Probing the Interactions of Phosphosulfomannans with Angiogenic Growth Factors by Surface Plasmon Resonance

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The binding interactions of the phosphosulfomannan anticancer agent PI-88 (**1**) with the angiogenic growth factors FGF-1, FGF-2, and VEGF were studied by surface plasmon resonance (SPR) on a BIAcore 3000 biosensor. Compared with heparin, PI-88 has at least 11-fold higher affinity for FGF-1 and at least 3-fold higher affinity for VEGF, but at least 13-fold lower affinity for FGF-2. To define the structural features of PI-88 that are important for growth factor binding, several analogues, such as dephosphorylated PI-88 and a sulfated pentasaccharide, were prepared. The binding interactions of these analogues with FGF-1, FGF-2, and VEGF were similarly studied by SPR, and structure−activity relationships were determined.

Introduction

There is now a compelling body of evidence in support of the hypothesis, first proposed in the early 1970s,[1] that tumors require a blood supply for growth and metastasis.[2] This has fueled the intense activity in recent years in search of inhibitors of angiogenesis, that is, the sprouting of new blood vessels from pre-existing ones surrounding a tumor.[3−5] The development of drugs that inhibit angiogenesis is an attractive approach for the treatment of cancer because such drugs should offer improved efficacy and reduced toxicity, without the potential for drug resistance.[6] Tumor angiogenesis is a complex, multistep cascade involving the activation of endothelial cells by heparan sulfate-binding, angiogenic growth factors (such as VEGF, FGF-1, and FGF-2), resulting in endothelial cell proliferation and migration and, ultimately, new blood vessel formation.[7]

The phosphosulfomannan agent known as PI-88 (**1**) was recently shown to be a promising inhibitor of tumor growth and metastasis[8,9] and is undergoing Phase II clinical trials in cancer patients.[10] PI-88 exerts antiangiogenic effects by inhibiting the interactions of angiogenic growth factors and their receptors with heparan sulfate.[8] In addition, PI-88 is a potent inhibitor of the enzyme heparanase, a glycosidase that cleaves the heparan sulfate side chains of proteoglycans that are a major constituent of the extracellular matrix (ECM) and basement membranes surrounding tumor cells.[8] Heparanase has been strongly implicated in angiogenesis: it is able to liberate active heparan sulfate-bound angiogenic growth factors from the ECM and is involved in the degradation of the ECM and subsequent tissue remodeling associated with the sprouting of new blood vessels.[11] The degradation of the ECM by heparanase is also crucial in the spread of tumor cells (metastasis) by allowing them to pass into the blood stream and lodge in remote sites, where they can form secondary tumors.[11,12]

In addition to its antiangiogenic effects, PI-88 inhibits the blood coagulation cascade by (i) inhibiting proteases in the intrinsic pathway, (ii) stimulating the release of tissue factor pathway inhibitor (TFPI), and (iii) activating the heparin cofactor II-mediated inhibition of thrombin. However, PI-88 does not interact with AT III and thus shows no anti-Xa or AT III-mediated anti-IIa activity.[13,14] In vivo studies in monkeys have shown that low doses of PI-88 stimulate the release of all heparan sulfate bound TFPI from the vascular cell wall.[14] Apart from its effect on coagulation, TFPI was recently shown to be an antiangiogenic agent[15] and an inhibitor of metastasis.[16] PI-88 is thus unique among the antiangiogenic drugs currently in development because of its novel, multiple modes of action.

PI-88 is a mixture of highly sulfated, monophosphorylated mannose oligosaccharides ranging in size from di- to hexasaccharide.[17,18] The major components are penta- (∼60%) and tetrasaccharides (∼30%). The aims of this study were to determine which component(s) or structural features of PI-88 contribute to its antiangiogenic activity and to identify which of the growth factors FGF-1, FGF-2, and VEGF had the greatest affinity for PI-88. Toward this end, various derivatives of PI-88 were prepared and similarly assayed for growth factor binding in order to determine how oligosaccharide chain length, phosphorylation, and degree of sulfation influence these interactions. Binding affinities were quantified with a surface plasmon resonance (SPR) solution affinity assay.

