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Original Report

Anticoagulant and Antiprotease Profiles of a Novel Natural Heparinomimetic Mannopentaose Phosphate Sulfate (PI-88)

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Summary: Heparinomimetic mannopentaose phosphate sulfate (PI-88) (Progen Industries Ltd. Brisbane, Australia), currently developed as an anticoagulant and antiproliferative agent, mainly is composed of a pentomannan. However, tetrasaccharide and disaccharide components are also present. The molecular profile and the anticoagulant potency of PI-88 are investigated in this study. Gel permeation chromatography and polyacrylamide gel electrophoresis analyses were carried out to determine the molecular profile and separation of components of PI-88, respectively. Potentiation of antithrombin III (ATIII) and heparin cofactor-II (HC-II) activity were measured using chromogenic substrate assay. In order to determine anticoagulant and antiprotease effects of PI-88, various global anticoagulant tests, such as the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), Heptest® (Haemachem Inc., St. Louis), ecarin clotting time (ECT), activated clotting time (ACT), and thromboelastography (TEG) were used. Anti-Xa and anti-IIa activities also were measured. The effect of PI-88 on the release of tissue factor pathway inhibitor (TFPI) was performed in nonhuman primates who received PI-88 and in endothelial cell culture systems. The relative susceptibility of PI-88 to heparinase I, protamine sulfate (PS), and platelet factor 4 (PF4) also was evaluated. The high-performance liquid chromatography profiles of PI-88 showed that its average molecular weight is approximately 2300 Da. Separation and gradient electrophoretic patterns of PI-88 showed that it is composed of five different fractions. This agent activates HC-II through inhibiting the thrombin generation but not inhibiting ATIII. Although PI-88 produced a concentration-dependent prolongation of all of the clotting tests, ECT gave the best correlation in the dose-response curve (ECT, $r^2 = 0.94$; TT, $r^2 = 0.84$; APTT, $r^2 = 0.69$). Heparinomimetic mannopentaose phosphate sulfate (PI-88) exhibited marked inhibition of FIIa, but not of FXa. Heparinase I failed to produce significant neutralization of PI-88 in all the assays used, whereas PS and PF4 partially neutralized the effects of this compound. Heparinomimetic mannopentaose phosphate sulfate (PI-88) produced fivefold increase in the TFPI levels at 15 minutes after intravenous (IV) injection to primates. The incubation of PI-88 in endothelial cell culture system also showed a strong effect on TFPI release. These results suggest that PI-88 exhibited strong antithrombotic and anticoagulant activity in addition to its known antiproliferative properties. Because of the molecular characteristics and the dual nature of the pharmacologic action of PI-88, this agent represents an attractive pharmacologic agent for the control of thrombotic and proliferative disorders. **Key Words:** Anticoagulant agent—Antithrombotic agent—Heparinase digestion—Low-molecular-weight-heparin—Molecular profile—Pentasaccharide—PI-88 (heparinomimetic mannopentaose phosphate sulfate)—Unfractionated heparin.

Unfractionated heparin (UFH) and its derivatives are among the most commonly used pharmaceutical agents in the prophylaxis and treatment of acute venous thrombosis, heart attacks, strokes, and interventional procedures. Unfractionated heparin usually is obtained from mammalian organs like beef lung and porcine, sheep, and beef intestinal mucosa. Low-molecular-weight-heparins (LMWHs), which are obtained from UFH by enzymatic degradation or chemical treatment, have become popular in the clinical setting as alternates to heparin because of their favorable risk benefit ratio and superior pharmacokinetic properties, including their longer half-lives and greater bioavailability (1). Because of the increased use of heparin in the production of LMWHs,

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there is a shortage of the raw materials to produce LMWHs. Substitutes for UFH and LMWHs have long been sought; therefore, some synthetic and biologic products have been developed as alternates to UFH and LMWHs (1).

