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Structure-activity relationship study of WSS25 derivatives with anti-angiogenesis effects

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Abstract WGEW, an α(1-4) linked glucan with an α(1-4) linked branch attached to C-6, was isolated from the rhizoma of *Gastrodia elata* Bl. WSS25, a sulfated derivative of WGEW, was reported to inhibit angiogenesis by disrupting BMP2/Smad/Id1 signaling pathway. However, the structure-activity relationship (SAR) for WSS25 is not known. To study the SAR, seven sulfated saccharides derived from WGEW degradation products, six sulfated polysaccharides with varying degrees of substitution, and four aminopropylated, carboxymethylated, phosphorylated, and acetylated derivatives of WGEW were prepared. A sulfated, unbranched product of polysaccharide was also obtained. The structural features of these derivatives were characterized by infrared spectroscopy and nuclear magnetic resonance spectroscopy. An HMEC-1 cell tube formation assay was employed to measure the anti-angiogenic effect of the derivatives. The results indicated that only sulfated polysaccharides with molecular weights of more than 41,000 Da could inhibit HMEC-1 cell tube formation. The inhibition effect was dependent on the presence of a sulfate group, since the tube formation was not blocked by aminopropylated, carboxymethylated, phosphorylated, or acetylated WGEW. A higher degree of sulfate substitution on the polysaccharide led to a stronger inhibitory effect, and the degree of sulfate substitution between 0.173 and 0.194 was found to be optimal. Interestingly, the WGEW side chain was not required for anti-tube formation activity. All these preliminary results may provide a clue for further modification of the core structure of WSS25 to discover polysaccharide derivatives as novel anti-angiogenic inhibitors.

Keywords Gastrodia elata · Sulfated polysaccharide derivative · Anti-angiogenesis · WSS25

Abbreviations

bFGF	Basic fibroblast growth factor
BMP2	Bone morphogenic protein 2
DMSO	Dimethyl sulfoxide
DS	Degree of substitution
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
GC-MS	Gas chromatography–mass spectrometry
HMEC-1	Human microvascular endothelial cells
HPGPC	High performance gel permeation chromatography
HS	Heparan sulfate
Id1	Inhibitor of DNA binding 1
IR	Infrared
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NMR	Nuclear magnetic resonance
QCM	Quartz crystal microbalance
SAR	Structure-activity relationship
TFA	Trifluoroacetic acid
VEGF	Vascular endothelial growth factor

Introduction

Angiogenesis is the physiological process of growing new blood vessels from pre-existing ones, which is a normal and vital process for growth, development, and the healing of wounds [1, 2]. Unfortunately, angiogenesis is also an essential

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step in the growth of tumors and their transition from a dormant state to a malignant one [3, 4]. Disrupting angiogenesis with inhibitors will lead to depriving oxygen and nutrient for the tumor and inhibiting its growth. Hence, anti-angiogenic therapy is now an important strategy for cancer treatment [5].

Heparan sulfate (HS) chains are negatively charged polymers composed of hexuronic acid and glucosamine disaccharide repeats [6]. Because of their negative charges, they bind multiple functional proteins including pro-angiogenic factors such as VEGF and FGF2, through which they can mediate angiogenic signaling. HS mimetics are widely reported to inhibit angiogenesis by blocking the interaction between angiogenic factors and their receptors [7, 8]. For example, the HS mimetic PI-88 can inhibit angiogenesis by disrupting interactions between HS and growth factors [9]. Other sulfated polysaccharides, such as sulfated laminarin, have also been shown to be potent angiogenesis inhibitors [10]. Overall, the HS-protein interaction is being interestingly targeted in the design of new drugs [11]. Recent evidence had indicated that there was an intimate relationship between the structures of HS polysaccharides and their anti-angiogenesis activities [12]. In our previous study, we found that the sulfated HS mimetic WSS25 could block angiogenesis by impeding the bone morphogenic protein 2 (BMP2)/Smad 1/5/8 signaling pathway [13]. However, the structure-activity relationship (SAR) of the sulfated polysaccharide is not well understood. In this study, we investigated the SAR of WSS25 to obtain more effective sulfated polysaccharides with more potent antiangiogenic activities.

Materials and methods

General methods of analysis

Infrared (IR) spectra (KBr or Nujol pellets) were obtained with a Perkin-Elmer 599B FTIR spectrophotometer. Elemental analysis was performed with a Carlo-Erba 1108 elemental analyzer. High performance gel permeation chromatography (HPGPC) was performed with an Agilent instrument that included a G1311A Quadra pump, a DAD G1315D RI detector, a G1362A dual λ absorbance detector, and GPC software. Nuclear magnetic resonance (NMR) spectroscopy was recorded on a Varian Mercury 400 NMR spectrometer. Gas chromatography–mass spectrometry (GC-MS) data were obtained using a Shimadzu QP-5050A apparatus equipped with a DB -1 capillary column (0.25 mm×30 m).

Preparation of the parent polysaccharide, WGEW, and its sulfated derivative, WSS25

The parent polysaccharide WGEW was extracted, isolated and purified from *Gastrodia elata*, and WSS25 was obtained from the sulfation of WGEW as described previously [14]. The degree of sulfate substitution was measured by elemental analysis.

Preparation of polysaccharides with various molecular weights (*MW*s) and their sulfated derivatives

WGEW was hydrolyzed in solutions with various concentrations of trifluoroacetic acid (TFA) (Table 1) for 1 h at 100 °C, evaporated to dryness, and dialyzed against distilled water for 24 h. The concentrated dialysate and the retentate were lyophilized, respectively. The *MW* of the degraded polysaccharide was estimated by HPGPC.

The sulfated derivatives of the degraded polysaccharides were prepared as described previously [14]. The degree of sulfate substitution was measured by elemental analysis.

