Gekko Sulfated Glycopeptide Inhibits Tumor Angiogenesis by Targeting Basic Fibroblast Growth Factor

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**Background:** We studied basic fibroblast growth factor (bFGF) to elucidate the anti-angiogenesis mechanisms of a sulfated glycopeptide from *Gekko swinhonis Guenther* (GSPP).

**Results:** GSPP inhibited tumor angiogenesis by blocking bFGF production, release from the extracellular matrix, and binding to its low affinity receptor.

**Conclusions:** GSPP inhibits tumor angiogenesis by targeting bFGF.

**Significance:** GSPP is a promising candidate for cancer therapy.

**SUMMARY**

Basic fibroblast growth factor (bFGF) is a therapeutic target of anti-angiogenesis. Here, we report that a novel sulfated glycopeptide derived from *Gekko swinhonis Guenther* (GSPP), an anticancer drug in traditional Chinese medicine, inhibits tumor angiogenesis by targeting bFGF. GSPP significantly decreased the production of bFGF in hepatoma cells by suppressing early growth response-1. GSPP inhibited the release of bFGF from extracellular matrix by blocking heparanase enzymatic activity. Moreover, GSPP competitively inhibited bFGF binding to heparin/heparan sulfate via direct binding to bFGF. Importantly, GSPP abrogated the bFGF-stimulated proliferation and migration of endothelial cells, whereas it had no inhibitory effect on endothelial cells in the absence of bFGF. Further study revealed that GSPP prevented bFGF-induced neovascularization and inhibited tumor angiogenesis and tumor growth in a xenograft mouse model. These results demonstrate that GSPP inhibits tumor angiogenesis by blocking bFGF production, release from the extracellular matrix, and binding to its low affinity receptor, heparin/heparan sulfate.
Angiogenesis, the process of new blood vessel formation from preexisting vasculature, is crucial for malignant tumor growth (1, 2). Recently, basic fibroblast growth factor (bFGF) was demonstrated to play important roles in angiogenesis and tumor growth (3-5). Blocking bFGF production markedly restricted tumor angiogenesis and growth (6). Moreover, compelling evidence indicates that high levels of bFGF are associated with tumor escape from anti-angiogenic therapy (7, 8). Thus, targeting bFGF may be an efficient strategy for suppressing angiogenesis and tumor growth (9).

The transcription factor early growth response-1 (Egr-1) is an immediate early gene that regulates bFGF transcription as an activator of bFGF gene promoter (10, 11).

bFGF is commonly sequestered within heparan sulfate proteoglycans in the basement membrane/extracellular matrix in an inactive manner (12, 13). Heparanase specifically degrades the heparin/heparan sulfate (HS) chains of heparan sulfate proteoglycans to release active bFGF sequestered in the basement membrane/extracellular matrix (14, 15). Once released, bFGF exerts its proangiogenic activity by binding to its specific FGF receptor located on the endothelial cell surface (16). This process critically requires the involvement of the low affinity receptor heparin/HS side chains of heparan sulfate proteoglycans to form the HS/FGF/FGF receptor ternary complex (17, 18). Therefore, blockade of the involvement of heparin/HS is a putative target for interfering with bFGF bioactivity (19-21). Suramin, a competitive inhibitor of heparin that binds to bFGF, is known to be a potent inhibitor of bFGF-induced angiogenesis (22, 23). Recently, PI-88, a complex mixture of highly sulfated oligosaccharides, progressed to phase II clinical trials as an adjuvant therapy for hepatocellular carcinoma after curative resection. This represents the development of heparin/HS mimetic to competitively interfere with the formation of the HS/FGF/FGF receptor complex (24).

Although *Gekko swinhonis Guenther* has been used as an anticancer drug in traditional Chinese medicine for hundreds of years, its underlying mechanism is poorly understood. We previously isolated a novel sulfated polypeptide, GSPP, from *Gekko swinhonis Guenther* and demonstrated its direct effects on the proliferation, differentiation, and migration of hepatoma cells (25-27). Here, we investigated the potent anti-angiogenic ability of GSPP in vitro and in vivo and found that bFGF signaling is involved in this process.

