Oligomannururate Sulfate, a Novel Heparanase Inhibitor Simultaneously Targeting Basic Fibroblast Growth Factor, Combats Tumor Angiogenesis and Metastasis

Huajun Zhao,¹ Haiying Liu,¹,² Yi Chen,¹ Xianliang Xin,¹ Jing Li,² Yongtai Hou,¹ Zhonghua Zhang,¹ Xiongwen Zhang,¹ Chengying Xie,¹ Meiyu Geng,¹,² and Jian Ding¹

¹Division of Anti-Tumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, P.R. China and ²Department of Pharmacology and Glycobiology, Marine Drug and Food Institute, Ocean University of China, Qingdao, P.R. China

Abstract

Inhibitors of tumor angiogenesis and metastasis are increasingly emerging as promising agents for cancer therapy. Recently, heparanase inhibitors have offered a new avenue for such work because heparanase is thought to be critically involved in the metastatic and angiogenic potentials of tumor cells. Here, we report that oligomannururate sulfate (JG3), a novel marine-derived oligosaccharide, acts as a heparanase inhibitor. Our results revealed that JG3 significantly inhibited tumor angiogenesis and metastasis, both in vitro and in vivo, by combating heparanase activity via binding to the KKDC and QPLK domains of the heparanase molecule. The JG3-heparanase interaction was competitively inhibited by low molecular weight heparin (4,000 Da) but not by other glycosaminoglycans. In addition, JG3 abolished heparanase-driven invasion, inhibited the release of heparan sulfate–sequestered basic fibroblast growth factor (bFGF) from the extracellular matrix, and repressed subsequent angiogenesis. Moreover, JG3 inactivated bFGF-induced bFGF receptor and extracellular signal–regulated kinase 1/2 phosphorylation and blocked bFGF-triggered angiogenic events by directly binding to bFGF. Thus, JG3 seems to inhibit both major heparanase activities by simultaneously acting as a substrate mimic and as a competitive inhibitor of heparan sulfate. These findings suggest that JG3 should be considered as a promising candidate agent for cancer therapy. (Cancer Res 2006; 66(17): 8779-87)

Introduction

Tumor invasion and metastasis is a multistep process that promotes the spread of the cancer from primary sites to distant locations. One of the critical steps involved in this process is the ability of tumor cells to degrade and penetrate the basement membrane and the extracellular matrix (1, 2). Heparan sulfate proteoglycans, along with other proteoglycans and structural proteins found in the extracellular matrix, play key roles in the self-assembly, insolubility, and barrier properties of the basement membrane and extracellular matrix (2–5). Heparanase, a mammalian endo-β-1,3-glucuronidase, capable of partially depolymerizing heparan sulfate chains at a limited number of sites, is thought to help promote cancer invasion and metastasis (6).

Heparanase is synthesized as a latent 65-kDa precursor that undergoes proteolytic cleavage, yielding 8- and 50-kDa subunits that heterodimerize to form a highly active enzyme. The enzymatic activity of heparanase is thought to participate in the cleavage of heparan sulfate chains from heparan sulfate proteoglycans, leading to extracellular matrix remodeling that may facilitate the cell invasiveness associated with cancer metastasis (7–11). In addition to its involvement in the egress of cells from the vasculature, heparanase is tightly involved in angiogenesis, where it acts to release heparan sulfate–sequestered heparin-binding angiogenic factors, such as basic fibroblast growth factor (bFGF) and possibly other endothelial cell growth factors, from the basement membrane and extracellular matrix (2, 12–14). Thus, it is perhaps unsurprising that heparanase expression levels are closely correlated with the metastatic and angiogenic potentials of tumor cells.

Because of its involvement in the development and metastasis of malignant tumors, heparanase has recently become an attractive target for the treatment of highly malignant tumors. Some studies have sought to use exogenous heparin or heparin mimetics to inhibit heparanase activity, based on the high-affinity interaction between the enzyme and its substrate and the limited degradation of these alternative substrates (15–19). However, these efforts have been complicated by the diverse functionality of polysaccharides, which limits their specificity. In addition, these substrate-based inhibitors tend to have high molecular weights that lead to potentially toxic anticoagulant activities and complications with regards to oral delivery and excretion. Thus, researchers have sought to identify smaller saccharides that may avoid these limitations while potently inhibiting heparanase activity. The first such molecule, phosphomannopentose sulfate (PI-88), showed good safety and tolerability in phase I trials and is now undergoing phase II clinical trials. As such, the emphasis on the key requirement of higher efficacy (specificity) and a better side effect profile is emerging as the new driving force in the development of new oligosaccharide-based heparanase inhibitors using glycosaminoglycans mimetic substrate as a prototypic molecule.