Preparation of sulfated polysaccharides with various degrees of sulfated substitution

Sulfated polysaccharides were prepared by the chlorosulfonic acid-pyridine method [15]. Briefly, the parent polysaccharide WGEW (500 mg) was stirred in 25 mL of dry formamide for 30 min at room temperature. 2 mL of sulfation reagent consisting of chlorosulfonic acid and pyridine was added. Various reaction times and ratios of sulfation reagent were used, as listed in Table 2. After the reaction was finished, the mixture was cooled and neutralized with NaOH. The products were isolated by dialysis against saturated $NaHCO_3$ for 1 day, dialyzed against distilled water for an additional 3 days, and finally lyophilized. The degree of sulfation was measured by elemental analysis.

Preparation of an aminated polysaccharide

The parent polysaccharide WGEW (800 mg) was dissolved in a stirred solution of NaOH (5 g) in water (40 mL) over 5 h. At this point, the mixture was added to 2 g of 3-chloropropylammonium chloride and the temperature was held at 80 °C for 24 h. After the reaction was finished, the mixture was poured into 160 mL of acidified ethanol (1 % HCl), and the precipitate was washed with 160 mLof 80 % alcohol. The precipitate was then dispersed in distilled water and extensively dialyzed for 3 days. The nondialyzate product was then dried by lyophilization to give the aminopropylated polymer products. The degree of substitution was measured by elemental analysis [16].

Preparation of a carboxymethylated polysaccharide

The parent polysaccharide WGEW (1 g) was suspended in 2-propanol (30 mL) at room temperature. After stirring for 30 min, 30 % NaOH (5 mL) was slowly added over 5 min,

Table 1 Molecular weight (*MW*), degree of substitution (DS) of the sulfated derivatives of the acid-degraded products of WGEW by various concentrations of TFA

Derivative	C_{acid} (mol/L)	*MW* before sulfation	DS [a]	*MW* after sulfation
M1S	0.05	180,000	1.05	220,000
M2S	0.1	130,000	1.22	200,000
M3S	0.2	75,000	1.27	140,000
M4S	0.4	41,000	1.21	66,000
M5S	0.6	14,000	1.05	31,000
M6S	1	12,000	1.24	21,000
M7S	2	2,700	1.35	980

[a]DS is calculated as $162\times\%W/(96–80\times\%W)$; $\%W$ is the content of SO_4^{2-}

and vigorous stirring was continued for 1 h. Then, chloroacetic acid (1.2 g) was added, and the mixture was stirred for another 3 h at 70 °C. At this point, the mixture was cooled, diluted with distilled water and neutralized with CH_3COOH. The product was dialyzed against distilled water for 3 days and then lyophilized [16].

Preparation of a phosphorylated polysaccharide

The parent polysaccharide WGEW (1 g) was suspended in 50 mL of dry formamide at room temperature. After stirring for 30 min, dry tributylamine (25 mL) was added, and the reaction mixture was stirred until the solution turned translucent. Then, polyphosphoric acid (24 mL) was added [17], and the reaction temperature was held at 100 °C for 4 h. After the reaction was finished, the mixture was poured into ethanol (200 mL), and then centrifuged at 8000 rpm. The precipitate was then dispersed in distilled water (5 mL) and neutralized with NaOH. A reduced pressure distillation was performed to get rid of the excess amine and the product was extensively dialyzed against distilled water for 3 days, followed by lyophilization.

Preparation of an acetylated polysaccharide

The parent polysaccharide WGEW (1 g) was suspended in 40 mL of dry dimethyl sulfoxide (DMSO) at room temperature and stirred for 24 h. The mixture was added in pyridine (6 mL) and acetic anhydride (5 mL), and the reaction temperature was held at 4 °C for 2 h. After the reaction was finished, distilled water (5 mL) was added [18]. The product was dialyzed against distilled water for 3 days and then lyophilized.

Table 2 Molecular weight (*MW*), degree of substitution (DS) of the sulfated derivatives of WGEW obtained with various reaction times and ratios of sulfation reagent

Derivative	[$ClSO_3H$/Pyridne]	Time (h)	DS[a]	*MW*
WGES1	1:1	2	0.141	240,000
WGES2	1:1	4	0.097	67,000
WGES3	2:1	2	0.194	180,000
WGES4	2:1	4	0.173	80,000
WGES5	3:1	2	0.220	138,000
WGES6	3:1	4	0.202	76,000

[a] DS is calculated as $162\times\%W/(96–80\times\%W)$; $\%W$ is the content of SO_4^{2-}

Preparation of the unbranched polysaccharide and its sulfated products

A solution of WGEW (200 mg) in 5 mL of PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/LNa_2HPO_4 and 0.24 g/L KH_2PO_4, pH 7.4) was treated with 15 μL of isoamylase (1000 U, I5284, Sigma, Steinheim, Germany) for 24 h at 37 °C. The enzymatic reaction was terminated by heating the solution to 100 °C for 20 min. The mixture was then centrifuged at 5,000 rpm and dialyzed against distilled water [19]. The retentate was lyophilized to give WGE-DE (91 mg).

The sulfated products of WGE-DE were prepared as described previously [12].

Methylation analysis of the unbranched polysaccharide WGE-DE

The vacuum-dried polysaccharide WGE-DE (10 mg) was methylated three times as described by Needs and Selvendran [20] with minor modifications. The polysaccharide was weighed precisely and dissolved in 3.0 mL of DMSO. 200 mg of dry NaOH was added, and the reaction mixture was sonicated for 10 min. After stirring at room temperature for 10 min, 1 mL of methyl iodide was added slowly. The sample was kept in the dark for 1 h, at which point 2.0 mL of distilled water was used to decompose the remaining methyl iodide. The methylated polysaccharides were extracted with 3×2 mL of chloroform and dried under low pressure on a rotary evaporator. The completeness of the methylation was confirmed by the disappearance of the hydroxyl absorption in the IR spectrum (Nujol). Partially methylated alditol acetates were then prepared and analyzed by GC-MS.