Petitou et al. (2) have designed and chemically synthesized a complex oligosaccharide that is 10 times more potent in vivo than both standard heparin and LMWH. The original pentasaccharide sequence was prepared from natural heparin via fractionation procedure (3). It also is remarkably devoid of certain detrimental side effects when compared with natural heparin. Most of the biological activities known to be associated with heparin are caused by the binding interactions between the anionic groups of the heparin molecule and the positively charged groups of a large variety of proteins (3). Antithrombin III (ATIII), which is the main physiologic inhibitor of coagulation factors, is one such protein. The interaction between heparin and ATIII is saccharide specific. Furthermore, it has been recognized that a structurally unique sequence of five sugars within the polysaccharide is capable of highly selective and tight binding to the protein ATIII. The action of ATIII is amplified several thousand-fold after binding to heparin (4,5). Currently, synthetic pentasaccharide is in advanced clinical development for the prevention of thromboembolism after orthopedic surgery. The results of a Phase IIb clinical trial (Pentathlon), comparing pentasaccharide to the standard of care, LMWH (enoxaparin), were recently reported (6).

On the other hand, a new agent, which is a natural pentamannose, namely heparinomimetic mannopentaose phosphate sulfate (PI-88), currently is being developed by Progen Industries Ltd. (Brisbane, Australia). This compound is relatively similar to the heparin pentasaccharide except for the degree of sulfation, which is higher in PI-88 (7). The other important feature of PI-88 is its strong ability to activate heparin cofactor-II (HC-II) but not ATIII. In contrast to pentasaccharide, PI-88 exhibited very potent tissue factor pathway inhibitor (TFPI) release effects in vivo. Thus, PI-88 is a mixture of highly sulfated oligosaccharides derived from the sulfation of phosphomannan, which is purified from the high molecular weight core produced by fermentation of the yeast, *Pichia holstii*. The main constituent is a pentamannose; however, small amounts of tetrasaccharide and trace amounts of hexasaccharide also are present (7).

The inhibition of the angiogenesis and tumoral cell proliferation properties of PI-88 was formerly determined (8). The heparanase enzyme plays a major role in the degradation of heparan sulfate side chains of the heparan sulfate proteoglycans in the extracellular matrix and in solubilizing the vascular basement membrane. These degradative activities are thought to be intimately involved in tumor cell migration and invasion (8). Heparinomimetic mannopentaose phosphate sulfate (PI-88) has been shown to be an inhibitor of tumor cell growth, metastases, and angiogenesis (neovascularization) by inhibition of the heparanase enzyme (9).

Besides these properties, PI-88 also has potent anticoagulant and antithrombotic effects. Together with HCII activation and TFPI release, this agent is, therefore, a relatively strong inhibitor of thrombogenesis. This agent appears to be potentially a stronger antithrombotic agent than many of the heparinomimetics such as dermatans and pentosan polysulfate. Keeping these perspectives in mind, the aim of this study was to determine the structural characteristics and the anticoagulant and antithrombotic potency of PI-88, the release of TFPI, and the in vitro neutralization of this agent.

MATERIALS AND METHODS

Materials

Heparinomimetic mannopentaose phosphate sulfate (PI-88), which is composed of sulfated mannopyranosyl units, was prepared by Progen Industries Ltd. (Brisbane, Australia). This compound is a phosphorylated mannose pentasaccharide, which is derived by the partial hydrolysis of phosphomannan from yeast, *Pichia holstii*.

Molecular profile of heparinomimetic mannopentaose phosphate sulfate (PI-88)

The average molecular weight of PI-88 was determined by the gel permeation chromatography (GPC) method (10). The entire run was coordinated via the System Interface Module, Rev. 2.2 (Millipore-Waters, Lexington, MA). This was connected in the series with a Waters 510 HPLC pump, an auto-injector (Waters 712 Wisp, Millipore-Waters, Lexington, MA), an ultraviolet (UV) Tunable Absorbance Detector (Waters 484, Millipore-Waters, Lexington, MA) and a Differential Refractometer (Waters 410, Millipore-Waters, Lexington, MA), respectively. The UV detector was set at an absorbance of 205 nm, whereas the Refractive Index (RI) detection was set at ambient temperature. The mobile phase used was a 0.3 M solution of sodium sulfate (pH 5.0), run at a steady rate of 0.5 mL/min, with the aid of an HPLC pump. All compounds were either dissolved or diluted with the above mobile phase to a final concentration of 10 mg/mL. Twenty μL of this solution was injected into the mobile phase using the auto-injector, separated by size exclusion chromatography columns, and finally passed through the two detectors (UV and RI). A run time of 65 minutes was chosen. The signal and data, thus generated, were compiled digitally into a chromatogram. Nineteen narrow-range calibrators (NRCs) obtained from Sanofi (Toulouse, France), were run under the above standardized conditions. The chromatograms of the unknown samples were processed using the above calibration curve to generate molecular weight profiles