With the availability in our laboratory of newly established in vitro assays for heparanase enzyme activity, together with the marine-derived carbohydrate library, a comprehensive screening program was undertaken in an effort to identify new heparanase inhibitors...
inhibitors. Oligomannurate sulfate (JG3), a newly semisynthesized, structurally novel sulfated oligosaccharide derived from marine oligomannururate blocks (Fig. 1A), stood out as a potential substrate-based heparanase inhibitor.

Here, we investigated the effects of JG3 on heparanase-driven tumor metastasis and angiogenesis, both in vitro and in vivo, and sought to elucidate its mechanisms of action. We found that JG3 inhibited heparanase activity via binding to the KKDC (KKFKNSTYRSSSVDC amino acids) and QPLK (QPRKTKAMLKK amino acids) domains within heparanase, inhibited the release of heparan sulfate–bound bFGF from the extracellular matrix, and simultaneously blocked bFGF–associated interaction with its receptor and subsequent signaling cascades. These actions confer a significant inhibitory action on tumor angiogenesis and metastasis, suggesting that JG3 should be considered as a novel candidate molecule for development as an anticancer agent.

Materials and Methods
Preparation and Semisynthesis of JG3
Preparation of JG3 precursor from polymannuronate using hydrogen peroxide. The polymannuronate blocks obtained from sodium alginate were dissolved in distilled water, to which hydrogen peroxide was added. The solution was maintained at 90°C with stirring for 2 hours. The reaction was terminated by adding Na2S2O5. After filtration, oligomannururate species were recovered by precipitation in ethanol. The pellet was washed with ethanol and dried. Oligomannururate mixtures were separated with the Bio-Gel P6 column (1.6 × 180 cm). Samples were collected (1-ml volume each) and analyzed following the carbazole reagent method and peaks were pooled and freeze-dried.

Preparation of JG3 using chlorosulfonic acid. The JG3 compound was obtained by semisynthesis following sulfate modification by reacting its precursor with ClSO3H in formamide. Briefly, oligomannururate was added to sulfitating reagents containing formamide and ClSO3H, and reacted for 3 hours. The pH of the products was adjusted to 7.0 with 4 mol/L NaOH and desalted with Sephadex G-10. The product peak was pooled and freeze-dried. The molecular weights of JG3 and its precursor were analyzed by high-performance gel permeation chromatography with a G3000PWxl column (300 × 7.8 mm; Tosoh, Tokyo, Japan).

Human Heparanase Activity
Human recombinant heparanase was expressed in insect cells and purified as previously reported (20) and its activity toward heparan sulfate–FITC was determined as described (11). Various concentrations of JG3 were added to the heparanase-heparan sulfate–FITC mixture first to react at 37°C for 2 hours.

Surface Plasmon Resonance Assay
The kinetics and specificity of the binding reactions between JG3 and heparanase or bFGF and the determination of the JG3 binding sequence on heparanase were carried out with the BIACore X surface plasmon resonance apparatus. Briefly, JG3, low molecular weight heparin, or peptide molecules were immobilized on CM5 sensor chips according to the Ligand Thiol protocol described in the BIACore Handbook. The unreacted surface moieties were blocked with ethanolamine. To correct for nonspecific binding and bulk refractive index changes, a blank channel (FC2) without oligosaccharide was employed as a control for each experiment. Sensorgrams for all binding interactions were recorded in real time and the blank channel readings were subtracted from the results before analysis. Changes in mass due to the binding response were recorded as resonance units. All binding experiments were done at 25°C with a constant flow rate of 10 μL/min HBS-EP. The sensor chip surface was regenerated with 60 μL of 2 mol/L NaCl in between experiments. For determination of association and dissociation rate constants, the real-time binding capacity was allowed to proceed to equilibrium. Binding kinetics and stoichiometry were determined by surface plasmon resonance using the BIACORE software 3.1.