Results

Preparation of Sulfated Oligosaccharides. PI-88 (**1**) was prepared as previously described.[18] Briefly, **2** was obtained by mild acid hydrolysis of *Pichia holstii* NRRL Y-2448 phosphomannan and then sulfated with excess sulfur trioxide pyridine complex (3 equiv per hydroxyl group). To investigate the effects of the level of sulfation, two preparations of undersulfated PI-88 with average degrees of sulfation of 2.8 and 2.2 sulfates

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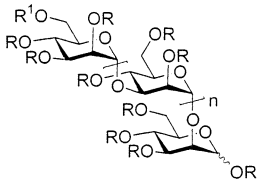

	n	R	R^1
1	0-4	SO_3Na or H	PO_3Na_2
2	0-4	H	PO_3Na_2
3	0	H	PO_3Na_2
4	1	H	PO_3Na_2
5	2	H	PO_3Na_2
6	3	H	PO_3Na_2
7	4	H	PO_3Na_2
8	0	SO_3Na or H	SO_3Na or H
9	1	SO_3Na or H	SO_3Na or H
10	2	SO_3Na or H	SO_3Na or H
11	3	SO_3Na or H	SO_3Na or H
12	0	H	H
13	1	H	H
14	2	H	H
15	3	H	H
16	0-4	H	H
17	0-4	SO_3Na or H	SO_3Na or H

Figure 1. Structures of the oligosaccharides used in this study.

per mannose unit (compared to 3.0−3.1 for normal PI-88) were prepared by employing, respectively, 1.0 and 0.75 equiv of sulfur trioxide pyridine complex per hydroxyl group. The average degree of sulfation for each of the sulfated oligosaccharides was estimated from the carbon-to-sulfur ratio obtained by microanalysis. The structures of the oligosaccharides used in this study are presented in Figure 1.

PI-88 can be only partially fractionated by gel permeation chromatography (GPC) on Bio-Gel P-6 into its constituent oligosaccharides of different chain lengths.[18] The individual oligosaccharide phosphates **3**−**7** present in the precursor **2** are also inseparable by GPC because of the presence of the phosphate group.[19] PI-88 derivatives of homogeneous chain length (**8**−**11**) were therefore prepared by sulfonation of the dephosphorylated oligosaccharides **12**−**15**. The latter were isolated by GPC as byproducts of the hydrolysis of *P. holstii* phosphomannan as previously described,[20] and all were >95% pure as judged by analytical HPLC and ^{1}H NMR spectroscopy.

To probe the effects of the phosphate group of PI-88, the precursor **2** was dephosphorylated with alkaline phosphatase as previously described[17] and the resulting mixture of oligosaccharides **16** was sulfonated to give "dephosphorylated PI-88" **17**. Analysis by capillary electrophoresis (CE) confirmed that the ratio of sulfated oligosaccharides in **17** was very similar to that in PI-88 (see Supporting Information).

Characterization of Sulfated Oligosaccharides. The degree of sulfation of the oligosaccharides was

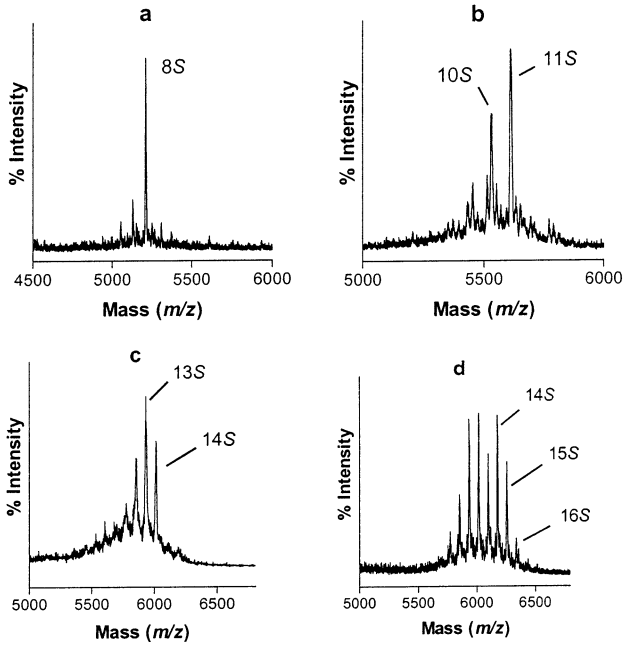

Figure 2. MALDI mass spectra of 1:1 complexes of sulfated oligosaccharides with the peptide $(RG)_{19}R$ with selected peaks identified: (a) disaccharide **8**; (b) trisaccharide **9**; (c) tetrasaccharide **10**; (d) pentasaccharide **11**. Numbers followed by an *S* indicate the number of sulfate groups. See the Supporting Information for a listing of observed masses along with calculated and theoretical masses of oligosaccharides.