of samples with number average (Mn), weight average (Mw), peak molecular weight (Mp), and polydispersity.

Separation of heparinomimetic mannopentaose phosphate sulfate (PI-88) on BioGel P6 column and polyacrylamide gel electrophoresis

The separation of PI-88 was performed on a fine-grade BioGel P6 column (2.5 × 96 cm) (BioRad, Hercules, CA) using a mobile phase of 0.2 M sodium chloride solution (pH 7.0) with a flow rate of 0.25 mL/min. The entire procedure washes in accordance with the low-pressure gel permeation chromatography (GPC) method. The fractions containing oligosaccharides were pooled and freeze dried. Other details of methodology were similar to that described earlier (11). Briefly, the polyacrylamide gel electrophoresis (PAGE) analysis was performed per the following procedures: the resolving gel buffer and lower chamber buffer contained 0.1 M boric acid, 0.1 M tris-(hydroxymethyl)-methylamine (Tris), 0.01 M disodium ethylenediaminetetraacetic acid (EDTA), pH 0.3. The stacking gel buffers contained 0.1 M boric acid, 0.1 M disodium EDTA, and 0.1 M Tris-hydrochloric acid (HCl), pH 6.3. The upper chamber buffer used was 1.24 M glycine, 0.2 M Tris. The front buffer used contained 100 g acrylamide (12%), 10 g bis, and 15 g sucrose in the final volume of 500 mL water. The stacking gel was 4.75 g acrylamide and 0.25 g Bis, in 100 mL of resolving buffer. The pH was brought to 6.3 by HCl, and, finally, the volume was adjusted to 100 mL with water. To prepare the gel, we mixed 5 mL front buffer, 30 μL 10% ammonium per sulfate (APS), 5 μL TEMED; and for the stacking gel, we took 2 mL stacking gel solution, 60 μL 10% APS, and 2 μL of TEMED. Five μL of samples (2–8 μg) blended with 5 μL of 50% sucrose solution was loaded on each well. The entire electrophoretic run was performed at 180 volts for up to 100 minutes using Bio-Rad Protean II vertical slab gel. The gel system was equipped with a power source and water was cooled with circulating water operating at 4°C. The gel was stained with Alcian Blue dye and destained with millipore water.

Pharmacologic profiles

Inhibition of thrombin generation

Thrombin (FIIa) and factor Xa (FXa) generation inhibition assays were performed according to the method previously described (12). These assays were performed on a fast kinetic centrifugal analyzer (ACL-300, Instrumentation Laboratory, Lexington, MA). Heparinomimetic mannopentaose phosphate sulfate (PI-88) was dissolved in physiological saline to the appropriate concentrations and placed in the sample cup carousel. Reagent position 1 contained 60 μL of the PI-88 solution and 60 μL of fibrinogen-deficient plasma (George King Biomedical, Overland Park, KS), diluted 1:8 in 100 mM Tris-HCl buffer (pH 8.5). Reagent 2 contained 60 μL of PI-88 solution and actin (Dade, Miami, FL) diluted 1:1 with Spectrozyme TH or Spectrozyme FXa (American Diagnostica, Greenwich, CT) for measuring intrinsic IIa or Xa generation. Thromboplastin C (Dade), diluted 1:6 with Spectrozyme TH or Spectrozyme FXa, was used for measuring the inhibition of thrombin and extrinsic generation. Optical density was recorded at 405 nm. The results were expressed as percentage of inhibition of an unsupplemented saline control.

