PI-88 and Novel Heparan Sulfate Mimetics Inhibit Angiogenesis

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ABSTRACT

The heparan sulfate (HS) mimic PI-88 is a promising inhibitor of tumor growth and metastasis expected to commence phase III clinical evaluation in 2007 as an adjuvant therapy for postresection hepatocellular carcinoma. Its anticancer properties are attributed to inhibition of angiogenesis via antagonism of the interactions of angiogenic growth factors and their receptors with HS. It is also a potent inhibitor of heparanase, an enzyme that plays a key role in both metastasis and angiogenesis. A series of PI-88 analogs have been prepared with enhanced chemical and biological properties. The new compounds consist of single, defined oligosaccharides with specific modifications designed to improve their pharmacokinetic properties. These analogs all inhibit heparanase and bind to the angiogenic fibroblast growth factor 1 (FGF-1), FGF-2, and vascular endothelial growth factor with similar affinity to PI-88. However, compared with PI-88, some of the newly designed compounds are more potent inhibitors of growth factor–induced endothelial cell proliferation and of endothelial tube formation on Matrigel. Representative compounds were also tested for antiangiogenic activity in vivo and were found to reduce significantly blood vessel formation. Moreover, the pharmacokinetic profile of several analogs was also improved, as evidenced primarily by lower clearance in comparison with PI-88. The current data support the development of HS mimetics as potent antiangiogenic anticancer agents.

KEYWORDS: PI-88, heparan sulfate mimetics, heparanase inhibitors, angiogenesis inhibitors, sulfated oligosaccharides

To grow and metastasize, tumors are critically dependent on angiogenesis1,2 (i.e., the growth of new blood vessels from pre-existing ones surrounding a tumor), and its inhibition is now well established as an important therapeutic strategy for cancer patients.3 Cell surface/extracellular matrix (ECM) heparan sulfate (HS) glycosaminoglycans are complex polysaccharides that are ubiquitous in nature and play important roles in the regulation of several aspects of cancer biology, including angiogenesis, tumor progression, and metastasis.4,5 The use of HS mimetics to modulate these processes is therefore a promising approach for new cancer therapeutics.4,6,7 This is illustrated by the sulfated oligosaccharide PI-88, which is progressing through clinical development as an antiangiogenic anticancer agent. The development of newly designed HS mimetics with enhanced properties is also discussed.
The sulfated oligosaccharide mixture known as PI-88 (1; Fig. 1)\(^8\) is an angiogenesis inhibitor currently undergoing phase II clinical trials in patients with advanced malignancies.\(^9,10\) PI-88 inhibits angiogenesis\(^8,11,12\) by blocking the interactions of angiogenic growth factors (principally fibroblast growth factor 1 [FGF-1], FGF-2, and vascular endothelial growth factor [VEGF]) and their receptors with HS.\(^8,9\) In addition, PI-88 is a potent inhibitor of heparanase,\(^8\) an endoglucuronidase that cleaves the HS side chains of proteoglycans that are a major component of the ECM. Heparanase plays a key role in both metastasis and angiogenesis by participating in the degradation of the ECM, vascular remodeling, and the release of HS-bound angiogenic growth factors from the ECM.\(^13,14\) PI-88 also stimulates the release of tissue factor pathway inhibitor, an endogenous antiangiogenic protein.\(^15\)

PI-88 is prepared\(^16\) by exhaustive sulfonation of the oligosaccharide phosphate fraction obtained from mild acid-catalyzed hydrolysis of the extracellular phosphomannan produced by the yeast Pichia holstii NRRL Y-2448.\(^17\) This mixture is composed primarily of the phosphorylated penta- (2) and tetrasaccharides (3), which account for \(\sim 60\) and 30\%, respectively, of the total oligosaccharide content; the remaining 10\% comprises phosphorylated di- to hexasaccharides.\(^18\)

### PI-88 PRECLINICAL AND CLINICAL STUDIES
PI-88 was identified as a potent inhibitor of both in vitro angiogenesis and heparanase activity\(^8\) by screening libraries of sulfated oligosaccharide HS mimetics. Subsequent in vivo studies confirmed its capacity to inhibit tumor growth, metastasis, and angiogenesis. For example, PI-88 inhibited the growth of invasive rat mammary adenocarcinoma cells by \(\sim 50\%\) and reduced lymph node and blood-borne metastases.\(^8\) PI-88 also diminished the malignant cell load in rodent models of human myeloid leukemia.\(^19\) More recently, PI-88 showed significant effects in distinct stages of tumorigenesis in the RIP1-Tag2 transgenic mouse model of pancreatic islet \(\beta\)-cell carcinoma\(^1,12\), reducing the number of early progenitor lesions and inhibiting tumor growth at later stages. These responses were associated with decreased cell proliferation, increased apoptosis, inhibition of angiogenesis, and a significant reduction in the number of invasive carcinomas.