Assaying the Release of bFGF from the Extracellular Matrix
The release of bFGF from 35-mm extracellular matrix–coated dishes was assayed as previously described (21). Briefly, human umbilical vascular endothelial cells (HUVEC) were cultured on 35-mm tissue culture dishes to reach confluence and maintained for an additional 10 days to allow for the production of a robust extracellular matrix. Two days later, the subendothelial extracellular matrix was exposed by dissolving the cell layer. The extracellular matrix remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish. The culture medium was replaced by serum-free medium before each treatment. For inhibition studies, heparanase (100 ng/mL) was incubated with or without the indicated concentration of JG3 on the extracellular matrix for 4 hours at 37°C. After incubation, aliquots of the media were examined for bFGF level with an ELISA kit (R&D Systems, Minneapolis, MN).

Matrigel Invasion Assay
Transwell chamber membranes (8 μm pore size; Costar, Cambridge, MA) were coated with 100 μL of 1 mg/mL Matrigel. FCS (10%) was added to the lower chambers. Various concentrations of JG3 were added to the upper chambers at the same time. Cells (1 × 10⁵ per well) were added to the upper chamber and allowed to invade for an appropriate time at 37°C in a CO₂ incubator. Cells that had not migrated were removed from the upper chamber with a cotton swab. The remaining cells were fixed, stained with
0.1% crystal violet, and photographs were taken under a microscope and measured at 595 nm after extraction with 10% acetic acid.

Cell Migration Assay
The migration assay was done as described in Matrigel invasion assay except that in the Matrigel-coated plates, HUVECs (5 × 104 per well) were seeded in the inner chamber in M199 containing 1% fetal bovine serum (FBS). The lower chamber contained the same medium with or without bFGF (10 ng/mL) preincubated with different concentrations of JG3 for 2 hours at 4°C. After incubation for 6 hours at 37°C, the migrated cells were fixed and stained with 0.1% crystal violet. HUVEC migration was quantitated by counting the number of cells in five random fields (×100) per insert.

Rat Aortic Ring Assay and bFGF-Induced Aortic Ring Assay
The aortas were harvested from 6-week-old Sprague-Dawley rats. Each aorta was cut into 1-mm slices and embedded in 70 μL Matrigel in 96-well plates. The aortic rings were then fed with 100 μL of M199 with or without different concentrations of JG3, and photographed on day 6. The quantity of microvessels is valued by relative area covered with microvessels using Image-Pro Express.

As for bFGF-induced aortic ring assay, the aortas were treated as described above, with the exception of pretreatment of bFGF (20 ng/mL) with different concentrations of JG3 for 2 hours at 4°C.

Chicken Chorioallantoic Membrane Assay
Groups of 10 fertilized chicken eggs were transferred to an egg incubator (Lyon, Chula Vista, CA) maintained at 37°C and 50% humidity and allowed to grow for 7 days. Gentle suction was applied at the hole located at the broad end of the egg to create a false air sac directly over the chicken chorioallantoic membrane, and a 1-cm2 window was removed from the egg shell immediately. Spongocell suspensions (0.25 × 0.25 × 0.25 cm) saturated with compounds or normal saline was placed on areas between preexisting vessels and the embryos were further incubated for 48 hours. The neovascular zones beside the sponge were photographed under a stereomicroscope (Leica, M55, Heerbrugg, Switzerland) and printed out as 5 × 7-in.2 prints.

Murine B16F10 Experimental Lung Metastasis Assay
C57/BL6 mice received a single ip. injection (0.2 mL/mouse) of JG3 (5 mg/kg, 20 mg/kg) or vehicle (normal saline) 20 minutes before i.v. inoculation of B16F10 melanoma cells (5 × 106 per mouse). Eleven days later, animals were sacrificed and lungs were fixed in Bouin’s solution and scored for the number of metastatic nodules on the lung surface.