**EXPERIMENTAL PROCEDURES**

*Reagents, cell lines, and mice*–GSPP was isolated and purified as described previously (25). The concentrations of GSPP used were 10, 100, and 200 μg/mL in all cellular experiments unless indicated otherwise. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins and maintained in MEM supplemented with 20% FBS (Hyclone). Endothelial cells were verified by the detection of CD31. Hepatoma cell lines Bel-7402 and HepG2 were obtained from Tianjin Medical University Cancer Research Institute. Adult bovine aortic endothelial cells were kindly provided by Dr. Zhang (Nankai University, China). Bel-7402, HepG2, and adult bovine aortic endothelial cells were maintained in DMEM supplemented with 10% FBS. Male nu/nu nude and C57BL/6 mice were obtained from the Chinese Medical Academy of Science and used in accordance with the National Institutes of Health Guidelines for Animal Care.

*Enzyme-linked immunosorbent assay (ELISA)*–ELISAs were performed to detect the levels of bFGF (R&D Systems), heparanase (TAKARA, Japan), and Egr-1 (USCN LIFE, China) according to the manufacturer’s instructions. The hepatoma cells were treated as follows. HepG2 and Bel-7402 cells were seeded at $5 \times 10^4$
cells/well in 12-well plates and cultured for 24 hours. The media were replaced with fresh media containing the indicated concentrations of GSPP, and the cells were cultured for 72 hours. Culture supernatants were collected at 24, 48, and 72 hours to determine bFGF levels. After 72 hours of culture, HepG2 cells were lysed and the levels of heparanase were measured. HepG2 and Bel-7402 cells were seeded at $3 \times 10^5$ cells/well in 6-well plates and cultured for 24 hours. The media were replaced with fresh media containing 100 μg/mL GSPP, and the cells were cultured for 24 hours. The cells were lysed by the addition of 300 μL cell lysis buffer (Sigma) at 0.5, 2, 12, and 24 hours for the detection of Egr-1.

**Real-time reverse transcription-PCR**—To measure the levels of bFGF, Egr-1, and GAPDH in HepG2 and Bel-7402 cells, the cells were treated in the same manner as for ELISA. Total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized from 2 μg total RNA using MMLV reverse transcriptase. Real-time PCR was carried out with 2 μL cDNA and SYBR Green Master Mix (Qiagen). The forward and reverse primers used for bFGF, Egr-1, and GAPDH were as follows: bFGF, 5′-GAG CGA CCC TCA CAT CAA-3′ and 5′-CGT TTC AGT GCC ACA TAC C-3′; Egr-1, 5′-CTG CAC GCT TCT CAG TGT TC-3′ and 5′-AGC AGT TTC AGC ACA TAC C-3′; GAPDH, 5′-AGG CAT CCA CAT GC-3′ and 5′-AGG GCC CAT CCA CAG TCT TC-3′. The thermal conditions were as follows: 3 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute. For semi-quantitative analysis, the target gene expression was normalized to that of GAPDH. Data are expressed as percentages compared to the control.

**Heparanase stimulation experiments**—To investigate the effect of GSPP on the release of bFGF from extracellular matrix triggered by heparanase, HepG2 cells were seeded at $1 \times 10^5$ cells/well in 12-well plates and incubated for 24 hours. The culture medium was replaced with DMEM, and the cells were cultured for another 24 hours. The cells were then washed twice with DMEM. Heparanase-1 or heparanase-2 was then added to a final concentration of 0.5 U/mL in 1 mL DMEM with or without 200 μg/mL GSPP and cultured for 2 hours at 37°C. The media were collected to detect bFGF levels using an ELISA kit. The cells were lysed by the addition of 250 μL cell lysis buffer. The cellular protein was extracted for western blot analysis.

**Western blot analysis**—HepG2 cells were treated for 72 hours as described in the procedures for ELISA. The cells were lysed and subjected to Western blotting. Aliquots of protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Blots were incubated with mouse monoclonal anti-total ERK1/2 antibody, anti-phosphorylated pERK1/2 antibody (1:1000; Cell Signaling Technology), and anti-actin antibody (1:5000; Sigma) overnight. Protein expression was detected using the ECL Western Blotting detection system.