Homogeneity and molecular weight of polysaccharides

The homogeneity and MW of polysaccharides were estimated by HPGPC with series-connected Ultrahydrogel™ 2000

and Ultrahydrogel™ 500 columns. The columns were equilibrated and eluted with mobile phase containing 0.4 g/L KH_2PO_4 and 7.32 g/L K_2HPO_4 at a flow rate of 0.5 mL/min. For *MW* estimation, the columns were calibrated using the T-series dextran standards of known molecular weights (T-700, T-580, T-300, T-110, T-80, T-70, T-40, T-9.3 and T-4, Pharmacia). The column temperature was kept at 30.0±0.1 °C. All samples were prepared in mobile phase as 0.2 % (w/v) solution, and 20 μL of solution was analyzed in each run [21].

NMR analysis of polysaccharides

The deuterium-exchanged polysaccharide samples (30 mg) were dissolved in 0.5 mL of D_2O (99.8 % D). The ^{13}C-NMR spectra were measured at room temperature on a Varian Mercury 400 NMR spectrometer, using acetone as the internal standard (31.50 ppm). All chemical shifts were referenced to Me_4Si (TMS).

Cell culture

Human microvascular endothelial cells (HMEC-1) were cultured in MCDB131 medium (Gibco BRL, U.S.A.) supplied with 15 % FBS (v/v), 2 mM L-glutamine, 10 ng/mL EGF (Shanghai PrimeGene Bio-Tech Co., Ltd., Shanghai, China) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, Gibco BRL, U.S.A) at 37 °C in a humidified atmosphere containing 5 % CO_2.

MTT assay

HMEC-1 cells were seeded at a density of 4.5×10^3 cells/well into a sterile 96-well plate and grown in a 5 % CO_2 atmosphere at 37 °C for 24 h. The polysaccharides to be tested were dissolved in PBS and diluted with culture medium to various concentrations. After treatment with the polysaccharides for 72 h, HMEC-1 cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, U.S.A.) assay. A solution of 5 mg/mL MTT was added to each well and incubated with the cells for 4 h in an incubator. The formazan was dissolved in DMSO after the medium was removed. Finally, the optical density was measured using a spectrophotometer (Thermo Multiskan MK3, German) at an absorption wavelength of 570 nm [22]. The inhibition rate was calculated as [(control - sample)/control]×100 %.

Tube formation assay

The tube formation assay was employed to determine the effects of different polysaccharides on angiogenesis *in vitro*. Briefly, a 96-well plate coated with 50 μL of Matrigel per well was allowed to solidify at 37 °C for 30 min. HMEC-1 cells (3×10^4 cells/well) were seeded into the plate and cultured for 12 h in MCDB131 media containing certain concentration of the polysaccharides. The enclosed capillary networks of tubes were photographed by a microscope (Olympus, IX51, Japan).

Results and discussion

MW and the degree of sulfate substitution of the polysaccharides

The parent polysaccharide WGEW is an α(1-4) linked glucan with an α(1-4) linked branch attached to the C-6 position (Fig. 1a), whereas WSS25 is a sulfated derivative of WGEW exclusively substituted at O-6 (Fig. 1b) [14]. A number of different methods could be used to obtain polysaccharides and sulfated derivatives with various *MW*s from the parent polysaccharide WGEW for SAR study, such as acid degradation [23], enzymatic degradation [24], and ultrasound-assisted degradation [25]. Acid degradation was chosen in this study to achieve homogeneous polysaccharides. After WGEW was hydrolyzed by various concentrations of TFA (Table 1), seven polysaccharides, named M1~M7 were obtained. As shown by the HPGPC chromatogram in Fig. 2, all of the glucans were homogeneous. The seven polysaccharides were then sulfated with the chlorosulfonic acid-pyridine method [14]. The molecular weights of the seven degraded polysaccharides and their sulfated derivatives (M1S~M7S) were shown in Table 1 along with the degrees of sulfate substitution.

Enzymatic hydrolysis was performed to elucidate the relationship between the presence of the side chain and bioactivity. WGEW (200 mg) was hydrolyzed with isoamylase to obtain WGE-DE (91 mg). The molecular weight of WGE-DE was estimated to be 3.0×10^3Da by HPGPC. After the WGE-DE was sulfated using chlorosulfonic acid-pyridine (1:2) to give WGE-DE-S, the degree of sulfate substitution was found to be 1.37 sulfate groups per residue.

A series of sulfated WGEW derivatives (WGES1-WGES6) was obtained by varying the molar ratio of chlorosulfonic acid to pyridine and the reaction time, as shown in Table 2. Interestingly, longer reaction time led to a lower degree of sulfate substitution.

All the sulfated derivatives of acidically or enzymatically degraded products of WGEW were confirmed successful by the appearance of a signal at 1268 cm^{-1} in the IR spectra compared with that of WGEW, and the position at which they were sulfated was assigned to be O-6 by comparison of the ^{13}C-NMR spectrum with that reported by Qiu *et al.* [14]. As a representative sulfated derivative, the IR and ^{13}C-NMR spectra of WGE-DE-S were shown in Fig. 3b and Fig. 4b.