Spontaneous Metastasis Assay and Tumor Growth Inhibition
Female athymic nude mice, ages 4 to 5 weeks, were anesthetized with chloral hydrate, and a 5-mm incision was made in the skin over the lateral thorax, as previously described (22). The mammary fat pads were exposed and an inoculum of human breast cancer MDA-MB-435 cells (5 × 105) was implanted into the tissue through a 27-gauge needle. At a volume of 100 to 200 mm₃, mice were divided into three experimental groups, specifically (a) untreated (n = 20); (b) JG3 5 mg/kg (n = 20); and (c) JG3 20 mg/kg (n = 20). JG3 was i.v. administrated through the tail vein once a week for 7 weeks thereafter. Tumors and body weight of mice were measured individually twice per week. Mice were sacrificed 5 days later after the final therapy. The lungs were removed, some were fixed with Bouin’s solution, and metastatic nodules were counted; the other lungs were fixed in 10% neutral-buffered formalin solution and stained with H&E-safranin.

Statistical Analysis
The significance of differences between means was assessed by Student’s t test or by ANOVA and post hoc Newman-Keuls tests. P values of <0.05 and 0.01 were considered as statistically significant.

Supporting Materials and Methods
For details about materials and equipment, cell culture, animals, construction of stably heparanase-expressing cell lines, preparation of the fluorescence-tagged heparanase substrate, cell proliferation assay, immuno-histochemistry, and Western blotting assay, see Supplementary data.

Results
JG3 Inhibits the Heparanase Enzymatic Activity via Binding to Heparin-Binding Domain on Heparanase
JG3 inhibits heparanase enzymatic activity in a cell-free system. The enzymatic activity of heparanase acts to specifically degrade the heparan sulfate chain (23). Using a size exclusion chromatography assay along with a fluorescence-labeled substrate (11), we examined the ability of JG3 to inhibit heparanase-mediated degradation of heparan sulfate. The resulting chromatographic profiles indicated that JG3 significantly and concentration-dependently inhibited heparanase enzymatic activity, with an IC₅₀ value of 6.55 ng/mL. Notably, 40 ng/mL JG3 yielded a maximal inhibitory efficacy of 97.8%, which is far more potent than a comparable molar concentration of low molecular weight heparin (60.3%; Fig. 1B and C).

JG3 specifically binds to the KKDC and QPLK epitopes on heparanase. As our results indicated that JG3 is capable of inhibiting heparanase activity, we next questioned whether JG3 acts as a competitive substrate of heparanase. We first assessed the JG3-heparanase interaction with a surface plasmon resonance assay. JG3 molecules immobilized on a sensor chip were concentration-dependently bound by heparanase, yielding an apparent dissociation constant (Kₐ) of 72.3 nmol/L (Fig. 2A). Scatchard plot analysis further revealed a clearly nonlinear dependence, implicating the possible involvement of two binding sites on heparanase molecule (Fig. 2B). We calculated the stoichiometry with the surface plasmon resonance software and the results indicated that each JG3 molecule could engage with three to four molecules of heparanase.

JG3 is a close structural homologue of endogenous glycosaminoglycans such as heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and hyaluronic acid, making it likely that these glycosaminoglycans might compete with JG3 for binding to heparanase. To test this possibility, we examined the effects of soluble glycosaminoglycans on the JG3-heparanase interaction in a competitive inhibition assay. Low molecular weight heparin significantly competed with JG3 for binding to heparanase; the IC₅₀ for low molecular weight heparin was 0.75 μg/mL, which was very similar to that of the JG3 interaction (Table 1). In contrast, heparan sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronic acid showed little or no inhibition of the JG3-heparanase interaction. Likewise, JG3 dose-dependently blocked the heparanase-low molecular weight heparin association, with an IC₅₀ value of 0.58 μg/mL (Fig. 2C). These results indicate that JG3 and low molecular weight heparin-heparan likely share, to a great extent, the overlapping binding site(s) within heparanase.

To confirm that JG3 binds to heparanase via the heparin binding sites, we tested three heparin-binding domains [KKDC, KLR (KKLVGTKVLMASVQGSKRRKLR amino acids), and QPLK] newly identified on the heparanase molecule (24) for their ability to bind JG3. As shown in Fig. 2D, JG3 bound the KKDC peptide dose-dependently over a concentration range of 100 to 300 μmol/L whereas no such binding was seen with the scrambled control. The KLR and QPLK peptides exhibited only very weak interactions with JG3, even at a higher concentration (300 μmol/L; Fig. 2E and F). However, as we might fail to record the weak but existent interaction with JG3 because the short QPLK peptide spanned only 10 amino acids, we did further experiments in which the QPLK peptide was immobilized on a sensor chip. The results of these experiments revealed that JG3 exhibited a low but detectable...
binding response to QPLK (Fig. 2G). Collectively, these results indicate that JG3 inhibits heparanase enzymatic by binding strongly to the KKDC epitope and more weakly to the QPLK epitope on heparanase.