estimated from the microanalysis data, and this showed that all were highly sulfated, with the exception of the two undersulfated PI-88 preparations. All sulfated oligosaccharides gave a single peak when analyzed by high-performance liquid chromatography (HPLC). The sulfated oligosaccharides were next analyzed by CE in reversed polarity mode using 5-sulfosalicylic acid (pH 3) as the background electrolyte with detection by indirect ultraviolet (UV) absorbance. The electropherograms of **8**−**11** showed a major symmetrical peak corresponding to highly sulfated species of homogeneous chain length and similar mass-to-charge ratios (see Supporting Information). These results are similar to those reported for reversed polarity CE analysis of sulfated cyclodextrins.[21]

The oligosaccharides **8**−**11** were characterized by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) of their non-covalent 1:1 complexes with the basic peptide $(RG)_{19}R$.[22] Mass spectral analysis of polysulfated oligosaccharides in the absence of such a positively charged peptide gives rise to very complex spectra due to facile in-source loss of SO_3 and the presence of multiple adducts with cations (such as Na^+ and K^+). The use of stable 1:1 non-covalent complexes in MALDI or electrospray ionization (ESI) mass spectrometry largely alleviates these problems.[23−25] The MALDI mass spectra of the 1:1 complexes obtained here (Figure 2) confirmed that the sulfated oligosaccharides were indeed mixtures of highly sulfated species. It was observed that the shorter the length of the oligosaccharide, the fewer the number of peaks in the spectrum, indicating a greater apparent degree of sulfation. The complete chemical sulfonation of oligosaccharides larger than disaccharide is often very difficult to accomplish,[26,27] and in this case only disaccharide **8**

appeared to be fully sulfonated. However, it is important to note that although desulfonation induced within the ionization source of the MALDI mass spectrometer is reduced by complexation with basic peptides, it is not removed completely. Whereas very little desulfonation occurs for small structures (as is seen in Figure 2a for disaccharide **8**), we and others[22] have observed that for longer sulfated oligosaccharides, in-source desulfonation is more pronounced, although the parent compound is always the most abundant by a factor of 2−3. Moreover, the response for oligosaccharides derived from heparin decreases with increasing chain length. Thus, the degree of incomplete sulfation seen here is probably slightly exaggerated.

Solution Affinity Assays for the Measurement of Binding Constants. Affinities for ligands binding to the growth factors were measured using a solution affinity assay.[28,29] In this method, the protein (P) is mixed with the ligand (L) and a binding equilibrium is established in solution. The mixture is then tested for its ability to bind to immobilized heparin.

For the equilibrium

$$P + L \rightleftharpoons P{\cdot}L$$

the equilibrium equation is

$$K_d = \frac{[L][P]}{[L{\cdot}P]} \quad (1)$$

Because

$$[L]_{total} = [L] + [L{\cdot}P] \quad (2)$$

and

$$[P]_{total} = [P] + [L{\cdot}P] \quad (3)$$

these can be substituted into eq 1 to give

$$K_d = \frac{([L]_{total} - [L{\cdot}P])([P]_{total} - [L{\cdot}P])}{[L{\cdot}P]} \quad (4)$$

Rearranging eq 4 and solving for [L·P] gives

$$[L{\cdot}P] = \frac{(K_d + [L]_{total} + [P]_{total})}{2} - \sqrt{\frac{(K_d + [L]_{total} + [P]_{total})^2}{4} - [L]_{total}[P]_{total}} \quad (5)$$

Substitution of eq 5 into eq 3 gives

$$[P] = [P]_{total} - \frac{(K_d + [L]_{total} + [P]_{total})}{2} + \sqrt{\frac{(K_d + [L]_{total} + [P]_{total})^2}{4} - [L]_{total}[P]_{total}} \quad (6)$$

The concentration of free protein in the equilibrium mixture, [P], can be measured by injecting the equilibrium mixture over a surface with immobilized heparin. Ligands that bind to the protein at the same site as heparin will prevent the protein from interacting with the surface. Thus, any response observed is due to free protein in the equilibrium mixture.

Under conditions of mass transport, the observed binding rate no longer reflects the interaction kinetics between protein and immobilized heparin, because the rate of transport of protein between bulk solution and the sensor chip surface is limited.[30] Therefore, under these conditions the initial binding rate, r, is proportional to the free protein concentration

$$r = k[P] \quad (7)$$

and the maximum binding rate, r_m, measured in the absence of ligand, is proportional to the total protein concentration.

$$r_m = k[P]_{total} \quad (8)$$

Combining eqs 7 and 8 gives

$$[P] = \frac{r}{r_m}[P]_{total} \quad (9)$$

Thus, provided the surface binding experiments are conducted under conditions of mass transport, the binding rate in the absence of ligand can be used to determine the free protein concentration in solution mixtures containing ligand.