**Surface plasmon resonance assay**—The kinetics and specificity of the binding reactions of bFGF to heparin or GSPP were determined with the BIAcore X100 Surface plasmon resonance apparatus. Briefly, recombinant human bFGF (PeproTech) was immobilized on a CM5 sensor chip (GE Healthcare) using an amine coupling kit (Pharmacia Biosensor AB) according to the manufacturers’ and Zhu et al.’s protocols (28). To determine the direct binding affinity of bFGF for GSPP and heparin, 15 μL GSPP (0.625, 1.25, 2.5, 5, or 10 mg/mL) and 15 μL heparin (0.625, 1.25, 2.5, 5, 10, or 20 mg/mL; Solarbio Biology, China) were injected into a buffer and flowed onto a chip with immobilized bFGF, respectively. To evaluate the inhibitory effect of GSPP on the binding of heparin to bFGF, 10 mg/mL heparin was added to the chip after GSPP was bound to the chip. Data are expressed as response units.
Solid-phase heparan sulfate–bFGF binding assay—The heparan degrading enzyme assay kit (TAKARA) was used to observe the interaction between bFGF and the mixture of HS/GSPP, although the protocol was modified from that recommended by the manufacturer. Briefly, 50 μL GSPP (final concentration: 0.1, 0.25, 0.50, 1.0, or 2.0 mg/mL) and 50 μL biotinylated HS were mixed for 1 minute and transferred into a bFGF-immobilized 96-well plate. The control group received 50 μL biotinylated HS and 50 μL PBS. The results are expressed as the percentages of the inhibitory effects of GSPP on the binding of biotinylated HS to bFGF.

Proliferation and migration of endothelial and hepatoma cells—To evaluate the effects of GSPP on endothelial cells, we used HUVECs in the presence of bFGF (2 ng/mL) as well as HUVECs in the absence of bFGF and adult bovine aortic endothelial (without the induction of bFGF). In order to detect the activities of bFGF and GSPP on endothelial cells, we serum-starved the cells (reducing serum levels to 1%) to induce a quiescent state in endothelial cells prior to the experiments and subsequently re-introduced full serum or bFGF in the course of the experiments. To evaluate the effects of GSPP on hepatoma cells, HepG2 and Bel-7402 cells were seeded at 2 × 10⁴ cells/well in 12-well plates, cultured for 72 hours, and counted. Trypan blue exclusion, transwell, and wound-healing assays were performed as described previously (25).

Confocal microscopy—HUVECs were seeded on coverslips in 12-well plates and cultured for 24 hours. The culture medium was replaced with fresh MEM plus 10% FBS and 200 μg/mL GSPP in the presence or absence of 2 ng/mL bFGF. The cells were cultured for 48 hours and washed twice with PBS. Immunofluorescence staining of phalloidin rhodamine (Invitrogen) was performed as described previously (25).

Tube formation assay—A HUVEC suspension (2 × 10⁵ cells/well) was added onto the Matrigel-coated (BD Biosciences) 12-well plates and incubated for 7 hours. Photographs were taken under a phase-contrast microscope.

Mouse aortic sprouting assay—Mouse aortas were harvested from 6-week-old C57BL/6 mice and cut into 1-mm slices in MEM medium. Aortic segments were embedded into Matrigel-coated plates and cultured in MEM supplemented with 20% FBS, 5 ng/mL bFGF, and GSPP. Photographs were taken on the ninth day.

Chicken chorioallantoic membrane assay—Groups of 10 fertilized chicken eggs were transferred to an egg incubator maintained at 38°C and 50% humidity, and allowed to grow for 6 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 immediately removed from the egg shell. Matrigel (50 μg) plus 5 ng/mL bFGF with or without GSPP were placed on the areas between pre-existing vessels. The embryos were incubated for another 48 hours. The neovascular zones beside the Matrigel were photographed under a stereomicroscope.

Tumor xenograft assay—Male nu/nu nude mice aged 4–6 weeks and weighing approximately 18–20 g were injected subcutaneously with hepatoma Bel-7402 cells (0.1 mL, 8 × 10⁶ cells). The mice were allocated into 4 groups according to treatment: (1) normal saline (control group); (2) GSPP 20 mg/kg; (3) GSPP 200 mg/kg; and (4) suramin 1 mg/kg. Drug administration began on the next day of inoculation, and each animal received the treatment drug once a day for 25 days via cavitas abdominalis injection. Tumors were measured individually once every 3 days in 2 dimensions (i.e., length and width) using calipers. The tumor volume (mm³) was calculated as V = length × width² / 2. On the 25th day, the animals were weighed and sacrificed. The implanted tumors were excised and weighed.

Immunohistochemical staining, microscopy,
and vessel density measurements—Sections (4 μm thick) from formalin-fixed paraffin-embedded tumor xenografts were prepared. After deparaffinization, primary rat monoclonal anti-mouse antibodies against CD34 (dilution 1: 10, Abcam) and bFGF (dilution 1: 20, Santa Cruz) were used to perform immunohistochemical analysis according to the manufacturers’ instructions. To evaluate microvessel density, tumor tissue sections were examined at low-power magnification to identify the areas with the highest density of CD34-positive vessels. The numbers of microvessels in 400× fields were counted. To quantify bFGF expression, the sections were photographed with a digital camera at 400× magnification. The quantification of bFGF density was analyzed by Image-Pro Plus image analysis software. For every section, the integral optical density of every visual field was calculated.