Serpin protease (antithrombin III and heparin cofactor-II) inhibition activity assay

Potentiation of ATIII and HC-II activity was measured using chromogenic substrate assays (13). In these assays, PI-88 was diluted in saline and incubated with purified HC-II (Diagnostica Stago, Gennevierres, France) or ATIII (Kabi, Stockholm, Sweden) and either thrombin or FXa. After 1 min incubation at 37°C, residual enzymatic activity was measured with a chromogenic substrate.

Anticoagulant activity of heparinomimetic mannopentaose phosphate sulfate (PI-88)

Global anticoagulant effect on native human whole blood

Activated clotting time (ACT) and thromboelastography (TEG) assessments were performed to determine the effects of PI-88 on native human whole blood.

Activated Clotting Time (ACT): Hemochron instrument and celite-ACT tubes were obtained from International Technidyne Corporation (Edison, NJ). Blood was drawn up to 2 mL in each set of syringes to obtain a final concentration of 25, 50, 75, and 100 μg/mL, and 0 μg/mL (saline control). Activated clotting times were performed as per the standard procedure, and the results were recorded.

Thromboelastography (TEG): Hellige Thromboelastograph-D Model (Haemoscope, Skokie, IL) was utilized to obtain thromboelastograms. Following a double-syringe technique, blood was drawn up to 1 mL in each of the respective syringes prefilled with the drug to obtain a final desirable concentration (6.25, 12.5, and 25 μg/mL, and 0 μg/mL-saline control). Thromboelastography graphs were obtained, and different TEG parameters such as reaction (r) time, clotting (k) time, total clotting (rk) time, maximal amplitude, and angle were calculated accordingly (14).

Anticoagulant activity in normal pooled human plasma

To determine the in vitro anticoagulant profile of PI-88, PI-88 was supplemented to pooled normal human plasma in a 1:10 dilution and then diluted serially with plasma from 100 μg/mL to 0 μg/mL and assayed immediately using all the tests. Prothrombin time (PT; Dade Thromboplastin C-Plus, Miami, FL); activated partial thromboplastin time (APTT; Platelin L; Organon Teknika Corporation, NC); Heptest® (Haemachem Inc.,

St. Louis, MO); thrombin time (TT; 5 unit; Fibrindex® Thrombin Human, Ortho Diagnostic, Raritan, NJ); and ecarin clotting time (ECT; Ecarin Reagent, American Diagnostica Inc., Greenwich, CT) were used. The PT, APTT, Heptest® TT (5 unit), and ECT tests were performed by using the standard protocols on fibrometer (Becton Dickinson, NY) (15).

Anti-protease activity assays

Plasma anti-FXa (Anti-Xa) and anti-FIIa (Anti-IIa) activities were determined by amidolytic assay (ACL 300 plus, Instrumentation Laboratory, Lexington, MA). The plasma anti-FXa assay was set up using the specific chromogenic substrate for FXa (Spectrozyme® FXa, American Diagnostica Inc., Greenwich, CT) and bovine FXa purchased from (Enzyme Research Lab., South Bend, IN) as reagents. The purified human thrombin was purchased from Ortho Diagnostic Systems, and Spectrozyme® Thrombin (American Diagnostica Inc.) was the chromogenic substrate used in the anti-IIa activity assay system.

In vitro neutralization of the effects of heparinomimetic mannopentaose phosphate sulfate (PI-88)

Heparinase I (EC 4.2.2.7, IBEX Corp., Montreal, Canada), protamine sulfate (PS; Laboratory Choay, Paris, France) and platelet factor 4 (PF4; Diagnostica Stago, Parsippany, NJ) were used for the neutralization study of PI-88. Heparinase I was added to achieve the final concentration of 0.1 U/mL to each concentration of drug. Twenty five μg/mL of PS and 10 μg/mL of PF4 were used for the neutralization study. Heparinomimetic mannopentaose phosphate sulfate (PI-88) was used in a concentration range of 0 to 100 μg/mL.