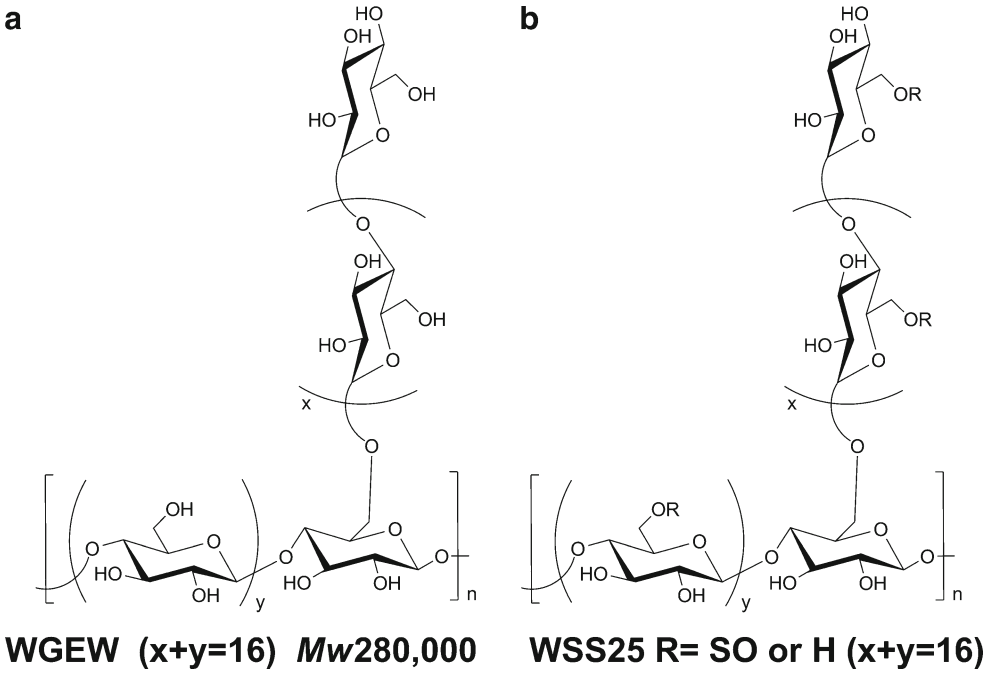

Fig. 1 Structures of WGEW (**a**) and its sulfated derivative WSS25 (**b**)

Polysaccharides substituted with other functional groups

To test the effects of other functional groups on HMEC-1 cell tube formation, WGEW was aminated, carboxymethylated, phosphorylated, and acetylated. The structures of all the derivative products were determined by IR, ^{13}C-NMR or elemental analysis.

In general, aminopropylated polysaccharides are easily absorbed as reported [26]. The aminopropylated derivative of WGEW was named WGEA. This derivative was detected as a homogeneous polysaccharide and its *MW* was estimated to be 5,700 Da (data not shown). An absorbance peak for the C-N stretching vibration appeared in the IR spectrum of WGEA at ca. 1375 cm^{-1}, indicating the incorporation of aminopropyl substituents (Fig. 3c) [26]. Moreover, as shown in Fig. 4c, the ^{13}C-NMR spectrum of this derivative (DS 0.17) was more complicated than that of WGEW. The additional signals at 20–50 ppm confirmed the presence of the aminopropyl groups. The signal at 70 ppm was ascribed to glucosyl units that were aminopropylated at O-6.

It has been widely accepted that carboxymethylation can enhance the solubility of polysaccharides in water. Various polysaccharides, such as cellulose, chitin, scleroglucan, and schizophyllan, can be used as starting materials for carboxymethylation [27]. WGEW was carboxymethylated in 2-propanol to produce the derivative WGEC. WGEC was detected as a homogeneous polysaccharide and its *MW* was estimated to be 73,000 Da. The IR spectrum (Fig. 3d) of WGEC showed a characteristic peak at ca. 1731 cm^{-1}, indicating that the carboxymethylation reaction was successful [26]. As shown in Fig. 4d, a signal at 177 ppm in the ^{13}C-NMR spectrum of WGEC confirmed the presence of a carboxyl group. The signal for the anomeric carbon was split, and the upfield peak was assigned to the O-6

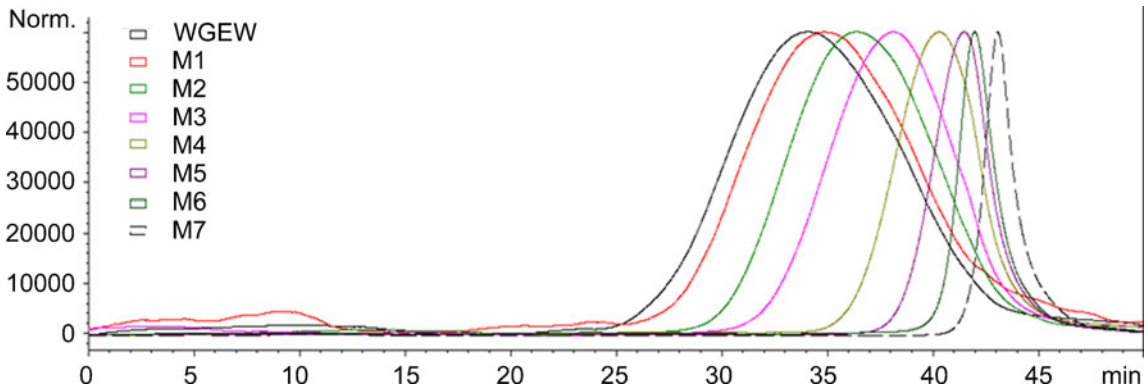

Fig. 2 HPGPC chromatograms of M1-M7 and WGEW. The parent polysaccharide WGEW was acidically degraded by various concentrations of TFA shown in Table 1 to give M1-M7. Then the HPGPC was performed to estimate their homogeneity and molecular weight