Preclinical data supported the decision to test PI-88 in the various clinical trials\(^20,21\) summarized in Table 1. In early trials PI-88 (as a solution in normal saline) was administered by intravenous infusion, although it is now administered by subcutaneous injection. As indicated in Table 1, PI-88 has been administered to close to 400 subjects (including healthy volunteers) as both a single agent and in combination with standard chemotherapy for a range of different cancers. These clinical studies have demonstrated that PI-88 has an excellent safety and tolerability profile, with few serious adverse events reported. Mild anticoagulation effects have been reported in all patients; however, the dose-limiting toxicity is immune-mediated thrombocytopenia.\(^20,25\) Immune-mediated thrombocytopenia has been observed in \(\sim 5\%\) of patients, usually occurs
during the first treatment cycle, and appears to be dose related. Platelet counts return to normal upon cessation of PI-88 treatment.

PI-88 also has shown evidence of patient benefit. For example, in a phase I trial in patients with advanced solid tumors, one patient with melanoma had a partial response, which was maintained for > 50 months, and nine patients, including five with melanoma, had stable disease for ≥ 6 months. These positive outcomes supported the commencement of a phase II trial in advanced melanoma patients as a single agent and subsequently in metastatic melanoma in combination with chemotherapy (dacarbazine). Also of note is the current phase II trial in postresection hepatocellular carcinoma, in which PI-88 is administered as an adjuvant therapy. It is this indication with high unmet need that has been targeted for phase III trials to commence in 2007.

NEW HS MIMETICS AS ANGIOGENESIS INHIBITORS

Given the encouraging clinical progress of PI-88 as an anticancer therapy, the design and synthesis of PI-88 analogs with improved properties was undertaken. PI-88 is a complex mixture of oligosaccharides and this presents major challenges in characterization, assay development, interpretation of structure–activity relationships, and in ensuring batch-to-batch consistency during manufacture. HS mimetics that are single chemical entities, or at least based on a single carbon backbone, would address the mixture problems. (It is well established that sulfonation of tri- or larger oligosaccharides rarely goes to completion and instead gives a randomly, but reproducibly, highly sulfated mixture. See Karoli et al and references cited therein.)

Previous studies of derivatives of the individual components of PI-88 had established that the pentasaccharide and tetrasaccharide components are the most biologically active. New compound design was thus based on anomerically pure (α anomer only), single pentasaccharide glycosides in which the reducing end phosphate has been replaced by sulfate for ease of synthesis and because it has minimal impact on biological activity (Fig. 1; generic structure 6). The aglycones (Fig. 1; R2 = primarily lipophilic groups) were chosen specifically to improve the pharmacokinetic properties and thus bioavailability.

The new compounds were prepared conveniently in four or five steps from the oligosaccharides 4 or 5, which are themselves amenable to total synthesis using readily available monosaccharide building blocks (unpublished results). The compounds were assessed initially for their affinity for the angiogenic growth factors FGF-1, FGF-2, and VEGF, and for their ability to inhibit heparanase activity. In general, most compounds displayed similar, and in some cases slightly better, activity to PI-88 in these assays (Table 2). Pharmacokinetic studies of the initial series of compounds using radiolabeled material showed that the addition of a suitable aglycone, especially a lipophilic one, could have a favorable effect on the pharmacokinetic profile. For example, the plasma clearance of compound 7 (PG501) was approximately three times slower than that of PI-88 (Table 2). Pharmacokinetic assessments of more promising compounds are being conducted in parallel with efficacy experiments in mouse tumor models (see below and are ongoing.