Statistical analysis—Statistical significance was analyzed by one-way ANOVA using SPSS 16.0 software. Comparisons with P values less than 0.05 were considered to be statistically significant.

RESULTS

GSPP decreased bFGF production in hepatoma cells and tumor xenograft tissues—We observed the expressions of bFGF mRNA and protein in hepatoma cells after treatment with different concentrations of GSPP for 72 hours, while no antiproliferative effects were detected in hepatoma cells after treatment with GSPP for 72 hours (data not shown). GSPP significantly decreased bFGF mRNA expression in a dose-dependent manner. Similarly, GSPP decreased bFGF protein levels in cell supernatants in a dose- and time-dependent manner. The addition of 100 or 200 μg/mL GSPP decreased mRNA expression and protein levels 2- to 3-fold compared to those in the controls (Fig. 1A, B). The expression of bFGF in tumor tissues decreased after treatment with 200 mg/kg GSPP (Fig. 1C).

GSPP suppressed Egr-1 mRNA and protein expression—To determine the molecular determinants by which GSPP inhibits bFGF expression, the effect of GSPP on the expression of Egr-1 in hepatoma cells was determined because Egr-1 is suggested to be an upstream activator of the bFGF gene promoter (10, 11). Egr-1 gene expression was detected in both hepatoma cell lines. GSPP decreased the levels of both Egr-1 mRNA and protein. The decreases were detected 30 minutes after treatment with GSPP in all hepatoma cell lines (Fig. 2A, B).

GSPP inhibited heparanase enzymatic activity—Heparanase has been reported to promote the release of bFGF from the extracellular matrix (14, 15). We first assessed the levels of heparanase in hepatoma cell culture supernatants after treatment with GSPP for 72 hours. However, GSPP had no apparent effect on heparanase levels (data not shown). We further assessed the effect of GSPP on the activity of heparanase. Heparanase-1 and heparanase-2 were used to induce the release of bFGF from the extracellular matrix. As expected, treatment with heparanase markedly increased the levels of bFGF in the culture media, indicating that both heparanase-1 and heparanase-2 stimulate bFGF release. Meanwhile, bFGF levels were not affected by either heparanase-1 or heparanase-2 after pretreatment with 200 μg/mL GSPP. This indicates that the heparanase-stimulated release of bFGF from the extracellular matrix was completely blocked by GSPP (Fig. 2C). Heparanase has been reported to initiate the activation of ERK1/2 (29, 30). In order to further confirm the effect of GSPP on heparanase activity, we assessed the heparanase-stimulated phosphorylation of ERK1/2. Exposure of HepG2 cells to heparanase-1 or heparanase-2 resulted in increased ERK1/2 phosphorylation. Encouragingly, pretreatment with 200 μg/mL GSPP reduced the heparanase stimulation of
ERK1/2 phosphorylation (Fig. 2D).

GSPP inhibited bFGF binding to heparin/HS—We performed surface plasmon resonance assay to test the hypothesis that GSPP directly interacts with bFGF. We found that GSPP showed high affinity for bFGF in vitro in a dose-dependent manner (Fig. 3A, C). To further determine whether GSPP inhibits the binding of bFGF to heparin, we assessed the binding affinity of heparin at different concentrations (range, 0.625–20 mg/mL) to immobilized bFGF and found that bFGF binds to heparin in a dose-dependent manner (Fig. 3B). GSPP directly bound to bFGF and competitively inhibited the binding of heparin to bFGF in a dose-dependent manner (Fig. 3D). The response unit was 2.5-fold lower for 10 mg/mL heparin binding to bFGF after pretreatment with 10 mg/mL GSPP.

The solid-phase binding assay was applied to ensure competitive binding between bFGF-HS and bFGF-GSPP. The binding ability of HS to bFGF decreased when it was previously mixed with GSPP. More importantly, the binding ability of HS to bFGF decreased with increasing GSPP concentration (Fig. 3E).