Under conditions of mass transport, a standard curve, relating initial binding rates or relative responses to protein concentration in the absence of ligand, will be linear and pass through the origin. A standard curve was generated by injecting 25 μL of standard solutions containing protein at various concentrations in buffer (HBS-EP buffer for FGF-1 and VEGF; HBS-EP buffer containing 0.3 M NaCl for FGF-2) at 5 μL/min. Prior to injection, standard solutions were maintained at 4 °C to maximize protein stability, and the surface binding experiments were performed at 25 °C. The surface was regenerated by injection of 40 μL of 4 M NaCl at 40 μL/min, followed by injection of 40 μL of buffer at 40 μL/min. Data analyses were carried out using the BIAevaluation 3.0 software.

Binding of Growth Factors to Immobilized Heparin. Standard curves relating the relative response value to the injected protein concentration were constructed, as shown in Figure 3 for VEGF. In all cases the curves are linear, indicating that the binding rate is proportional to the protein concentration and thus suggesting that the binding experiments were conducted under conditions of mass transport. Furthermore, the standard curves also pass through the origin (within experimental error), suggesting that treatment of binding curves using eqs 7−9 was valid for solution affinity experiments measured on this chip. For FGF-2, however, relative response values obtained from the binding curves were converted to protein concentration using a standard curve measured with each solution affinity experiment.

Interaction of Growth Factors with Sulfated Oligosaccharide Ligands. Representative binding curves obtained from solution affinity experiments using VEGF with the sulfated tetrasaccharide **10** are shown in Figure 4a. These binding curves exhibit the expected drop in response level as the concentration of **10** increases. In Figure 4b, the analysis of one of these binding experiments, following conversion of binding

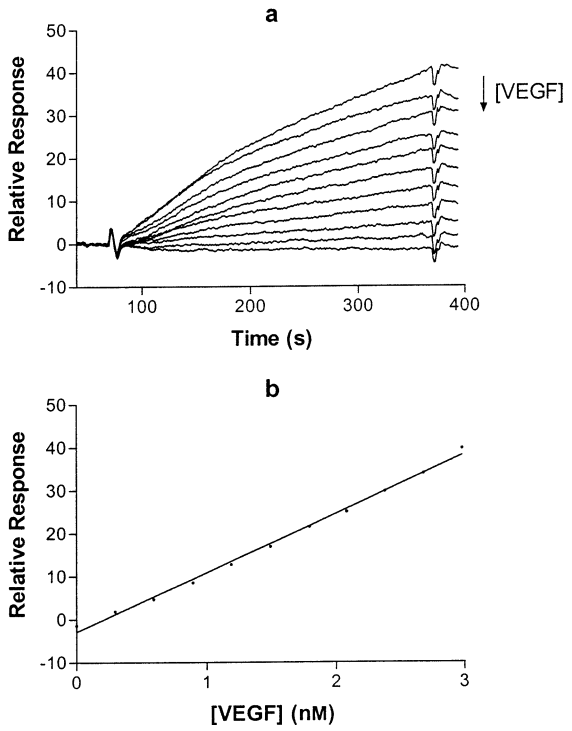

Figure 3. Representative protein standard curve determined for VEGF: (a) SPR sensorgrams demonstrating the change in binding response (in RU) upon injection of various concentrations of VEGF from 0 to 2.98 nM in HBS-EP running buffer over a heparin surface; (b) protein standard curve constructed by plotting the observed binding response against the protein concentration.