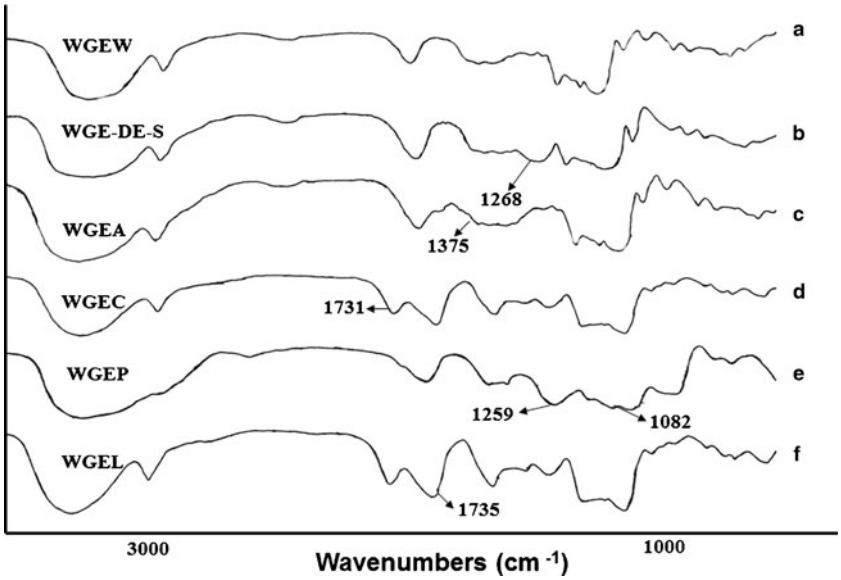

Fig. 3 IR spectra of **a** WGEW, **b** WGE-DE-S, **c** WGEA, **d** WGEC, **e** WGEP, and **f** WGEL. The parent polysaccharide WGEW was enzymatically degraded and then sulfated (WGE-DE-S), directly aminopropylated (WGEA), carboxymethylated (WGEC), phosphorylated (WGEP), or acetylated (WGEL). Then IR spectra were recorded and main characteristic signals were shown with arrows

substitution. By comparison with the ^{13}C-NMR spectrum of WGEW (Fig. 4a), the signal at 61.7 ppm was assigned to an O-6 substituted carbon. The peak at 81.1 ppm was ascribed to C-4 on residues with carboxymethyl substitution at C-6.

Many recent papers have reported on the phosphorylated polysaccharides with important biological activities, such as anti-tumor properties [28]. The phosphorylated derivative WGEP was obtained as a homogeneous polysaccharide with an estimated *MW* of 2,200 Da. Compared to the O-H stretching peak in the IR spectrum of WGEW, the IR spectrum of WGEP (Fig. 3e) had a more broad and intense band at ca. 3300 cm^{-1} because of the presence of phosphoryl groups [17]. The absorbance bands at ca. 1259 cm^{-1} and ca. 1082 cm^{-1}, which correspond to the P-O stretching vibration and the P-O-P stretching vibration, respectively, further indicate that the phosphorylation reaction was successful. However, the ^{13}C-NMR spectrum (Fig. 4e) was not very different from that of WGEW (Fig. 4a), indicating that the extent of phosphorylate substitution was quite low.

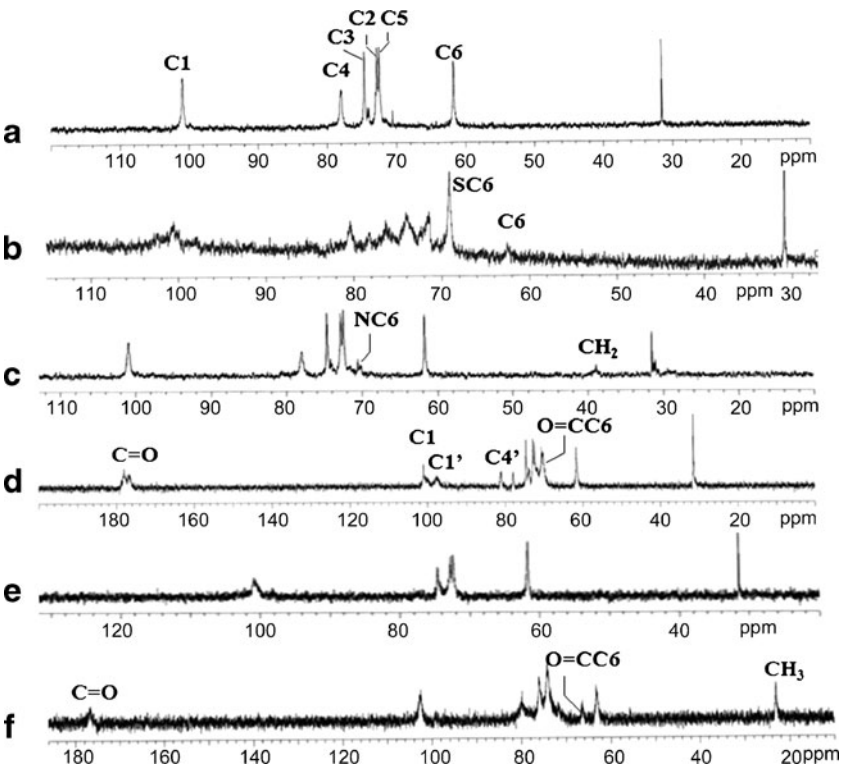

Fig. 4 ^{13}C-NMR spectra of **a** WGEW, **b** WGE-DE-S, **c** WGEA, **d** WGEC, **e** WGEP, and **f** WGEL. The parent polysaccharide WGEW was enzymatically degraded and then sulfated (WGE-DE-S), directly aminopropylated (WGEA), carboxymethylated (WGEC), phosphorylated (WGEP), or acetylated (WGEL). Then the ^{13}C-NMR spectra of the compounds were recorded

Acetylation may increase the bioactivity of polysaccharides [18]. To test this possibility using the WGEW scaffold, the acetylated derivative WGEL was prepared. WGEL was detected as a homogeneous polysaccharide, and its *MW* was estimated to be 6,000 Da. The IR spectrum of WGEL (Fig. 3f) showed a characteristic peak at ca. 1735 cm^{-1}, indicating that the acetylation reaction was successful [26]. As shown in Fig. 4f, the signal at 175 ppm in the ^{13}C-NMR spectrum of WGEL confirmed the presence of an acetyl group. By comparison with the ^{13}C-NMR spectrum of WGEW (Fig. 4a), the signal at 64.7 ppm was assigned to an O-6 substituted carbon. The signal at 21.4 ppm gave further evidence for the presence of acetyl group.