GSPP abrogated the stimulatory effects of bFGF on endothelial cells in vitro—To investigate the effects of GSPP on the proliferation and migration of endothelial cells stimulated by bFGF, we conducted 3 independent studies: HUVECs with bFGF induction, HUVECs without bFGF induction, and adult bovine aortic endothelial cells without bFGF induction. bFGF is a critical factor for the activation of HUVECs but is unnecessary for the activation of adult bovine aortic endothelial cells. There were 1.62-fold more cells in HUVEC treatment with bFGF than without bFGF. Meanwhile, the cell numbers of bFGF-stimulated HUVECs were reduced as much as 1.5-fold after treatment with 200 μg/mL GSPP, compared to the control. These results suggest that 200 μg/mL GSPP almost completely blocks the bFGF-stimulated proliferation of HUVECs. Meanwhile, we evaluated the effect of GSPP on HUVECs without bFGF induction. Interestingly, the addition of GSPP did not reduce the proliferation of HUVECs compared with the control. We further evaluated the inhibitory effect of GSPP on adult bovine aortic endothelial cells. Similarly, GSPP had no effect on the proliferation of adult bovine aortic endothelial cells, even at higher concentrations of up to 200 μg/mL (Fig. 4A).

We performed a transwell assay to investigate the effect of GSPP on bFGF-induced endothelial cell migration. bFGF (2 ng/mL) significantly promoted the migration of HUVECs. This promotion was attenuated by GSPP at concentrations of 100 and 200 μg/mL. However, GSPP has no inhibitory effect on the migration of HUVECs or adult bovine aortic endothelial cells without bFGF stimulation. The inhibitory effect of GSPP on endothelial cell migration was further confirmed by a wound-healing assay in which only HUVEC pretreatment with bFGF showed a decrease in migration after the addition of GSPP (Fig. 4C, E). In contrast, GSPP did not have the same effects on HUVECs or adult bovine aortic endothelial cells in the absence of bFGF. Analysis of cytoskeletal protein showed that bFGF increased actin stress fiber formation. In contrast, treatment with GSPP prevented the bFGF-induced stress fiber formation (Fig. 4F).

GSPP inhibited bFGF-induced angiogenesis—The inhibitory effect of GSPP on the bFGF bioactivity of endothelial cells prompted us to examine the involvement of GSPP in bFGF-induced angiogenesis more closely. The in vitro tube formation of HUVECs on Matrigel was greatly affected by the addition of GSPP, exhibiting shortened tubule length, a decreased number of tubules, reduced tubule area, and a decreased number of branch points (Fig. 5A). Such suppression was also found in the aortic sprouting assay, where the neovascularization of aortic segments was evaluated in 3-dimensional models (Fig. 5B).
Interestingly, in the chick chorioallantoic membrane assay, we found that the ability of GSPP to inhibit angiogenesis was much more pronounced when bFGF was used as an angiogenic stimulator. This implies a possible interaction between GSPP and bFGF in this process (Fig. 5C).

**GSPP inhibited tumor growth and tumor angiogenesis in vivo**—A xenograft mouse model of hepatocellular carcinoma was performed to analyze the efficacy of GSPP in vivo. GSPP (200 mg/kg) significantly decreased tumor volumes (Fig. 6A). The tumor weight in the GSPP-treated group was reduced by 23.37% and 48.7% with 20 and 200 mg/kg, respectively (Fig. 6B). Similar results regarding the tumor volume and tumor weight were found in suramin-treated group. In order to evaluate the adverse effects of GSPP and suramin, we measured the final weights of the mice. There was no significant difference between the control and GSPP-treated groups, whereas a significant difference was found between the control and suramin-treated groups. The weight of the suramin-treated group decreased (Fig. 6C). Considering the hypervascular nature of hepatocellular carcinoma, we subsequently evaluated microvessel density in the tumor sections. As a result, GSPP markedly reduced microvessel density in tumors compared with the control (Fig. 6D).

**DISCUSSION**

bFGF, a ubiquitous angiogenic and heparin-binding growth factor, plays important roles in angiogenesis and tumor growth. The proangiogenic activity of bFGF can be inhibited by different strategies such as reducing bFGF production by tumor cells, affecting the release of active bFGF from the extracellular matrix, and interfering with FGF-FGF receptor recognition (3, 8). Here, we evaluated the effects of GSPP on bFGF through different mechanisms.