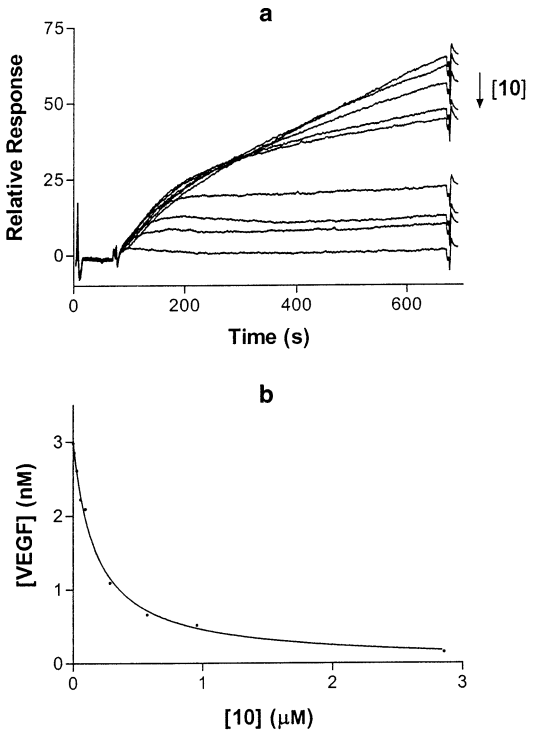

Figure 4. Representative K_d measurement of tetrasaccharide **10** binding to VEGF: (a) SPR sensorgrams showing the change in binding response (in RU) upon injection of 2.98 nM VEGF mixed with various concentrations of tetrasaccharide **10** from 0 to 2.86 μM in HBS-EP running buffer over a heparin surface; (b) binding curve constructed by plotting free VEGF concentration against total concentration of tetrasaccharide **10**. Values for free VEGF concentration were calculated as described under Experimental Section.

data to free VEGF concentration according to eq 9 and calculating binding parameters according to eq 6, is shown. In the data analysis, $[P]_{total}$ was floated during the fitting procedure to allow for any variations in sample preparation. The K_d values measured for all of the ligands binding to the growth factors are summarized in both tabular and graphical formats in the Supporting Information.

Discussion

Previous studies have demonstrated that PI-88 is a potent inhibitor of angiogenesis in an in vitro model.[8] The assay, in which a fragment of human placental blood vessel embedded in a fibrin gel is induced to sprout microvessels, is largely dependent on endogenous FGF-2 and, to a lesser extent, FGF-1 and VEGF action.[31] Interestingly, in this assay the polyanionic, antiangiogenic agent suramin[32] was a 25-fold less active inhibitor than PI-88, whereas heparin was completely inactive. PI-88 was also shown to inhibit the interactions of FGF-1 and FGF-2 with heparan sulfate on the surface of murine fibroblasts.[8]

In the current study, we have tested the binding of PI-88 and its various derivatives to the angiogenic growth factors FGF-1, FGF-2, and VEGF, using an SPR assay based on solution affinity. The derivatives of PI-88 were analyzed by both CE and MALDI-MS. The MALDI-MS data suggest species **9**, **10**, and **11** contain a mixture of sulfated products, but this was not apparent in the CE analysis. Although loss of sulfo groups is markedly reduced by the complexing of sulfated species with basic peptides, some loss may still occur during the MALDI-MS analysis. However, the size of the peaks observed (Figure 2) is indicative of incomplete sulfonation during production and a mixture of variously sulfated products. In our hands the resolution these sulfated species afforded by the MALDI-MS technique appears to be superior to that of the CE analysis.

K_d measurements using SPR rely on the relative stability of the sensor chip surface, and therefore the solution affinity assay method, which utilizes immobilized heparin, was chosen for K_d determination. Immobilization of the protein molecules onto the surface was not desirable because the FGFs are relatively unstable.[33] The principle of the SPR assay is that a solution, at equilibrium, of the growth factor and a ligand is passed over a sensor chip containing immobilized heparin. As the unbound growth factor binds to the heparin, an increase in the SPR response is detected and its concentration can thus be determined. A decrease in the free growth factor concentration as a function of the ligand concentration allows for the calculation of the dissociation constant, K_d. Furthermore, a decrease in binding of the growth factor to the immobilized heparin is observed only if the ligand binds to the growth factor in the heparin binding site.

The assumption underlying these results is that the ligand molecules bind to FGF-1, FGF-2, and VEGF with 1:1 stoichiometry. Although it has previously been reported that multiple binding sites exist for FGF-1 and FGF-2 on heparin,[34,35] it is assumed here that these bind to proteins independent of each other and that binding

of one protein molecule to heparin does not alter the affinity for binding of a second protein to the heparin. In the case of VEGF, which is dimeric, the protein is assumed to act as a single entity, forming a single binding surface for interaction with large ligands such as heparin. When the ligands are small, the half sites on each monomer are assumed to interact with ligand independently of each other.