Following these positive findings, additional compounds were synthesized and tested. Cell-based assays indicative of antiangiogenic activity were added to the suite of tests. In human umbilical vein endothelial

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**Table 1 Summary of PI-88 Clinical Trials**

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<th>Status</th>
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<td>Healthy volunteers (IV)</td>
<td>Completed</td>
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<tr>
<td>I</td>
<td>Healthy volunteers (SC crossover)</td>
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<td>22</td>
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<tr>
<td>I</td>
<td>Advanced cancers (IV)</td>
<td>Completed</td>
<td>14</td>
</tr>
<tr>
<td>I</td>
<td>Advanced cancers (IV), Asian population</td>
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<td>9</td>
</tr>
<tr>
<td>I</td>
<td>Advanced cancers</td>
<td>Completed</td>
<td>42</td>
</tr>
<tr>
<td>Ib</td>
<td>Advanced cancers (combination)*</td>
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<td>II</td>
<td>Multiple myeloma</td>
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<td>II</td>
<td>Advanced melanoma</td>
<td>Completed</td>
<td>44</td>
</tr>
<tr>
<td>II</td>
<td>Advanced prostate cancer (combination)*</td>
<td>Ongoing</td>
<td>Up to 90</td>
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<tr>
<td>II</td>
<td>Metastatic melanoma (combination)*</td>
<td>Ongoing</td>
<td>Up to 118</td>
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<tr>
<td>II</td>
<td>Advanced lung cancer (NSCLC; combination)*</td>
<td>Ongoing</td>
<td>99</td>
</tr>
<tr>
<td>II</td>
<td>Post-resection hepatocellular carcinoma*</td>
<td>Ongoing</td>
<td>172</td>
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*In combination with docetaxel.
†First-line treatment in combination with dacarbazine.
‡Adjuvant therapy.
§PI-88 administered as a SC injection unless otherwise indicated.
IV, intravenous; SC, subcutaneous.
cell (HUVEC) and dermal human microvascular endothelial cell (dHMVEC) proliferation assays, all compounds potently inhibited FGF-2–induced endothelial cell proliferation. However, inhibitory activity against FGF-1 or VEGF–induced HUVEC proliferation was compound specific (i.e., although PI-88 and some of the compounds had poorer activity against these growth factors, others exhibited potent inhibitory activity similar to those generated in the FGF-2–induced HUVEC proliferation assays; Fig. 2). Endothelial cell tube formation on Matrigel using either HUVEC or dHMVEC was potently inhibited by several compounds in comparison to PI-88 which only had modest activity in this assay (Fig. 3).

Two representative compounds 7 (PG501) and 8 (PG500) together with PI-88 were chosen for evaluation in two in vivo mouse models of angiogenesis: the AngioChamber and AngioSponge (vivoPharm Pty,墨尔本，澳大利亚). The inhibitory effect of PI-88 and PG500 series compounds on endothelial cell tube formation in the in vitro Matrigel assay. Human umbilical vein endothelial cells were plated onto 96-well plates precoated with Matrigel and a range of concentrations of each compound (0, 10, 50, and 100 μM) and cultured for 24 hours. Similar data were obtained using dermal human microvascular endothelial cells. Tube formation was examined by phase-contrast microscopy and images were collected using an Olympus C5050 digital camera. Tube formation inhibition was quantitated manually from images by recording the total number of nodes connecting three or more tubules. Results are expressed as percentage inhibition compared with control.

### Table 2 Heparanase Inhibitory Activity In Vitro, Angiogenic Growth Factor Binding (surface plasmon resonance solution affinity assay), and Selected Pharmacokinetic Parameters in Male Sprague-Dawley Rats for PI-88 and Selected Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵢ Heparanase (nM)</th>
<th>Kᵢ GFG-1 (nM)</th>
<th>Kᵢ GFG-2 (nM)</th>
<th>Kᵢ VEGF (nM)</th>
<th>t½ (rat) (h)</th>
<th>CL (rat) (mL/h/kg)</th>
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<tr>
<td>PI-88 (1)*</td>
<td>240</td>
<td>0.24</td>
<td>130</td>
<td>1.3</td>
<td>0.83</td>
<td>250</td>
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<tr>
<td>PG501 (7)*</td>
<td>310</td>
<td>0.14</td>
<td>68</td>
<td>1.5</td>
<td>1.1</td>
<td>84</td>
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<tr>
<td>PG500 (8)*</td>
<td>340</td>
<td>0.14</td>
<td>86</td>
<td>1.7</td>
<td>0.83</td>
<td>199</td>
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<tr>
<td>PG517</td>
<td>280</td>
<td>0.27</td>
<td>125</td>
<td>23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PG518</td>
<td>330</td>
<td>0.11</td>
<td>123</td>
<td>21</td>
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<td>—</td>
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<tr>
<td>PG522</td>
<td>345</td>
<td>0.06</td>
<td>81</td>
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<td>11</td>
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*Data from Karoli et al. 25

It is well established that sulfonation of tri- or larger oligosaccharides rarely goes to completion and instead gives a randomly, but reproducibly, highly sulfated mixture. Assays were performed as described by Karoli et al. 25

Kᵢ, ??_; Kᵢ, ??_; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; t½, ??_; CL, Clearance.

