteolytic lesions are indicated by arrows. (B) The number of osteolytic lesions and (C) the total osteolytic lesion area (in square millimeters) in right and left tibia, femur, and humerus of the animals determined by the x-ray radiography. Each dot represents the lesion number or the lesion area in the individual mice; the columns represent the median of the lesion number or of the lesion area in a treatment group. ** \(p < 0.01\) and *** \(p < 0.001\) according to Mann-Whitney U test with Bonferroni correction. PBS, phosphate-buffered saline.
A statistically significant difference was obtained only with 10 mg/kg of heparin or OS-HMW (Fig. 2B). Reducing the molecular weight of both OS and NSOS K5PS preparations seemed to lower their antimetastatic activity (Fig. 2B).

O-Sulfated K5PS Inhibits Metastasis of Breast Cancer Cells to Bone

To confirm the potential of the K5PS HMW preparations to inhibit metastasis in another in vivo setting, human breast cancer MDA-MB-231 cells ($1 \times 10^5$) were injected into the left cardiac ventricle of athymic nude mice. Between days 1 and 21, the animals were treated daily with or without test compounds. On day 22, the animals were x-rayed and the radiographs were analyzed for osteolytic bone lesions appearing as radiolucent areas in the fore limbs (humerus) and in the hind limbs (femur and tibia; Fig. 3A). All the animals receiving NSOS-HMW developed obvious lesions, whereas only 67% of the OS-HMW–treated animal had obvious lesions. Both the lesion number (Fig. 3B) and the total osteolytic lesion area (in square millimeters) in the right and left tibia, femur, and humerus per animal (Fig. 3C) were significantly decreased (by ~56% and ~86%, respectively) in the OS–HMW group when compared with the nontreated animals (Kruskal-Wallis test followed by Mann-Whitney $U$ test). Furthermore, total tumor area per animal measured from sections of tibia and femur stained with H&E (Fig. 4A) was significantly lower in OS-HMW–treated animals compared with the control group (Fig. 4B). The dose of the test compound OS-HMW used nearly completely prevented the tumor growth in the hind limbs. No tumor growth was observed in 50% of the mice in this group (Fig. 4A).

The percentage of cachectic animals at the end of the study as determined by a curved spine, dry skin, and a weight reduction of at least 10% was 31% in the nontreated animal group, 27% in the NSOS-HMW group, and 0% in the OS–HMW group.

O-Sulfated K5PS Inhibits Cancer Cell Invasion and In Vitro Activity of Heparanase

There are multiple possible mechanisms underlying the antimetastatic activity of O-sulfated K5PS. For example, we have demonstrated previously that O-sulfated K5PS is a potent inhibitor of FGF-stimulated cancer cell growth.14 To address other possible mechanisms with a role in metastasis, K5PS preparations were tested for

![Figure 4](image-url)
their ability to inhibit invasion of mouse melanoma B16-BL6 cells through Matrigel-coated membranes. Matrigel is rich in HS proteoglycans and other basement membrane constituents, and it is used routinely to study cellular interactions with the basement membrane. At a concentration of 5 μg/mL, both OS-HMW and NSOS-HMW compounds clearly inhibited (by ~50%) the invasion of B16-BL6 cells, but the LMW saccharides had little or no anti-invasive effect (Fig. 5A).

Heparanase activity is regarded as a key element in the invasive capacity of tumor cells, and B16-BL6 melanoma cells are known to express high levels of heparanase.25 Our results indicated that both OS-HMW and NSOS-HMW were efficient inhibitors (~90% inhibition) of heparanase activity in vitro (Fig. 5B). OS-LMW was the weakest inhibitor of the heparanase activity (~40% inhibition), whereas NSOS-LMW and heparin inhibited the activity by ~75%.

O-Sulfated K5PS Inhibits Adhesion of Melanoma Cells to Endothelial Cells and to Adhesion Receptors

One possible mechanism underlying the antimetastatic activity of OS-HMW is the inhibition of tumor cell adhesion to vascular endothelial cells. Therefore, the effect of the polysaccharides on adhesion of B16-BL6 melanoma cells to rat endothelial Ax cells was tested. All four K5PS compounds showed moderate inhibitory activity similar to that of heparin (Fig. 6A).