**JG3 Inhibits Heparanase-Associated Invasiveness**

**JG3 inhibits the invasion of NIH 3T3 cells stably expressing heparanase.** As heparanase expression has been consistently correlated with the metastatic potential of tumor cells, we used a Matrigel chamber to examine the effects of JG3 on heparanase-associated cell invasion in two newly generated NIH 3T3 cells lines stably expressing heparanase (Fig. 3A and B). As shown in Fig. 3C, the heparanase-expressing cells abundantly migrated through the Matrigel to the lower chamber following stimulation with 10% FBS. Exposure to 100 μg/mL JG3 markedly reduced the number of migrated cells, suggesting that JG3 suppresses heparanase-associated migration.

**JG3 inhibits the invasiveness of MDA-MB-435 human breast cancer cells in vitro.** To confirm and extend the above findings, we examined whether JG3 abrogated the invasiveness of MDA-MB-435 human breast cancer cells, which have high-level expression of endogenous heparanase (8). In the absence of JG3, the MDA-MB-435 cells freely invaded the Matrigel and passed into the lower chamber. Treatment of cells with 1 to 100 μg/mL JG3 significantly and dose-dependently reduced the number of migrated cells, with 100 μg/mL JG3 yielding 55.7% inhibition compared with the untreated control (Fig. 3D). Collectively, these findings indicate that JG3 inhibits heparanase-associated invasiveness.

**JG3 Combats the Release of bFGF from the Extracellular Matrix and, Thus, Angiogenesis**

**JG3 combats the release of bFGF from the extracellular matrix.** As heparanase is believed to release active bFGF and other heparan sulfate–bound angiogenic growth factors from sequestration in the basement membrane/extracellular matrix, leading to acceleration of proangiogenic factors-driven angiogenesis, we examined the effects of JG3 on the release of bFGF from the extracellular matrix. ELISA measurements of bFGF spontaneously released into the media of HUVEC-derived extracellular matrix revealed that cultures treated with 100 ng/mL heparanase showed significantly higher levels of bFGF (24.94 pg/mL) whereas JG3 dose-dependently inhibited such a release, with 0.1 μg/mL to 19.53 pg/mL and 0.5 μg/mL to 10.56 pg/mL, respectively. Of note, with the concentration mounting to 1 μg/mL, JG3 completely blocked the bFGF release (Fig. 4A).

**JG3 is antiangiogenic both ex vivo and in vivo.** We next tested the ability of JG3 to abolish angiogenesis in ex vivo and in vivo models. In an ex vivo aorta sprout outgrowth assay, we found that new microvessels began to grow when control cultures were incubated for 6 days resulted in notable suppression of microvessel formation.

Table 1. The inhibitory effects of JG3 and glycosaminoglycans on JG3-heparanase interaction (IC₅₀)

<table>
<thead>
<tr>
<th>JG3</th>
<th>LMWH</th>
<th>HS</th>
<th>CS</th>
<th>HA</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (μg/mL)</td>
<td>0.64</td>
<td>0.75</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

Abbreviations: LMWH, low molecular weight heparin; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronic acid.
with inhibition rates of 18.0%, 45.0%, and 77.0%, respectively, versus the control (Fig. 4B). Similar results were obtained in an in vivo chorioallantoic membrane model. As shown in Fig. 4C, the normal branching pattern of blood vessels formed over a 9-day incubation in the control group, whereas treatment with 1,000 μg/egg JG3 completely blocked this angiogenesis.

**JG3 Abrogates bFGF-Induced Angiogenesis via Direct Binding of bFGF**

bFGF-mediated angiogenesis, a key event in tumor metastasis, requires the function of heparin/heparan sulfate (25, 26). As JG3 is a close homologue of heparin/heparan sulfate, we examined whether the antiangiogenic effects of JG3 occur via competitive inhibition of the interaction between heparin/heparan sulfate and bFGF.