Anti-angiogenesis activity analysis

In this study, a typical *in vitro* angiogenesis model, the tube formation of HMEC-1 cells on Matrigel was employed to test the anti-angiogenesis activities of various polysaccharides.

Seven glucans with different *MW*s (M1-M7) were obtained from WGEW by acid degradation. As shown in Fig. 5, the anti-angiogenesis activities exhibited by the sulfated derivatives of these seven saccharides and WSS25 were compared. HMEC-1 cells in the control group (Fig. 5a) formed a network of tubes within 12 h. In contrast, the tube formation was disrupted by the sulfated derivatives WSS25 (Fig. 5b), M1S (Fig. 5i), M2S (Fig. 5h), M3S (Fig. 5g) and M4S (Fig. 5f) at a concentration of 25 μg/mL. Such inhibition was not observed for the sulfated derivatives M5S (Fig. 5e), M6S (Fig. 5d), and M7S (Fig. 5c) at the same concentration. These results indicated that the sulfated derivatives had an anti-angiogenesis effect only at *MW*s of more than 41,000 Da. This effect may be related to the configuration of the sugar chain. The bioactivity of polysaccharides is closely related to the length of the sugar chain [29]. Hence, the longer sugar chains may have acted as a pocket, whereas the shorter chains may have been not long enough to achieve the necessary structural orientation.

Six sulfated derivatives with different DS (WGES1-WGES6) were obtained by changing the reaction conditions for the sulfation of the parent polysaccharide, WGEW. An MTT assay was employed to test the cytotoxicity of WGES1-WGES6 towards HMEC-1 cells. As shown in Fig. 6II, the MTT results suggested that, except for WGES5, the inhibition rates of these derivatives were less than 20 % at a concentration of 200 μg/mL. However, at a concentration of 500 μg/mL, the inhibition rates exhibited by these derivatives were more than 20 %, except for WGES2 and WGES3. This preliminary study confirmed that the anti-angiogenesis activities of these products were not significant at low concentration. Thus, the anti-angiogenesis activities were measured at 400 μg/mL. Compared with a blank control (Fig. 6Ig), the anti-angiogenesis activities of the

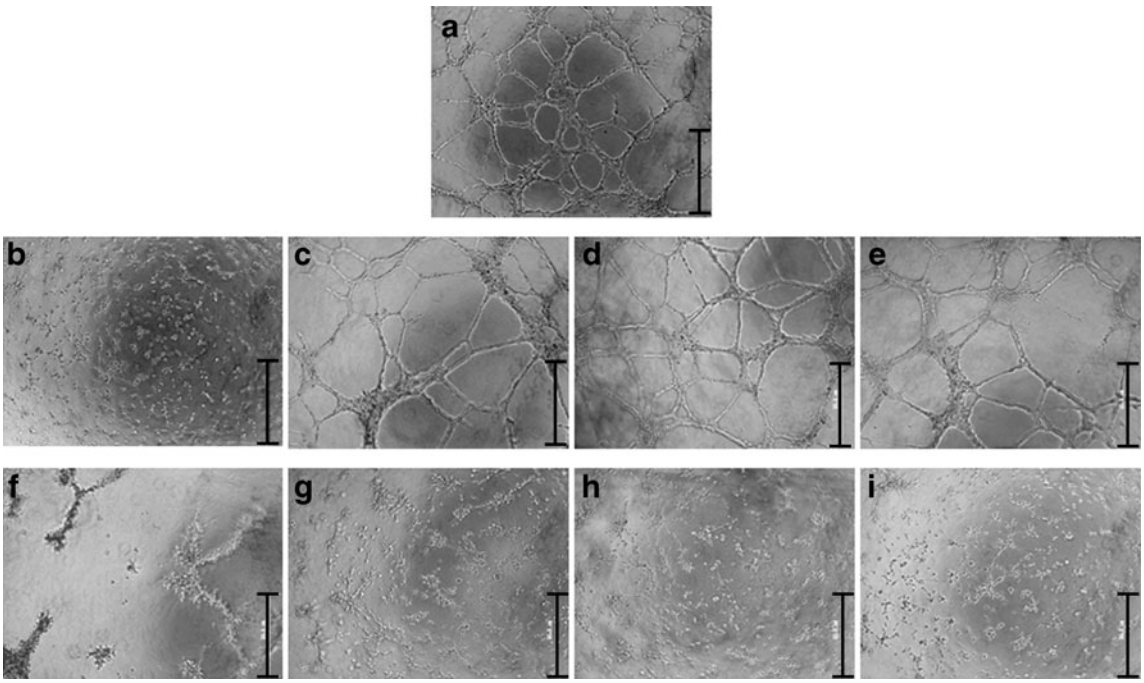

Fig. 5 Effects of *MW* on the anti-angiogenesis activity of the sulfated products. HMEC-1 cells without (**a**) or with (**b**) 25 μg/mL of WSS25, and sulfated derivatives of polysaccharides with various lengths (C, M7; D, M6; E, M5; F, M4; G, M3; H, M2; I, M1) were seeded into a 96-well plate precoated with Matrigel for 12 h. Images of each well were captured as described in the "Materials and Methods" section