To study the antiadhesive effects of the K5PS HMW preparations in more detail, the adhesion of the B16-BL6 cells to endothelial cell–cell adhesion molecules was assessed. OS-HMW clearly inhibited the adhesion of the cells to ICAM-1 and P-selectin but not to E-selectin (Fig. 6B). The inhibition of B16-BL6 cells to P-selectin achieved by NSOS-HMW was similar to OS-HMW, but NSOS-HMW failed to block melanoma cell adhesion significantly to either ICAM-1 or E-selectin (Fig. 6B).

O-Sulfated K5PS Has Little or No Anticoagulant Activity

To study the anticoagulant potency of K5PSs, the anti-FIIa and anti-FXa activities of each compound were tested. Whereas dalteparin showed the expected activity and NSOS-HMW showed some activity, the other compounds largely were devoid of anti-FIIa and anti-FXa activity (in the order of OS-LMW < OS-HMW < NSOS-LMW; Table 1). Moreover, the effects of the test compounds on aPTT were tested as a function of saccharide concentration (Table 1). OS-HMW had a weak prolonging effect on aPTT, and the concentration of OS-HMW required to prolong aPTT to 100 seconds was ~1.3 times greater than that of NSOS of comparable molecular weight, and ~2.4 times greater as compared with dalteparin.

DISCUSSION

We have demonstrated previously that various chemically NOS species of K5PS exert high antimetastatic activity in the mouse B16-BL6 melanoma cell metastasis model.17 In the present report, we demonstrate...

Figure 5 The effect of disaccharide units of capsular polysaccharide from E. coli K5 (K5PSs) on melanoma cell invasion and heparanase activity. (A) B16-BL6 mouse melanoma cells with or without test compounds (5 μg/mL) were plated onto cell culture inserts layered with Matrigel and incubated for 72 hours. The number of cells invaded onto the lower surface of the insert was scored under a microscope. The data represent mean ± standard deviation of three independent experiments performed in triplicate. (B) Recombinant heparanase (40 ng/mL) with or without test compounds (1 μg/mL) was incubated with sulfate-labeled extracellular matrix for 4 hours. The reaction mixture was subjected to gel filtration on Sepharose CL-6B and the amount of sulfate-labeled heparan sulfate degradation fragments was determined by counting the radioactivity eluted in fractions 15 to 30 (0.5 < Kav < 0.8). Each experiment was performed three times and the variation in elution positions (Kav values) did not exceed ± 15%. OS, O-sulfated saccharide; HMW, high molecular weight; LMW, low molecular weight; NSOS, N,O-sulfated saccharide.
that N-acetylated, O-sulfated K5PS is at least an equally (or even more) potent antimetastatic agent as NOS K5PS. Both OS-HMW and NOS-HMW preparations inhibited the metastasis of mouse B16-BL6 melanoma cells, although the inhibition by the latter compound was not statistically significant due to high variation within the treatment groups. Furthermore, OS-HMW, but not NSOS-HMW, inhibited metastasis of human MDA-MB-231 breast cancer cells to bone.

The antimetastatic activity of OS-HMW preparation may be mediated via several mechanisms. We have reported previously that OS-HMW is more efficient than NS-HMW or NSOS-HMW K5PS in suppressing FGF-stimulated cancer cell growth.\textsuperscript{14} It seems apparent that the effect on melanoma cell metastasis has to take place before or during the early extravasation and tissue invasion steps, given that the OS-HMW compounds did not inhibit melanoma cell growth in vitro.

Table 1 Effect of K5PSs on Coagulation In Vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>aFXa (IU/mg)</th>
<th>aFIIa (IU/mg)</th>
<th>aPTT\textsubscript{100 s} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS-HMW</td>
<td>3</td>
<td>2</td>
<td>12.3</td>
</tr>
<tr>
<td>OS-LMW</td>
<td>0.1</td>
<td>—</td>
<td>134</td>
</tr>
<tr>
<td>NSOS-HMW</td>
<td>53</td>
<td>37</td>
<td>9.0</td>
</tr>
<tr>
<td>NSOS-LMW</td>
<td>10</td>
<td>8</td>
<td>22.5</td>
</tr>
<tr>
<td>Dalteparin</td>
<td>217</td>
<td>85</td>
<td>5.2</td>
</tr>
</tbody>
</table>