**JG3 inhibits bFGF-induced HUVEC angiogenesis in vitro and ex vivo.** In vitro, we found that JG3 effectively inhibited bFGF-induced HUVEC proliferation and migration, as shown by 

![Image](https://example.com/image.jpg)

Figure 3. The inhibitory action of JG3 on the invasion of stably heparanase-expressing NIH 3T3 cells and MDA-MB-435 cells. A, Western blotting analysis of heparanase expression in pcDNA3.1/myc-HisC-hpses-kozak vector-transfected NIH 3T3 cells. NIH 3T3-hpse-11 (1) and NIH 3T3-hpse-4a (2) are the high heparanase-expressing cells and low heparanase-expressing cells, respectively. B, analysis of heparanase activity following the enzymatic activity assay described in Materials and Methods. C, the inhibitory effect of JG3 (100 μg/mL) on invasion of serum-stimulated stably heparanase-expressing cells in vitro. Top, representative photograph of three independent experiments with similar results (magnification, ×100). D, the inhibitory effect of JG3 on invasion of serum-stimulated MDA-MB-435 cells in vitro. In vitro invasion capacity was assessed after 39 hours as described in Materials and Methods and recorded under a microscope. Columns, mean of three independent experiments; bars, SD. Top, representative images of three independent experiments with similar results (magnification, ×200).
correlated with heparanase secretion in the well-characterized murine B16F10 experimental lung metastasis model (28), providing a good context for our analysis of the in vivo efficacy of JG3 against metastasis. Intravenous inoculation of B16F10 melanoma cells generated a significant metastatic effect in the mouse model. In contrast, mice treated with 5 and 20 mg/kg JG3 showed dramatically decreased numbers of B16F10 metastases (123 ± 28 and 37 ± 14, respectively, versus 208 ± 55 in untreated controls) yielding inhibition rates of 40.9% and 82.2%, respectively (Fig. 6A). Notably, the animals tolerated the tested dosages well, showing no signs of toxicity or body weight loss during the experiments.

**JG3 suppresses lung metastasis and angiogenesis of MDA-MB-435 orthotopic xenografts in athymic mice and suppresses primary tumor growth.** To further confirm that JG3 can decrease metastasis, we examined the effects of this agent in a parallel heparanase-associated metastasis model (i.e., athymic mice bearing human breast cancer MDA-MB-435 cells, ref. 22). Orthotopic injection of human breast cancer MDA-MB-435 cells into the mammary fat pads of female athymic nude mice caused a significant increase in the number of pulmonary metastatic nodules (315 ± 56). However, weekly i.v. administration of JG3 for 7 weeks (5 and 20 mg/kg) caused a dramatic and dose-dependent decrease in the number of pulmonary metastatic nodules (126 ± 49 and 37 ± 10, respectively), yielding evident inhibition rates of 60.0% and 88.3%, respectively (Fig. 6B). Moreover, JG3 treatment dose-dependently suppressed primary tumor growth, with 20 mg/kg JG3 yielding a treated/control (%) of 37.6% (Fig. 6C). In addition, immunohistochemical analyses revealed that treatment of mice with 20 mg/kg JG3 also remarkably decreased the mean number of microvessels in MDA-MB-435-derived primary tumors versus controls (18 ± 2 versus 38 ± 5, respectively), with an inhibition rate of 52.6% (Fig. 6D). As above, the JG3-treated mice survived well and showed no signs of toxicity or body weight loss throughout the experiments. Collectively, these results suggest that JG3 can inhibit tumor angiogenesis, metastasis, and tumor growth in vivo.

**Discussion**

Mammalian endoglycosidase heparanase is the predominant enzyme responsible for degradation of heparan sulfate activity thought to play a decisive role in tumor cell invasion during cancer metastasis and angiogenesis (1, 2, 29–31). Furthermore, heparanase facilitates tumor invasion by collapsing the extracellular matrix, leading to release of angiogenic factors and induction of an angiogenic environment (31, 32). These actions make heparanase a promising target for cancer therapy; heparanase inhibitors are expected to exert anticancer effects by suppressing both angiogenesis and tumor cell metastasis. In the present study, we found that a newly identified sulfated oligosaccharide, JG3, potently and dose-dependently inhibited tumor angiogenesis and metastasis by acting as a noncleavable substrate mimetic (data not shown) and inhibiting heparanase activity.