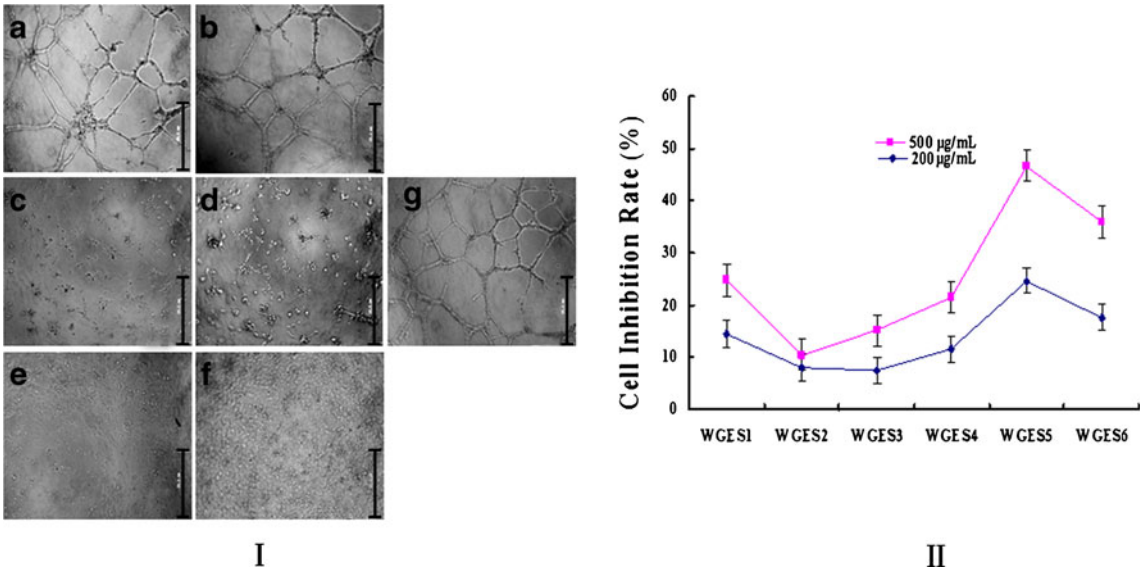

Fig. 6 Effects of DS on the anti-angiogenesis activity of the sulfated products. I HMEC-1 cells (3×10^4 cells/well) and 400 μg/mL of sulfated polysaccharides with various DSs (**a**, WGES1; **b**, WGES2; **c**, WGES3; **d**, WGES4; **e**, WGES5; **f**, WGES6; **g**, no polysaccharide) were seeded into a 96-well plate precoated with 50 μL of Matrigel and cultured for 12 h in MCDB131 media. Images of each well were taken as described in the "Materials and Methods" section. II. HMEC-1 cells were seeded into a 96-well plate. After incubation for 24 h, WGES1~WGES6 were added to produce final concentration of 200 or 500 μg/mL. The cell inhibition rates were then determined by the MTT assay 72 h later

six sulfated polysaccharides with different DS obviously varied in a DS-dependent manner. WGES1 (Fig. 6Ia) and WGES2 (Fig. 6Ib), which had a low DS, could not inhibit HMEC-1 cell tube formation at this concentration. WGES3 (Fig. 6Ic) and WGES4 (Fig. 6Id), which had a moderate DS, demonstrated significant anti-angiogenesis activities, but even greater anti-angiogenesis effects were seen for WGES5 (Fig. 6Ie) and WGES6 (Fig. 6If), which had a higher DS. However, because the HMEC-1 cell growth inhibition rates exhibited by WGES5 and WGES6 were more than 30 %, the derivatives most suitable for use as anti-angiogenesis agents at 400 μg/mL had a DS of 0.173–0.194, when excluding the possibility that the anti-angiogenesis effects were due to the inhibition of the HMEC-1cells growth.

WGE-DE was obtained after specific enzymatic degradation of WGEW. Compared to a blank control (Fig. 7a), the sulfated derivatives of WGE-DE strongly inhibited HMEC-1 cell tube formation at a concentration of 25 μg/mL (Fig. 7b). The results indicated that the branched chain might not be required for its anti-angiogenesis activity. However, the other derivatives of WGEW, such as WGEA, WGEC, WGEP, and WGEL, exhibited little or no anti-angiogenesis activity, suggesting that the sulfate group might be necessary for producing anti-angiogenesis activity.

BMP2/Smad/Id1 signaling pathway plays important role in the process of angiogenesis. The transcriptional factor Id1 is required for angiogenesis. The Id1-Id3 knockout mice display vascular malformations, and tumor failed to grow and/or metastasize in $Id1^{+/-}$ $Id3^{-/-}$ mice along with poor vascularization [30]. Further investigation showed that the angiogenic defect of tumor in Id-mutant mice was due to impaired mobilization and recruitment of endothelial precursor cells [31]. BMPs belong to a subfamily of TGFβ, while accumulated evidences indicate that there is an important relationship between BMPs and Id1. Stimulation of Id1 expression by BMP is sufficient and necessary for activation of endothelial cells [32]. WSS25 was the first sulfated polysaccharide found to inhibit tumor growth by disrupting angiogenesis mainly through binding BMP2 and further blocking BMP2/Smad/Id1 signaling [13].