Although extensive research has focused on the mechanisms involving how HS mimetic affect the bioactivity of bFGF, little attention has been given to how HS mimetic affect the expression of bFGF in cancer cells. Interferon alpha downregulates bFGF expression in many human cancer cells (31). In addition, interferon alpha can inhibit the growth of hepatocellular carcinoma by inhibiting angiogenesis (32). Interestingly, we found that GSPP directly inhibits the expressions of bFGF mRNA and protein in hepatoma cells. Similar results were observed in tumor tissues. Although there are significant data indicating the roles of bFGF in tumor growth and angiogenesis, less is known about the regulation of bFGF gene expression. Egr-1 is reported to regulate bFGF transcription by activating activity upstream of the bFGF gene promoter (11, 12, 33). Inhibition of Egr-1 expression significantly attenuates the expression of bFGF (34). These observations prompted us to investigate the effect of GSPP on Egr-1 levels. GSPP significantly inhibited Egr-1 expression. GSPP suppressed Egr-1 expression within 30 minutes, which was sustained for up to 24 hours. Taken together, the results of previous studies and our present results suggest that this inhibitory effect of GSPP on bFGF production could occur in part via decreased Egr-1.

Heparanase has been reported to promote the ERK activation and the release of bFGF from extracellular matrix (29, 30). In the present study, treatment with heparanase markedly increased the levels of bFGF in the culture media, while bFGF levels were not affected by either heparanase-1 or heparanase-2 after pretreatment with 200 μg/mL GSPP. This indicated that the heparanase-stimulated release of bFGF from the extracellular matrix was completely blocked by GSPP. In order to further confirm the effect of GSPP on the heparanase activity, we hypothesized that whether GSPP affect the heparanase activity on ERK1/2 activation. We found that exposure of HepG2 cells to...
heparanase-1 or heparanase-2 resulted in increased phosphorylated ERK1/2. However, GSPP attenuated the stimulation. These data on the effect of heparanase on ERK1/2 further supported the notion that GSPP inhibits the heparanase enzymatic activity.

To determine whether GSPP directly interacts with bFGF, GSPP was added into a chip immobilized with bFGF before heparin was added in the surface plasmon resonance assay. GSPP directly bound to bFGF and subsequently inhibited heparin binding to bFGF in a dose-dependent manner. To test the hypothesis that GSPP binds to bFGF in competition with HS, GSPP and HS were mixed in advance and transferred into plates precoated with bFGF in solid-phase combined assay. Interestingly, the binding ability of HS to bFGF decreased with increasing GSPP concentration.

To further determine the inhibitory effect of GSPP on bFGF, 3 independent cell systems were studied to explore the possible mechanisms (i.e., HUVECs induced by bFGF, HUVECs without bFGF induction, and adult bovine aortic endothelial cells, which can proliferate without induction with bFGF). Interestingly, GSPP suppressed the proliferation and migration of HUVECs in the presence of bFGF. In contrast, GSPP showed no effect on HUVECs and adult bovine aortic endothelial cells in the absence of bFGF, indicating that the inhibitory effect of GSPP on endothelial cells is aimed at bFGF. This is the most sought-after identity in developing anti-angiogenesis drugs because it provides discrimination between targets and non-targets, thus reducing unintended side effects.

We further investigated the effect of GSPP on angiogenesis both in vitro and in vivo. GSPP inhibited bFGF-induced tube formation in Matrigel and aorta sprouting outgrowth. In addition, GSPP significantly reduced blood vessels in response to bFGF in chick chorioallantoic membranes. Treatment with 200 mg/mL GSPP significantly decreased microvessel density in tumor tissues compared with the control. Moreover, GSPP significantly suppressed tumor volume and tumor weight.

Suramin, a polysulfated naphylurea, interferes with FGF signaling by mimicking heparin, and is effective in several types of tumors (3). However, the high dosage of suramin required for clinical efficacy produces substantial side effects. Interestingly, in the present study, no significant difference was observed between suramin and GSPP with respect to decreased tumor volume or microvessel density, while a significant difference was found in the final mouse body weights. The body weight in the suramin-treated group decreased compared to that of the GSPP-treated group, indicating that GSPP has no adverse effect on mouse weight. Because the molecular weight of suramin is 1429 Da while that of GSPP is more than 2000 kDa, the dosage of suramin used in this study is 1 mg/kg while that of GSPP was 200 mg/kg.

Taken together, GSPP demonstrates anti-angiogenic activity both in vitro and in vivo. This can be attributed to 3 distinct mechanisms: (a) blocking the bFGF production (b) inhibiting the release of bFGF from the extracellular matrix, and (c) directly binding to bFGF and competitively inhibiting the binding of bFGF to its low affinity receptor heparin/HS. Based on these data, GSPP should be considered a novel candidate agent for anti-angiogenic target therapy in tumor treatment.