All coagulation tests were performed using commercially available assay kits (IL Test\textsuperscript{14} Heparin assay kit or aPTT lyophilized silica kit). aFXa and aFIIa activities were determined by monitoring the level of paranitroaniline released in the enzyme reaction at 405 nm. aPTT\textsubscript{100 s} was determined by preparing dose response curves as a clotting time (range 50–230 seconds) versus saccharide concentration. The concentration required to prolong aPTT to 100 second was estimated from the dose response curves. All the measurements were made according to International Low-Molecular-Mass Heparin standard (European Pharmacopoeia Commission). K5PSs, disaccharide units of capsular polysaccharide from E. coli K5; aFXa, anti-factor Xa; aFIIa, anti-factor IIa; aPTT, activate partial thromboplastin time; OS, O-sulfated saccharide; HMW, high molecular weight; LMW, low molecular weight; NSOS, N,O-sulfated saccharide; ICAM, intercellular adhesion molecule.
nor did they retard in vivo metastasis formation if administered after the tumor cells had disappeared from the circulation (data not shown). Such a hypothesis is supported further by the finding that radiolabeled B16-BL6 cells are cleared rapidly from the circulation with less than 25% of the initial inoculum remaining 15 minutes after the injection.26

To address the effect of OS-HMW on tissue invasion, two different approaches were used. In the first approach, the capacity of OS-HMW to inhibit the invasion of heparanase-expressing B16-BL6 cells through Matrigel-coated membranes was determined, whereas the second approach was directed to assessing the ability of OS-HMW to inhibit the enzymatic activity of heparanase. In both assays, OS-HMW showed clear inhibitory activity, indicating that the antimetastatic effects of OS-HMW may be mediated, at least partially, by inhibition of heparanase activity. This finding is in concert with our earlier report demonstrating that N-acetylated, O-sulfated K5PS is susceptible to cleavage by both human hepatoma- and platelet-derived heparanase.27 Furthermore, O-sulfate groups in K5PS were shown to be essential for substrate recognition by heparanases, whereas N-sulfate groups were not required for recognition, nor did they interfere with the enzymatic activity.

The antimetastatic activity of OS-HMW may, in addition, involve adhesion of cancer cells to vascular endothelia via adhesion receptors. Our results show that binding of B16-BL6 cells to ICAM-1 and P-selectin, but not to E-selectin, was diminished by treatment with OS-HMW. It has been demonstrated that heparin inhibits adhesion of leucocytes to activated endothelial cells expressing ICAM-1 and E-selectin,28 possibly via binding to ICAM-1, given that heparin is not believed to be able to bind E-selectin.29,30 Furthermore, heparin has been reported to inhibit adhesion of cancer cells to P-selectin.29,30 P-selectin is also expressed by platelets, and several studies demonstrate that heparin can exert its antimetastatic activity via preventing the formation of cancer cell–platelet emboli complexes.31–33 Such complexes are masked from the cytotoxic activity of natural killer cells, and on the other hand, bind avidly to vascular endothelia.34,35 Thus, the antimetastatic activity of OS-HMW may well involve various P-selectin–mediated mechanisms.

Given that the clinical applicability of heparin in cancer therapy is limited because of its strong anticoagulant activity, especially if doses higher than those used in the prophylaxis or treatment of thrombosis are required, we determined the anticoagulant activity of K5PSs using three different assays. OS-HMW showed ~43 times lower anti-FIIa activity and ~72 lower anti-FXa activity than dalteparin. Furthermore, the concentration of OS-HMW required to prolong aPTT to 100 second was ~2.4 times greater compared with that of dalteparin. Importantly, results from all three coagulation assays indicate that OS-HMW is largely devoid of anticoagulant activity. Taken together, these findings suggest that OS-HMW is a potential antimetastatic agent with low anticoagulant activity.

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Q2: AU: Please verify all chemical compound names throughout text for accuracy and consistency. All chemical compound acronyms/abbreviations should be defined at first mention in text and within each figure and table, if applicable.

Q3: AU: HS correct as defined at first mention?

Q4: AU: Do you mean the manuscript is in preparation or that the article appears in this issue of this journal (by Vismara et al, entitled "Structural Modification Induced in Heparin by a Fenton-Type Depolymerization Process." If the latter, we will insert a new reference to this companion article and renumber references accordingly.

Q5: AU: Please clarify definitions. Does OS mean simply "O-sulfated" or "O-sulfated saccharide"? If the former, "OS" will be used throughout after this first mention instead of the phrase "O-sulfated."

Q6: AU: Please supply city/state (or city/country if not in U.S.) location of manufacturer.

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Q14: AU: Please supply city/state (or city/country if not in U.S.) location of manufacturer.

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Q16: AU: Please supply complete name and type of instrument.

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