The structural components responsible for heparanase inhibition by sulfated oligosaccharides are not well known, but oligosaccharide chain length seems to be critical and a high degree of sulfation and...
 Recently shown to represent the active site on heparanase (24), epitopes on the heparanase molecule. The KKDC epitope was interaction revealed that JG3 could bind the KKDC and QPLK sequences mediating the heparin/heparan sulfate-heparanase binding with peptides representing the proposed consensus JG3 that overlap with those of heparin. Further examination of JG3 heparanase molecule might provide two distinct binding sites for molecular weight heparin led us to hypothesize that the heparanase-JG3 association was dramatically blocked by low observation that the Scatchard plot yielded a nonlinear profile and JG3 will help understand the exact action mode of JG3. Our competitively inhibit the heparanase-JG3 association.

The elucidation of the binding sites on heparanase molecule for JG3 will help understand the exact action mode of JG3. Our observations that the Scatchard plot yielded a nonlinear profile and the heparanase-JG3 association was dramatically blocked by low molecular weight heparin led us to hypothesize that the heparanase molecule might provide two distinct binding sites for JG3 that overlap with those of heparin. Further examination of JG3 binding with peptides representing the proposed consensus sequences mediating the heparin/heparan sulfate-heparanase interaction revealed that JG3 could bind the KKDC and QPLK epitopes on the heparanase molecule. The KKDC epitope was recently shown to represent the active site on heparanase (24), strongly suggesting that JG3 blocks heparanase activity by acting as a substrate mimic.

Heparanase activity has been implicated in several key events, including tumor cell invasion (2, 8) and angiogenesis. In terms of invasiveness, heparanase-induced cleavage of heparan sulfate chains from heparan sulfate proteoglycan facilitates tumor cell invasion, and vice versa (12, 14). Our present results showed that JG3 significantly and dose-dependently abrogated the invasiveness of heparanase-expressing NIH 3T3 cells and naturally heparanase-secreting MDA-MB-435 cells by blocking heparanase activity. Besides the involvement in tumor invasion, extracellular matrix degradation by heparanase also triggers the release of heparin-binding angiogenic factors sequestered by heparan sulfate proteoglycan, such as bFGF (33). Any compounds with the combination of high inhibition of heparanase and low release/potentiation of extracellular matrix–bound growth factor might promise their potential antiangiogenic and antimetastatic therapeutics. Encouragingly, JG3 efficiently counteracted the initial release of latent bFGF sequestered in heparan sulfate proteoglycans from the extracellular matrix. A note of caution could be raised with regard to the therapeutic use of sulfated oligosaccharides in general and JG3 in particular, in that a large body of literature has documented the capability of heparin and other sulfated oligosaccharides to facilitate (as opposed to inhibit) growth factor release. Notably, in one recent study, PI-88 was also observed to enhance release of the heparan sulfate binding growth factor bFGF, and yet PI-88 inhibited bFGF-mediated cell signaling (34). However, modified heparin by glycol-splitting as N-acetylated as PI-88 failed to release bFGF from extracellular matrix and, thus, its mitogenic activity (35). All these striking disparities, in particular of JG3 from other sulfated oligosaccharides including PI-88, are probably ascribed to their distinct structures in nature.

![Figure 5](https://www.aacrjournals.org/8785/CancerRes2006;66:(17).September1,2006)

**Figure 5.** Effects of JG3 on bFGF-induced HUVECs angiogenesis. A, JG3 concentration-dependently inhibits bFGF-induced HUVECs proliferation. The histogram summarized the relative inhibition of [3H]thymidine incorporation in HUVECs on treatment with different concentrations of JG3 compared with the bFGF control. B, JG3 treatment abolishes bFGF-induced HUVECs migration through a Transwell chamber. The histogram displays the average number of migrated cells per field. C, the effect of JG3 on bFGF-induced microvesSEL outgrowth arising from rat aortic ring. A representative photograph of three independent experiments with similar results (magnification, ×100). D, Western blotting analysis of JG3 on the phosphorylation of bFGF receptor (FGFR) and ERK1/2. E, binding sensorgrams of bFGF to JG3 immobilized on sensorchip using surface plasmon resonance. The concentrations of bFGF are, from bottom to top, 52, 104, 208, and 416 nmol/L, respectively.