The solution affinity assay was used to measure dissociation constants for the interactions of FGF-1, FGF-2, and VEGF with PI-88 and, for comparison, with the glycosaminoglycans heparin, LMWH, and heparan sulfate and the polyanionic drug sucrose octasulfate. K_d values reported in the literature for heparin binding to FGF-1 range from ~1 nM[36] and 5.0 nM[37] to 180 nM.[38] For heparin binding to FGF-2, the reported values range from ~1 nM[36] and 2.2 nM[39] to 71 nM.[38] The values obtained depend on the method used to determine them, the ionic strength of the buffer, the size and source of the heparin, and the source of the growth factor. The K_d values measured in this study for heparin binding to FGF-1 and FGF-2 (2.1−5.8 and 4.4−6.1 nM, respectively) are in good agreement with those previously reported. Neither the type of BIAcore chip nor the method of immobilization of heparin onto the surface of the chip had any effect on the K_d measured for heparin binding to FGF-1 and FGF-2. For example, for FGF-1, when heparin was immobilized to a C1 chip (BIAcore) by reductive amination using adipic acid dihydrazide,[40] solution affinity K_d values of 1.2−4.0 nM were obtained, whereas heparin immobilized to a CM5 chip (BIAcore) using the same immobilization method resulted in K_d values of 4.7−5.4 nM.[41] The K_d for heparin binding to VEGF was at least 4-fold higher than that for FGF-1, which was measured using the same buffer conditions. All three growth factors have reduced affinity for low molecular weight heparin (LMWH) compared to heparin. The affinity of binding was reduced by around 5-, 15-, and 25-fold, respectively, for FGF-1, FGF-2, and VEGF. The binding to sucrose octasulfate was quite weak, the affinity being in the micromolar range.

PI-88 bound very tightly to FGF-1 (K_d = 281−444 pM), FGF-2 (K_d = 82−140 nM), and VEGF (K_d = 1.9−6.9 nM), with affinities that compare favorably with the glycosaminoglycans tested and are considerably better than for sucrose octasulfate. Compared with heparin, PI-88 has at least 11-fold higher affinity for FGF-1 and at least 3-fold higher afffinity for VEGF, but at least 13-fold lower affinity for FGF-2.[42]

To elucidate the features of PI-88 that are important for its interaction with the growth factors, several derivatives of PI-88 were investigated. These ligands differ in the degree of sulfation, the absence or presence of a nonreducing end phosphate group, and the number of saccharide units. Reduced levels of sulfation in PI-88 resulted in reduced affinities for the growth factors, as expected. The largest effect was observed for FGF-1, in which a reduction in the degree of sulfation from 3.1 to 2.2 sulfates per saccharide resulted in at least a 6-fold reduction in affinity. The inhibitory activity of PI-88 in the in vitro angiogenesis assay, heparanase assay, and metastasis and tumor growth assays was previously shown to fall with reduced levels of sulfation.[8]

The effect of replacing the nonreducing end phosphate group of PI-88 with another sulfate group was also examined. There is evidence in the literature that proteins may discriminate between phosphate and sulfate groups by different networks of hydrogen bonds (due to the markedly different orientations adopted by a sulfate or a phosphate upon formation of a hydrogen bond).[43−45] This discrimination is well illustrated in the case of AT III, where replacement of the nonreducing terminal sulfate group of the AT III-binding pentasaccharide with a phosphate group results in an analogue of no significant activity.[46] Replacement of the phosphate group in PI-88 with sulfate had a slight effect on binding to VEGF but had an insignificant effect on FGF-1 and FGF-2 binding affinities. This suggests that the phosphate group is not essential for PI-88 activity in these growth factors.

Finally, a series of derivatives of PI-88 of defined carbohydrate chain length (and lacking a phosphate group) was examined. Drawing from the previous observation that replacement of the phosphate with a sulfate has little effect on PI-88 activity, the results obtained were assumed to be indicative of the actual components of PI-88 itself. For FGF-2 and VEGF, the affinity for the sulfated oligosaccharides increased as oligosaccharide chain length increased from 2 to 5 saccharide units, whereas for FGF-1 the affinity plateaued at a length of 4 saccharide units. For all three growth factors, the binding affinity for the disaccharide was reduced by 2−3 orders of magnitude compared to the pentasaccharide. The trend observed is in keeping with previous studies of sulfated oligosaccharides in which increasing chain length correlates favorably with increased biological activity.[8,13,47] The results indicate that the two dominant components of the PI-88 mixture, that is, the penta- and tetrasaccharide components, are responsible for the bulk of the antiangiogenic activity.

In conclusion, the binding studies of PI-88 and its derivatives with FGF-1, FGF-2, and VEGF by the SPR solution affinity assay demonstrated that PI-88 binds very tightly to these angiogenic growth factors, particularly FGF-1. PI-88 shows a greater affinity for FGF-1 and VEGF than glycosaminoglycans such as heparin, LMWH, and heparan sulfate or polyanionic drugs such as sucrose octasulfate. On the other hand, PI-88's affinity for FGF-2 is less than heparin's but is of the same order as those of LMWH and heparan sulfate. The binding is highly dependent on the degree of sulfation and the chain length but not on the nonreducing end phosphate group. These results provide some insight into how PI-88 exerts its biological activity and may assist in the design of more potent angiogenesis inhibitors with greater specificity.