Measurement of tissue factor pathway inhibitor release via heparinomimetic mannopentaose phosphate sulfate (PI-88)

Both tissue factor pathway inhibitor antigen levels in primate plasma and the supernatant of human umbilical vein endothelial cell (HUVEC) culture were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kit from American Diagnostica Inc. The primate studies were carried out in male (n = 2) and female (n = 2) nonhuman primates (*Macaca mulatta*) (16). In the intravenous (IV) studies, after ketamine immobilization, each primate was administered a dose of 1 mg/kg of PI-88. Blood samples were drawn at baseline, 15, 30, and 60 minutes after the PI-88 administration. In the subcutaneous studies, each primate was given 5 mg/kg of PI-88. Blood samples were collected at baseline, 60, 180, and 300 minutes after the PI-88 administration. In the HUVEC culture, 100, 250, 500, and 1000 μg/mL of the final concentrations of PI-88 were used. After one hour incubation at 37°C, the supernatants of the incubated cultured samples were collected to quantitate the released TFPI levels caused by this agent. Saline served as control.

Data analysis

All data were analyzed by an analysis of variance (one-way ANOVA), a multiple regression analysis, and a student t-test, as appropriate, using the Microsoft 2000 Excel Statistic Program.

RESULTS

Structure and molecular profile of heparinomimetic mannopentaose phosphate sulfate (PI-88)

Figure 1 shows the chemical structure of PI-88, which has the molecular formula C30H35Na18O77PS16, with an average molecular weight of 2300 Da. The R represents the sulfate group (sodium sulfate [SO_3Na] or hydrogen [H]). The HPLC profiles of PI-88, as detected by UV and RI, are shown in Figures 2 and 3, respectively. The molecular weight of PI-88 was found to be 2155 Da by UV response, whereas via RI, it was found to be only 1970 Da.

The separation and the purity of different fractions of PI-88 were performed because this compound is a mixture of various components. Figure 4 shows the separation and gradient electrophoretic pattern of PI-88 fractions, namely PK1, PK2, PK3, PK4, and PK5. This figure also shows the electrophoretic patterns of PI-88 mixture and heparin standards. Clear molecular weight-dependent fractions and their purity, in terms of homogeneity, is evident with various molecular mass fractions.

Pharmacologic profiles

Thrombin generation inhibition

Thrombin generation inhibition via both intrinsic and extrinsic pathways by PI-88 is shown in Figure 5. The

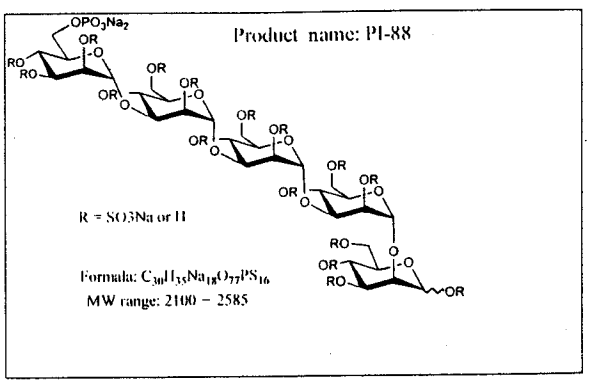
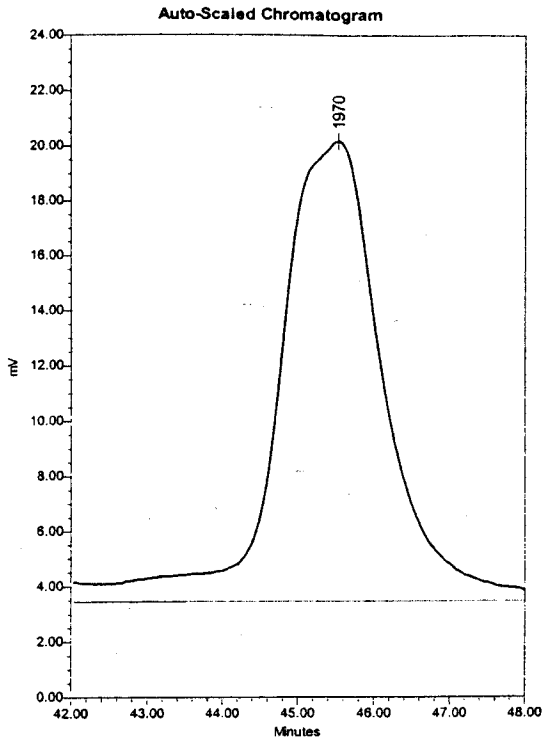