- **P < 0.01, versus control.**
- **P < 0.05; **P < 0.01, versus bFGF-treated group.
Besides its responsive role in the tumor invasion, heparanase is also tightly involved in tumor angiogenesis. Our results revealed that JG3 almost completely abolished rat aortic sprout growth and chorioallantoic membrane angiogenesis, significantly abrogating bFGF-induced HUVEC angiogenesis at noncytotoxic concentrations both in vitro and ex vivo. Thus, JG3 seems to act bifunctionally, directly blocking angiogenesis by degrading heparan sulfate proteoglycans in the extracellular matrix while also directly trapping bFGF. This is substantially supported by our finding that JG3 multivalently binds bFGF ($K_d = 189$ nmol/L) and acts as a competitive inhibitor of heparin by competing for the same binding site(s) on bFGF. A previous study showed that inhibiting formation of the "bFGF-bFGF receptor-heparan sulfate" ternary complex blocked bFGF-associated signal transduction (36–38). Thus, we propose that JG3 competitively binds bFGF and effectively blocks bFGF receptor binding, leading to the observed JG3-induced decrease in bFGF receptor and ERK tyrosine phosphorylation. Although different mechanisms are likely responsible for angiogenesis in our various models, these results seem to indicate that JG3 inhibits angiogenesis by simultaneously inhibiting heparanase activity and heparan sulfate effector functions.

Although no currently available animal model recapitulates all heparanase-associated functions, the highly heparanase-expressing murine B16F10 experimental lung metastasis model and human breast cancer MDA-MB-435 cells orthotopically xenografted into athymic nude mice can simulate many of the heparanase-driven pathologic processes. The results from these models revealing that JG3 (20 mg/kg) inhibited lung metastasis of B16F10 and MDA-MB-435 cells in mice by 82.2% and 88.3%, respectively, further supported this theory. This in vivo antimetastatic mechanism of JG3 lies greatly in its ability to interrupt heparanase-associated pathologic events. This efficacy is highly significant and is comparable to other commonly known antimetastatic agents. In addition, the in vivo angiogenesis inhibition we observed may be the comprehensive reflection of two defined mechanisms but serving the same outcome (i.e., JG3 simultaneously blocked heparanase activity and limited the availability of heparan sulfate–binding growth factor bFGF). In this context, the nature of the in vivo primary tumor growth arrest of JG3, albeit remaining unclear, is due to, at the very least in part, to a combinatorial result of heparanase-mediated and bFGF-associated events. One, of course, cannot exclude some other responsible factors unraveled.
In sum, we herein show for the first time that JG3, a novel sulfated oligosaccharide, has both in vitro and in vivo antiangiogenic and antimetastatic effects, which may be attributed to its ability to simultaneously inhibit the heparanase cascade and bFGF signaling. JG3 treatment could trigger in vivo tumor growth arrest in mouse tumor models and showed very low toxicity, likely due to its weak anticoagulant activity. Additional studies will be needed to characterize the three-dimensional structure of the heparanase-JG3 interaction and identify the nature and size requirements of JG3 and its analogues for heparanase, with the aim of structure-assisted development of new carbohydrate-based cancer therapeutics, in particular those simultaneously targeting heparanase and proangiogenic factors.

Acknowledgments

Received 4/14/2006; revised 6/13/2006; accepted 7/6/2006.

Grant support: National Basic Research Program of China, grant 2003CB716400. 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.

References


3 Approximately 13-fold less than heparin, and nearly 3-fold less than PI-88; unpublished data.
Oligomannururate Sulfate, a Novel Heparanase Inhibitor Simultaneously Targeting Basic Fibroblast Growth Factor, Combats Tumor Angiogenesis and Metastasis

Huajun Zhao, Haiying Liu, Yi Chen, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/17/8779

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2006/09/11/66.17.8779.DC1

Cited articles
This article cites 38 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/17/8779.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/66/17/8779.full.html#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.