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**FOOTNOTES**

This work was supported by grants from the National Science Foundation of China (No.30801495), Education Committee of Tianjin (No. ZD-0715), Health Bureau of Tianjin (No. 30772484) and Tianjin Medical University (No. 2010ky03). The abbreviations used are: bFGF, basic fibroblast growth factor; HS, heparan sulfate; GSPP, gekko sulfated glycopeptide; HUVECs, human umbilical vein endothelial cells; ELISA, Enzyme-linked immunosorbent assay.

**FIGURE LEGENDS**

**FIGURE 1.** GSPP decreased bFGF production in hepatoma cells and tissues. (A) Expression of bFGF mRNA in hepatoma cells. HepG2 and Bel-7402 cells were treated with GSPP (0, 10, 100, or 200 μg/mL) for 72 hours. RNA was isolated and reverse transcribed, and bFGF mRNA levels were assessed by quantitative real-time PCR. The relative levels of bFGF mRNA are expressed as percentages compared to the control (control equal to 1). (B) Levels of bFGF protein in hepatoma cell supernatants. HepG2 and Bel-7402 cells were treated as described in (A) for 24, 48, or 72 hours. The supernatants were collected for ELISA analysis. (C) Expression of bFGF in tumor tissues. Male nu/nu nude mice were inoculated subcutaneously with hepatoma Bel-7402 cells; on the day following inoculation, mice were treated with GSPP (20 or 200 mg/kg) or suramin (1 mg/kg) once a day for 25 days via cavity abdominalis injection. At the end of the experiment, the implanted tumors were sectioned. The expression of bFGF was determined by immunohistochemistry, and the integral optical density of each section for bFGF was analyzed by Image-Pro Plus image analysis software. Left, images are shown with the bFGF positive areas (400×). Right, quantification of bFGF-positive areas. Data are expressed as mean ± SD. * P < 0.05 and ** P < 0.01 versus control. IOD, integral optical density.

**FIGURE 2.** GSPP inhibited Egr-1 production and abrogated heparanase enzymatic activity in hepatoma cells. (A) Egr-1 mRNA expression in hepatoma cells. HepG2 and Bel-7402 cells were treated with GSPP (100 μg/mL) for 0.5, 2, 12, or 24 hours. Egr-1 mRNA levels were assessed by quantitative RT-PCR. The relative levels of Egr-1 mRNA are expressed as percentages compared with the control (control equal to 1). (B) Egr-1 protein levels in hepatoma cells. HepG2 and Bel-7402 cells were treated as described in (A). The cells were lysed and collected for ELISA analysis. (C) Release of bFGF from extracellular matrix mediated by heparanase. HepG2 cells cultured in DMEM were incubated with heparanase-1 or heparanase-2 (final concentration, 0.5 U/mL) for 2 hours with or without 200 μg/mL GSPP. Then, the levels of bFGF were examined by ELISA. Data are expressed as mean ± SD. * P < 0.05, ** P < 0.01 versus control and # P < 0.01 versus GSPP-untreated group. (D) Phosphorylation of ERK1/2. HepG2 cells were treated as described in (C). Then, cellular protein was
extracted and incubated with anti-pERK1/2 and anti-total ERK1/2 antibodies, respectively. HPSE, heparanase.

**FIGURE 3.** Effects of GSPP on the binding affinity of bFGF-heparin/heparan sulfate. (A) Surface plasmon resonance analysis of GSPP binding to immobilized bFGF. The response unit of GSPP binding to bFGF was plotted against time. (B) Scatchard plot of heparin–bFGF binding in surface plasmon resonance analysis. Heparin was administered at 0.625, 1.25, 2.5, 5, 10, or 20 mg/mL. (C) Scatchard plot of GSPP–bFGF binding in surface plasmon resonance analysis. GSPP was administered at 0.625, 1.25, 2.5, 5, or 10 mg/mL. (D) Scatchard plot of heparin–bFGF binding after pretreatment with GSPP in surface plasmon resonance analysis. Heparin (10 mg/mL) was added to the chip after GSPP (0.625, 1.25, 2.5, 5, or 10 mg/mL) was bound to the chip with immobilized bFGF. (E) Solid-phase heparan sulfate–bFGF binding assay. The results are expressed as the percentage of inhibitory effects of GSPP on the binding of biotinylated heparan sulfate to bFGF. Data are expressed as mean ± SD.