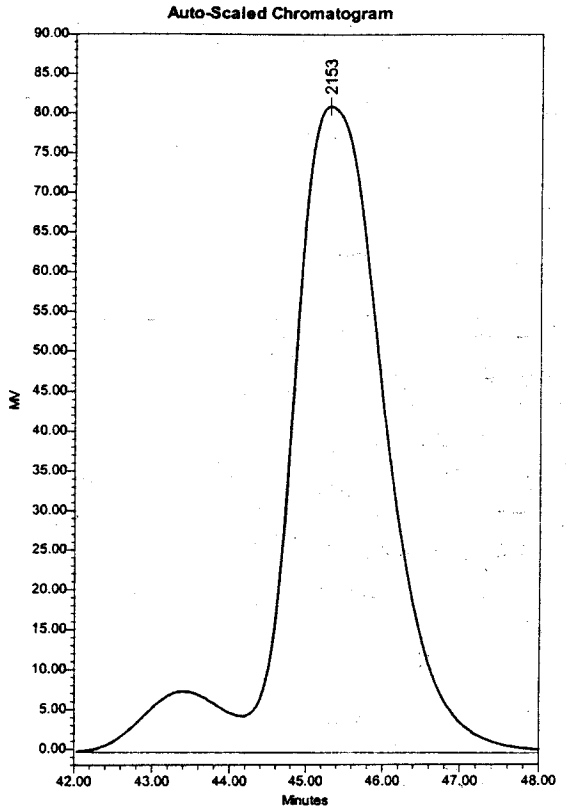
FIG. 1. Chemical structure and molecular weight of PI-88. Heparinomimetic mannopentaose phosphate sulfate (PI-88) is prepared by sulfation of a phosphorylated mannose pentasaccharide (PM5), which is obtained by the partial hydrolysis of the phosphomannan from the yeast *Pichia holstii*.



GPC results

	Mn	Mw	MP	Mz	Mz+1	Polydispersity
1	2011	2160	1970	2370	2678	1.073973

FIG. 2. Gel permeation chromatography (GPC): ultraviolet detector chromatogram of PI-88. The experimental details are described in the Methods section of this article.



GPC results

	Mn	Mw	MP	Mz	Mz+1	Polydispersity
1	2071	2160	2153	2262	2382	1.043071

FIG. 3. Gel permeation chromatography (GPC): refractive index chromatogram of PI-88. The experimental details are described in the Methods section.

mechanism of action of PI-88 on thrombin generation inhibition is based on the inhibition of intrinsic coagulation pathway. The inhibitory concentration at 50% (IC_{50}) values of PI-88 on thrombin generation inhibition, based on these experiments, are shown in Figure 6. It was speculated that by blocking the activity of coagulation factors that play a role in this pathway, PI-88, at a concentration of greater than 6.25 μg/mL, resulted in the inhibition of thrombin generation.

Serpin protease inhibition activity assays

The results of ATIII and HC-II–mediated thrombin generation inhibition assays showed that the antithrombotic activity of PI-88 is caused mainly by the HC-II–mediated inhibition of thrombin (Fig. 7). Heparinomimetic mannopentaose phosphate sulfate (PI-88) did not show any interaction with ATIII. Figure 6 shows that PI-88 did not result in ATIII–mediated FXa and FIIa generation. Figure 8 shows the IC_{50} values (μg/mL) of PI-88 regarding the HC-II and ATIII-mediated inhibition of thrombin by PI-88. A concentration-dependent response of PI-88 clearly is evident.

Anticoagulant activity of heparinomimetic mannopentaose phosphate sulfate (PI-88)

Global anticoagulant effect on native human whole blood

In native whole blood, PI-88 produced potent anticoagulant responses in the ACT. At 25, 50, 75, and 100 μg/mL, there were statistically significant differences between different concentrations and saline control ($P < 0.001$). At 50 to 100 μg/mL, PI-88 produced levels of anticoagulation, which are seen during cardiovascular surgical procedures (Fig. 9).

