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

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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.

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-1,4-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, 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 that not only exhibit high potency but are also selective and orally available.
inhibitors. Oligomannurane sulfate (JG3), a newly semisynthesized, structurally novel sulfated oligosaccharide derived from marine oligomannurane 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 (KKFKSTYRSSVDC amino acids) and QPLK (QPRKTAKLK 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 polymannurane using hydrogen peroxide. The polymannurane 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 Na2S2O3. After filtration, oligomannurane species were recovered by precipitation in ethanol. The pellet was washed with ethanol and dried. Oligomannurane mixtures were separated by 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 precipitated in ethanol. The pellet was washed with ethanol and 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, oligomannurane was added to sulfating reagents containing formamide and ClSO3H, and reacted for 3 hours. High-performance gel permeation chromatography with a G3000PWxl 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, oligomannurane was added to sulfating 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 Application Handbook. The untreated 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 recorded. For direct assessment of dissociation constants, the association phase 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 × 10⁶ 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
plates. The aortic rings were then fed with 100 μL of 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. Sponge (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-in² prints.

Murine B16F10 Experimental Lung Metastasis Assay
C57BL/6 mice received a single i.p. 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 × 10⁴ 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 × 10⁶) 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, immunohistochemistry, 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-heparanase 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, KKL (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 to the heparanase molecule (24) for their ability to bind JG3. As shown in Fig. 2D, JG3 bound the KKDC peptide dose-dependently to the heparanase molecule (24) for their ability to bind JG3. As shown in Fig. 2D, JG3 bound the KKDC peptide dose-dependently to the heparanase molecule (24) for their ability to bind JG3. As shown in Fig. 2D, JG3 bound the KKDC peptide dose-dependently to the heparanase molecule (24) for their ability to bind JG3.

JG3 Combats Tumor Angiogenesis and Metastasis
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 cell 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 2 days. Treatment with JG3 (1, 10, and 100 μg/mL) for 6 days resulted in notable suppression of microvessel formation.

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

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<th>JG3</th>
<th>LMWH</th>
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<td>IC₅₀ (μg/mL)</td>
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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 [3H]thymidine incorporation (Fig. 5A) and Transwell chamber assays (Fig. 5B), respectively. Ex vivo, our results revealed that treatment with JG3 (200 μg/mL) potently suppressed microvessel outgrowth from bFGF-stimulated rat aortic rings, as compared with untreated controls (Fig. 5C).

**JG3 overcomes bFGF-induced phosphorylation of bFGF receptor and extracellular signal-regulated kinase 1/2 in HUVECs.** bFGF receptor phosphorylation activates a number of downstream cytoplasmic signaling cascades that are central to angiogenesis (27). Of these downstream molecules, extracellular signal-regulated kinase (ERK)-1/2, a relatively well-characterized signaling molecule involved in bFGF-mediated endothelial cell proliferation and migration, is believed to be amplified during tumor-associated angiogenesis. We thus investigated the effects of JG3 on phosphorylation of bFGF receptor and ERK1/2. Treatment of HUVECs with bFGF (20 ng/mL) triggered extensive phosphorylation of bFGF receptor and ERK1/2, as compared with the control, whereas cells treated with JG3 (2, 20, and 200 μg/mL) showed significantly less phosphorylation of bFGF receptor and ERK1/2 in response to bFGF (Fig. 5D). These findings suggest that JG3-induced decreases in phospho-bFGF receptor and subsequent ERK1/2 account for the inhibitory effects of JG3 on bFGF-induced angiogenesis.

**JG3 specifically and multivalently binds the heparin-binding domain of bFGF.** Because bFGF-associated signaling is dependent on heparin/heparan sulfate chaperone (27), we hypothesized that the inhibitory effects of JG3 on bFGF signaling could occur via blockade of the interaction between heparin/heparan sulfate and bFGF. To test this hypothesis, we used surface plasmon resonance assays to investigate the binding between JG3 and bFGF. Our results revealed that JG3 exhibited a relatively high, multivalent affinity for bFGF, with a $K_d$ value of 189 nmol/L (Fig. 5E). Stoichiometric analysis further showed that each JG3 molecule could bind two to three bFGF molecules. Furthermore, JG3 can competitively and dose-dependently inhibit the interaction of bFGF with low molecular weight heparin immobilized on a sensor chip (data not shown), suggesting that JG3 and heparin largely share the same binding domain on bFGF.

**JG3 Inhibits Tumor Angiogenesis, Metastasis, and Tumor Growth in vivo**

As heparanase expression is closely related with the metastatic potential of tumor cells and our results indicated that JG3 efficiently abolished heparanase-mediated actions in vitro, we next investigated the efficacy of JG3 against tumor metastasis in vivo. JG3 suppresses lung metastasis in a murine B16F10 experimental metastasis model. Metastasis has been positively
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 F 28 and 37 F 14, respectively, versus 208 F 55 in untreated controls) yielding inhibition rates of 40.9% and 82.2%, respectively (Fig. 6 A).

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 F 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 F 49 and 37 F 10, respectively), yielding evident inhibition rates of 60.0% and 88.3%, respectively (Fig. 6 B). 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...
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-secretory 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-acetylatedheparin failed otherwise 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.** 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. Left, 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 nM, 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 chorionicallantoic 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 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.

Figure 6. JG3 inhibits tumor metastasis, angiogenesis, and tumor growth in vivo. A, effect of JG3 on lung metastasis of murine B16F10. Left, representative photograph of metastatic nodules on lungs. The histogram shows the inhibitory efficacy of JG3 on the number of pulmonary colonies. Columns, mean of a typical experiment; bars, SE. B, effect of JG3 on lung metastasis of MDA-MB-435 breast carcinoma orthotopic xenografts in nude mice. Top, representative photograph of metastatic nodules on lungs with H&E staining (magnification, >200). The histogram shows the inhibitory action of JG3 on the number of pulmonary metastatic nodules. Columns, mean of a typical experiment; bars, SE. C, effect of JG3 against primary tumor growth of MDA-MB-435 breast carcinoma. D, effect of JG3 against primary tumor angiogenesis. Left, typical photograph of primary tumor sections with immunohistochemistry staining. Arrows, sites where microvessels grow (magnification, >200). The histogram represents the inhibition of JG3 on the number of microvessels. Similar results were obtained from at least two separate experiments: *, \(P<0.05\); **, \(P<0.01\), versus the control.
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.

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References


3 Approximately 13-fold less than heparin, and nearly 3-fold less than PI-88; unpublished data.
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