Experimental Section

General. Samples for ^{1}H NMR analysis were dissolved in D_2O, and the spectra were recorded at 298 K on a Varian Unity 400 spectrometer. CE was performed in reverse polarity mode on a Beckman P/ACE 5000 system equipped with a P/ACE UV absorbance detector, using 10 mM 5-sulfosalicylic acid (pH 3) as the background electrolyte, as previously described.[18] Samples were prepared in water at a concentration of 1.0 mg/mL. Analytical HPLC was performed on a Waters Alliance 2690 separations module equipped with a Waters 2410 refractive index detector, using a Phenomenex PolySep-GFC-P2000

(300 × 7.8 mm) column and 0.1 M $NaNO_3$ as the mobile phase. The flow rate was 0.8 mL/min, and the column temperature was set at 35 °C. Microanalyses (C, H, S) were performed by Gribbles Analytical Laboratories (Notting Hill, Australia) or by the Australian National University's Microanalytical Services Unit (Canberra, Australia). All reagents used were of analytical grade and were used without further purification. All water used was purified in-house to USP Purified Water standard. Bio-Gel P-2 was obtained from Bio-Rad. Recombinant human FGF-1 (140 amino acid residue, N-terminally truncated form), recombinant human FGF-2 (146 amino acid residue, N-terminally truncated form), and recombinant human VEGF (165 amino acid form) were purchased from R&D Systems, Inc. (Minneapolis, MN). Each of these protein preparations contained 50 μg of bovine serum albumin (BSA) per 1 μg of growth factor. Heparin (from bovine lung or bovine intestinal mucosa, average molecular weight ~12 kDa), LMWH (from porcine intestinal mucosa, average molecular weight ~3 kDa), heparan sulfate (from bovine kidney, average molecular weight ~15 kDa), heparin−albumin−biotin, and albumin−biotin were purchased from Sigma (St. Louis, MO). Sucrose octasulfate, potassium salt, was obtained from Toronto Research Chemicals (Toronto, Canada). PI-88 (**1**) was prepared by the sulfonation of **2** as previously described.[18] The oligosaccharides **2** and **12**−**15** were obtained by hydrolysis of *P. holstii* NRRL Y-2448 phosphomannan as previously described.[20] The oligosaccharides **16** were obtained by dephosphorylation of **2** with alkaline phosphatase as previously described.[17]

General Procedure for the Sulfonation of Oligosaccharides. A stirred mixture of the oligosaccharide (20 mg) and sulfur trioxide pyridine complex (3 mol equiv per OH group) in dimethylformamide (DMF) (3 mL) was heated at 60 °C for 6 h, by which time the product had separated out as an oil (except disaccharide **8**, which was precipitated with ethanol). The DMF was decanted, and the residue was washed with EtOH (3 × 5 mL) and then dissolved in water (1 mL). The pH was adjusted to 9.0−9.5 with 1 M NaOH, and the solution was concentrated. The crude product was then purified by chromatography on a column of Bio-Gel P-2 (1.5 × 100 cm) equilibrated with 0.1 M ammonium bicarbonate. The appropriate fractions were pooled and lyophilized to give the sulfated oligosaccharides as white, amorphous solids. Alternatively, the crude product was purified by diafiltration (Millipore Minitan, 1000 NMWCO membrane) against 8 diavolumes of 1 M NaCl followed by 5 diavolumes of water and then lyophilized. The products were characterized by CE and MALDI-MS, and the average degree of sulfation was determined by microanalysis. Undersulfated oligosaccharides were prepared according to a similar procedure using either 1 or 0.75 equiv, respectively, of sulfur trioxide pyridine complex per hydroxyl group.