Analysis of the TEG parameters indicated that all TEG parameters (r time, k time, rk time, maximum amplitude, and angle) were concentration-dependently increased (Table 1). The whole blood was not coagulable at concentrations greater than 25 μg/mL.

Anticoagulant activity in pooled normal human plasma

In human plasma, PI-88 produced concentration-dependent anticoagulant effects in PT, APTT, Heptest®,

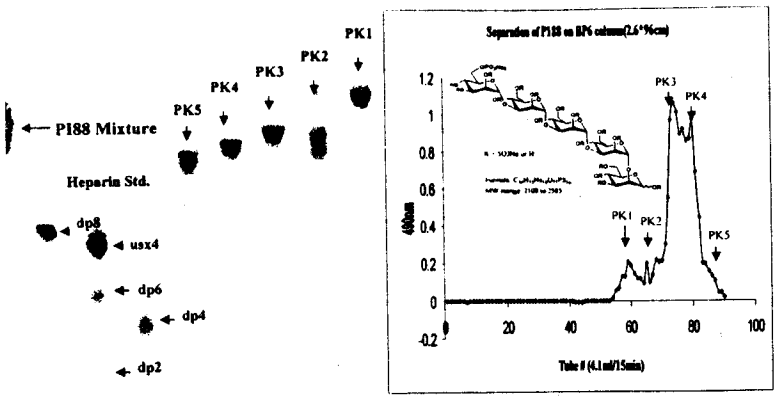


FIG. 4. The separation and purity determination of various fractions of PI-88 by polyacrylamide gel electrophoresis (PAGE) analysis. Details of the experimental conditions are described in the Methods section.

TT (5U), and ECT (Fig. 10). Heparinomimetic mannopentaose phosphate sulfate (PI-88) supplemented to pooled normal human plasma has a very weak effect on the prothrombin time. A 1.5-fold increase in the APTT was noted with 3.1 μg/mL of PI-88. This effect on the APTT was comparable with the effects observed in LMWH, such as enoxaparin. Thrombin time results were markedly more potent with the PI-88. The ecarin clotting time assay gave a good correlation between the clotting time and the concentration of PI-88 ($r^2 = 0.94$). When these three assays were compared, ECT produced the strongest effect on prolongation of clotting time (ECT, $r^2 = 0.94$; TT, $r^2 = 0.84$; APTT, $r^2 = 0.69$). These results show that the monitoring of levels of PI-88 in human plasma can be done via ECT. Heptest®, which is a clot-based assay, can measure both anti-Xa and anti-IIa activities of the compounds, whereas at the lower concentrations (< 2 μg/mL), this test reflects predominantly the anti-Xa activity. Heparinomimetic mannopentaose phosphate sulfate (PI-88) produces a mild effect when tested via Heptest®, especially at the high concentrations (Fig. 10).

The levels of anti-Xa and anti-IIa activity usually are measured to determine the effect of UFH. However, to measure the effect of LMWHs, anti-Xa activity is determined. In contrast to other LMWHs, PI-88 did not show any anti-Xa activity. However, it produced a concentration-dependent anti-IIa activity (Fig. 11). This anti-IIa activity is mainly due to HC-II mediated thrombin inhibition (Fig. 7).

In vitro neutralization of the effects of PI-88

Heparinase I, which is obtained from *flavobacterium heparinum*, failed to produce significant neutralization of PI-88 in all assays (PT, APTT, TT, Heptest®, and ECT; $P > 0.5$). Protamine sulfate is a traditional agent used to reverse the anticoagulant effect of UFH. Platelet factor 4 is an endogenous chemokine (a highly electropositively charged tetrameric protein) that is able to neutralize the effects of heparin. However, neither PS nor PF4 completely neutralized the effects of PI-88. Both of them produced partial assay-dependent neutralization.