In this study, a series of WSS25 derivatives was prepared with different methods and preliminary SAR with anti-angiogenesis effects was studied with tube formation assays. Since those sulfated polysaccharide derivatives had the same core structure as that of WSS25, the mechanism by which they inhibit angiogenesis was deduced to be similar as that by WSS25. We mainly studied the influence of various derivative groups, degree of sulfate substitution, molecular weight, and side chain on their anti-angiogenesis activity. Sulfation was found to be required for anti-angiogenesis activity because unsulfated WGEW, aminopropylated WGEA, carboxymethylated WGEC, phosphorylated WGEP, and acetylated WGEL exhibited little or no activity on cells tube formation. This is consistent with

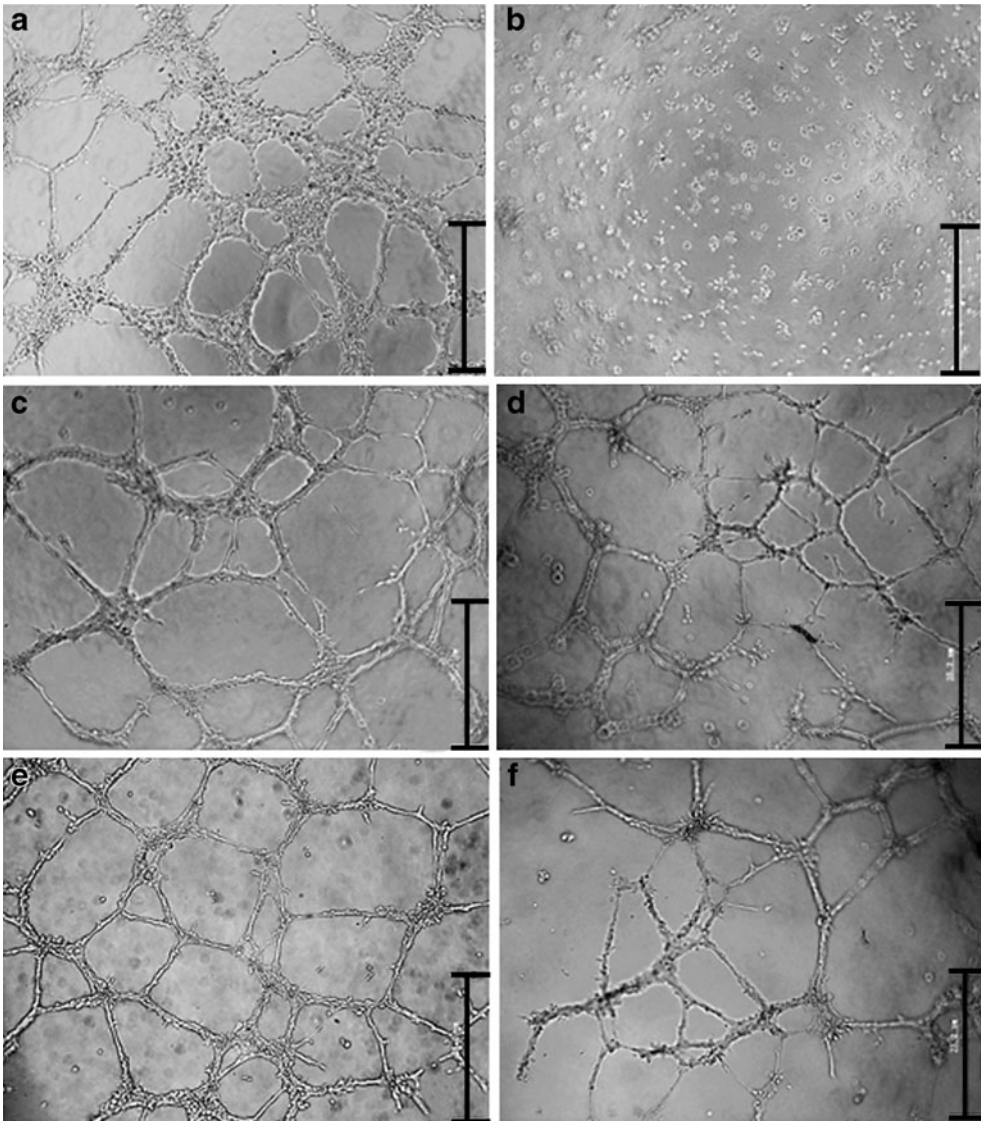

Fig. 7 Effects of functional groups on the anti-angiogenesis activity of the derivatives. HMEC-1 cells were seeded on a 96-well plate precoated with Matrigel and incubated for 12 h without **a** or with **b** 25 μg/mL of WGE-DE-S, **c** 1 mg/mL of WGEA, **d** 1 mg/mL of WGEP, **e** 1 mg/mL of WGEC, or **f** 1 mg/mL of WGEL. Images of each well were captured as described in the "Materials and Methods" section

the findings reported before that sulfation was essential for antiangiogenic activity exhibited by maltohexaose sulfate [33]. The existence of optimal degree of sulfation suggested that there might be a balance between the ability of binding BMP2 and the cytotoxicity exhibited by sulfated WGEW. The existence of the special molecular weight range and no side chain on the sulfated polysaccharide with anti-angiogenesis effects indicated that only sulfated polysaccharide with linear chain long enough could bind and constrain BMP2 binding its receptor. All our present findings provide preliminary SAR for further modification of the core structure of WSS25 to discover polysaccharide derivative as novel anti-angiogenic inhibitor. Indeed, it was a preliminarily non-quantitative result, since the anti-angiogenesis effects of the polysaccharides evaluated with tube formation assays were comprehensive results of interference of many other signaling such as VEGF and bFGF except BMP2. The exact SAR study of binding of BMP2 with the method using quartz crystal microbalance (QCM) technique is still ongoing.

Conclusions

The SAR study of WSS25 indicated that sulfate groups are required for anti-angiogenesis activity. The length of the saccharides and the DS of the sulfated serivatives also affected the bioactivity. To achieve the best anti-angiogenesis effects, the *MW* of the glucan needed to be higher than 41,000 Da, and a DS between 0.173 and 0.194 was found to be optimal for avoiding the cytotoxic effects. The results also suggested that C-6 branching was not necessary for this activity.

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