**FIGURE 4.** Effects of GSPP on bFGF-stimulated endothelial cells. (A) Trypan blue exclusion assay. HUVECs and adult bovine aortic endothelial cells were seeded at 2 × 10⁴ cells/well in 24-well plates, treated with GSPP (0, 10, 100, or 200 μg/mL) with or without 2 ng/mL bFGF, and cultured for 48 hours. Then, viable cells were counted. (B) Wound healing assay. Wounds were generated after cell confluence. Injury width was measured after treatment with GSPP for 24 hours. (C) Transwell assays. HUVECs and adult bovine aortic endothelial cells were allowed to migrate across the membrane of the transwell insert in response to 10% FBS in the presence or absence of 2 ng/mL bFGF. The number of migrated cells in the lower surface of the filter was quantified in 10 random fields (200×) per insert. Left, representative images of migrated HUVECs in transwell assay (200×). Right, quantification of migrated HUVECs. (D) Effect of GSPP on HUVEC morphology and stress fiber formation. Top, fluorescent images of actin filaments (600×). DAPI (blue), actin (red), as above. Bottom, light microscope images of HUVECs (400×). Data are expressed as mean ± SD. *P < 0.05 versus control, **P < 0.01 versus control, and #P < 0.01 versus HUVECs without induction of bFGF.

**FIGURE 5.** GSPP inhibited bFGF-induced angiogenesis. (A) In vitro tube formation assay. HUVEC suspension (2 × 10⁵ cells/well) was added onto Matrigel-coated plates in culture medium with or without GSPP (10, 100, or 200 μg/mL), and the capillary structure formation was observed after 7 hours. Left, images of tube formation (40×). Right, quantification of inhibitory ratios of tube branches. (B) Mouse aortic sprouting assay. Left, images of mouse aortic sprouting assay (400×). Right, quantification of inhibitory ratios of sprouting. Mouse aortic segments were embedded in Matrigel and cultured for 9 days. (C) Chick chorioallantoic membrane assay. Chick chorioallantoic membrane models were incubated with Matrigel plus 5 ng/mL bFGF for 48 hours in the presence or absence of GSPP. Left, images of neovascular zones (16×). Right, quantification of inhibitory ratios of neovascular zones. Quantification of blood vessels was performed in 10 random fields per sample. Data are expressed as mean ± SD. **P < 0.01 versus control.

**FIGURE 6.** GSPP inhibited tumor growth and tumor angiogenesis in vivo. Male nu/nu nude mice were treated as described in Figure 1. (A) Effect of GSPP on tumor volume. The lengths and widths of tumors were measured individually every 3 days. Left, representative image of an excised tumor.
Right, tumor growth curves for hepatocellular carcinoma mouse xenograft model. (B) Effect of GSPP on tumor weight. On the 25th day, the implanted tumors were excised and weighted. (C) Effect of GSPP on mouse weight. Mice were weighed at the beginning and end of the experiment. Data are expressed as the difference between final and initial weights. (D) Tumor microvessel density. The implanted tumors were sectioned and stained against CD34. The numbers of CD34-positive blood vessels in 10 high-power fields were counted. Left, immunohistological staining of tumor sections (400×). Blood vessels are shown as CD34 positive (arrows). Right, quantification of blood vessels. Data are expressed as mean ± SE. * $P < 0.05$ versus control and ** $P < 0.01$ versus control.
Figure 2

A

HepG2

Bel-7402

Relative mRNA

(Egr-1)

Control 0.5 2 12 24

Time (hours)

B

Control

GSPP 100 μg/mL

Time (hours)

Egr-1 level (ng/mL)

Control 0.5 2 12 24

Time (hours)

C

bFGF (pg/mL)

HPSE-1 0.5 U/mL - - - + +

HPSE-2 0.5 U/mL - + + - -

GSPP 200 μg/mL - - + - +

D

pERK 1/2

ERK1/2

Actin

HPSE-1 0.5 U/mL - - - + +

HPSE-2 0.5 U/mL - - + + - -

GSPP 200 μg/mL - + - - + +
Figure 3
Figure 6

A

B

C

D

Suramin 1 mg/kg
GSPP 200 mg/kg
GSPP 20 mg/kg
Control

Tumor volume (mm³)

Days of administration

Control
GSPP 20 mg/kg
GSPP 200 mg/kg
Suramin 1 mg/kg

Tumor weight (mg)

GSPP (mg/kg)

Change of mice weight (g)

GSPP (mg/kg)

Vessel No./field

GSPP (mg/kg)

suramin

Control
GSPP 20 mg/kg
GSPP 200 mg/kg
Gekko Sulfated Glycopeptide Inhibits Tumor Angiogenesis by Targeting Basic Fibroblast Growth Factor
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