MALDI-MS. Sulfated oligosaccharides were analyzed by MALDI-MS using the procedure described by Venkataraman et al.[22] The basic peptide $(RG)_{19}R$ was prepared as the trifluoroacetate salt by Auspep (Melbourne, Australia). Approximately 50−100 mg of AG-1 X8 anion-exchange resin in the hydroxide form (Bio-Rad, Sydney, Australia) was added to an ice-cold aliquot (100 μL) of 50 μM peptide. The resulting suspension was centifuged briefly and maintained in an ice bath. An aliquot of peptide (1 μL) was mixed with 10 mg/mL caffeic acid dissolved in 50% acetonitrile (8 μL) and 5−100 μM sample (1 μL). One microliter was spotted onto a stainless steel sample plate and allowed to dry. MALDI-MS spectra were acquired in the linear mode by using a PerSeptive Biosystems (Applied Biosystems, Melbourne, Australia) Voyager reflectron time-of-flight instrument fitted with a 337-nm nitrogen laser. Delayed extraction was used to increase resolution (22 kV, grid at 93%, guide wire at 0.15%, pulse delay of 150 ns, low mass gate at 2000, 50 shots averaged). Mass calibration was achieved by external calibration with the peptide calibration mixture provided by the manufacturer. The mass of the oligosaccharide was deduced by subtracting the observed mass of the $(RG)_{19}R$ peptide in that spectrum.

SPR Analysis. SPR measurements were performed on a BIAcore 3000 (BIAcore, Uppsala, Sweden) operated using the BIAcore control software. HBS-EP buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, and 0.005% (v/v) surfactant P20] and the streptavidin-coated sensor chips were purchased from BIAcore.

Immobilization of Heparin onto Sensor Chips. Prior to immobilization of heparin to the streptavidin chip, the chip was pretreated with three 5 μL injections of 50 mM NaOH in 1 M NaCl at 5 μL/min to remove any nonspecifically bound contaminants, followed by a rinse with 40 μL of HBS-EP buffer at 40 μL/min. Heparin−albumin−biotin was then immobilized in two of the four flowcells by injection of 5 μL of a 1 μg/mL solution prepared in H_2O. A single injection resulted in an increase in response of 60−200 response units (RU), depending on the flowcell. Subsequent injections of heparin−albumin−biotin at 1−50 μg/mL did not result in further immobilization. The remaining two flowcells were used as negative control flowcells, and albumin−biotin was immobilized in these, using the above method, resulting in a response increase of 360−730 RU.

To test the integrity of the heparin immobilized on the chip, 25 μL of 1.29 nM FGF-1 in HBS-EP buffer was injected at 5 μL/min. The chip was deemed to be suitable for use in experiments if FGF-1 binding resulted in a response increase of >25 RU. No binding was observed in the negative control flowcell, indicating that the observed binding response was due specifically to the interaction between protein and immobilized heparin. Injection of 25 μL of 0.5 nM FGF-2 or 2.98 nM VEGF resulted in a response increase of 25 or 40 RU, respectively. The surface was regenerated by injection of 40 μL of 4 M NaCl at 40 μL/min, followed by injection of 40 μL of HBS-EP buffer at 40 μL/min.

The protein preparations used in this study contained a 50-fold excess of BSA (supplied in this form by the manufacturer). However, in a control experiment, no binding response was observed when a solution of 1 μg/mL BSA was injected using the conditions described above.

K_d Measurements by Solution Affinity. For each K_d measurement, 100 μL solutions were prepared containing 1.29 nM FGF-1, 0.5 nM FGF-2, or 2.98 nM VEGF and various concentrations of the ligand (diluted from a 10 mg/mL stock solution prepared in H_2O) in buffer on ice. In the case of FGF-1 and VEGF, the buffer used was HBS-EP buffer. For FGF-2, the concentration of NaCl in the HBS-EP buffer was increased to 0.3 M to eliminate nonspecific background binding that was observed in the negative control flowcell. Prior to injection, samples were maintained at 4 °C to maximize protein stability. For each assay mix, 25−50 μL of solution was injected at 5 μL/min and the relative response or initial binding rate was measured. All surface binding experiments were performed at 25 °C. The surface was regenerated by injection of 40 μL of 4 M NaCl at 40 μL/min, followed by injection of 40 μL of buffer at 40 μL/min.

Data analysis was carried out using the BIAevaluation 3.0 software. For FGF-1 and VEGF, the initial binding rates were determined by measuring the slope of the binding curve in the first ~100 s. However, K_d values determined from binding rates measured elsewhere on the curves gave the same results. The binding rates were converted to free protein concentration using eq 9. For FGF-2, relative responses were converted to free protein concentration using a standard curve generated for each K_d experiment. K_d values were calculated by plotting free protein concentration against ligand concentration and fitting eq 6.

Supporting Information Available: Table and plots of K_d values for sulfated oligosaccharide ligands binding to FGF-1, FGF-2, and VEGF as measured by solution affinity using eq 6, capillary electropherograms for sulfated oligosaccharides **1**, **8**−**11**, and **17**, and table of MALDI-MS data (observed and theoretical masses) for sulfated oligosaccharides **8**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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