**Figure 2** Concentration that inhibits 50%([IC₅₀](#)) values of PI-88 and PG500 series compounds in the growth factor–induced human umbilical vein endothelial cell proliferation assay. Cells were coincubated for 72 hours with either fibroblast growth factor 1 (FGF-1), FGF-2, or vascular endothelial growth factor (VEGF) in the presence of a control or multiple concentrations of compound (5 to 50 μM). Similar data were obtained using dermal human microvascular endothelial cells. Proliferation was measured using a nonradioactive colorimetric method, CellTiter96 AQueous Assay from Promega.

**Figure 3** The inhibitory effect of PI-88 and PG500 series compounds on endothelial cell tube formation in the in vitro Matrigel assay. Human umbilical vein endothelial cells were plated onto 96-well plates precoated with Matrigel and a range of concentrations of each compound (0, 10, 50, and 100 μM) and cultured for 24 hours. Similar data were obtained using dermal human microvascular endothelial cells. Tube formation was examined by phase-contrast microscopy and images were collected using an Olympus C5050 digital camera. Tube formation inhibition was quantitated manually from images by recording the total number of nodes connecting three or more tubules. Results are expressed as percentage inhibition compared with control.

**Figure 4** AngioChamber assay: mean capsule wet weights (milligrams). AngioSponge assay: mean CD31 counts per view field. Test compounds were dosed at 30 mg/kg/day. (A) Vehicle control (no fibroblast growth factor [FGF]-2); (B) vehicle control + FGF-2; (C) PI-88 + FGF-2; (D) PG500 + FGF-2; (E) PG501 + FGF-2; (F) positive control + FGF-2.
Q10 Ltd., Adelaide, Australia\textsuperscript{(Q10)} assays. The mice were treated with twice-daily subcutaneous injections at 15 mg/kg, a dose selected on the basis of extensive preclinical experience with PI-88. In the AngioChamber assay,\textsuperscript{27} angiogenesis is induced by FGF-2 addition to a subcutaneously implanted, perforated polytetrafluoroethylene \textsuperscript{Q11} chamber and results in the formation of a fibrous capsule around the chamber. Efficacy is assessed by measurement of capsule wet weight and visual observation in situ. As shown in Fig. 4, all test compounds showed significant inhibition of FGF-2–induced fibrous capsule formation.

In the AngioSponge assay,\textsuperscript{28} a hydrated gelfoam impregnated with FGF-2 and agarose is implanted subcutaneously, resulting in slow release of FGF-2, which stimulates angiogenesis. Angiogenesis is measured by blood vessel counting after CD31 immunostaining, and blood vessel morphology can be assessed qualitatively. As shown in Fig. 4, all test compounds inhibited angiogenesis significantly compared with both the positive control and the control without added FGF-2. PG500 and PG501\textsuperscript{Q12} both showed less intense CD31 staining, with most blood vessels having no lumen.

The positive results in both cell-based assays and in vivo angiogenesis assays, coupled with the improvements seen in pharmacokinetic parameters, have led to the testing of selected compounds in mouse tumor models, supported by pharmacokinetic studies. It is anticipated that these ongoing studies will help in the selection of new HS mimetic clinical candidate(s) for cancer.

**CONCLUSIONS**

The HS mimetic angiogenesis inhibitor PI-88 has demonstrated a good safety and tolerability profile and clinical benefit to patients with various cancers. It is set to commence phase III trials as an adjuvant therapy in postresection hepatocellular carcinoma patients. A new series of HS mimetic PI-88 analogs has been synthesized with improved chemical and biological properties. The new compounds consist of single, defined oligosaccharides with specific modifications designed to improve their pharmacokinetic properties. Some compounds in this series are significantly more potent than PI-88 in cell-based assays. Moreover, representative compounds have demonstrated potent antiangiogenic activity in vivo and an improved pharmacokinetic profile. The current data support the additional development of this series of HS mimetics as potent antiangiogenic anticancer agents.

**ACKNOWLEDGMENTS**

We thank Dr. Ralf Brandt (\textit{vivo}Pharm Pty Ltd, Adelaide) for the AngioChamber and AngioSponge assays.

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Q11

Q12

Q13
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<th>Price</th>
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Subtotal:

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