Measurement of tissue factor pathway inhibitor release by heparinomimetic mannopentaose phosphate sulfate (PI-88)

Figure 12 shows the effect of IV administration of 1 mg/kg of PI-88 to monkeys ($n = 4$) on the TFPI levels. Heparinomimetic mannopentaose phosphate sulfate (PI-

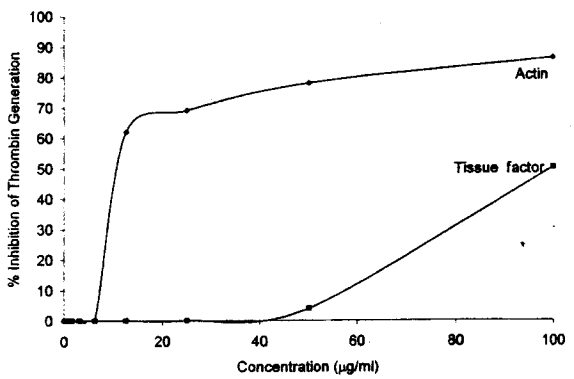


FIG. 5. The mechanism of action of PI-88 on thrombin generation inhibition is based on the inhibition of the intrinsic coagulation pathway.

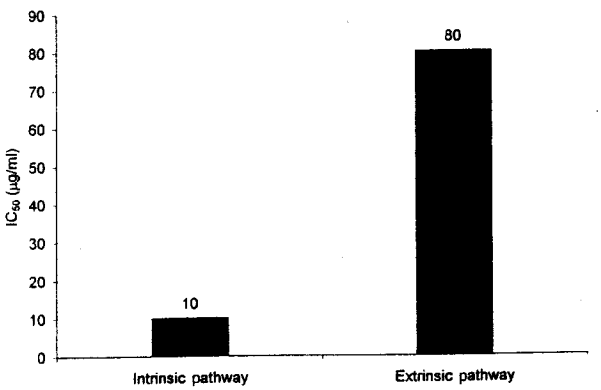


FIG. 6. IC_{50} values of PI-88 on thrombin generation inhibition. The IC_{50} value shows the concentration of PI-88, which inhibits the thrombin generation by 50%.

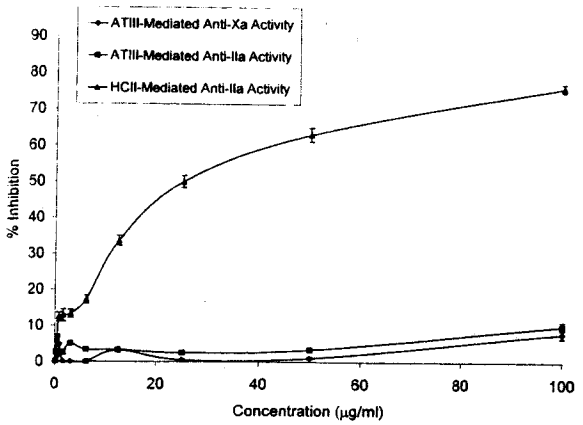


FIG. 7. Serine protease inhibition activity of PI-88. The antithrombotic activity of PI-88 mainly is due to the HC-II–mediated inhibition of thrombin. Heparinomimetic mannopentaose phosphate (PI-88) did not show any interaction with ATIII.

88) produced a significant increase (> fivefold) in the TFPI levels as early as 15 minutes after administration. This TFPI release effect is comparable with the effects of heparin and LMWHs (17). Because of the nonavailability of a TFPI standard for the primates, the results of this study are expressed in terms of number of fold increase over the baseline. Figure 13 shows the effect of subcutaneous (SC) administration of 5 mg/kg of PI-88 to monkeys ($n = 4$). This SC administration resulted in a maximum release of TFPI at 1 hour; however, at 5 hour, TFPI levels remained higher than the baseline (two–fivefold increase).

Tissue factor pathway inhibitor release via PI-88 in HUVEC culture was also performed in this study. It is interesting to note that the TFPI level was increased in a concentration-dependent manner by PI-88 in this culture system (Fig. 14). These in vivo and ex vivo studies clearly suggest that like other glycosaminoglycans, PI-88 is also capable of releasing endogenous TFPI levels.

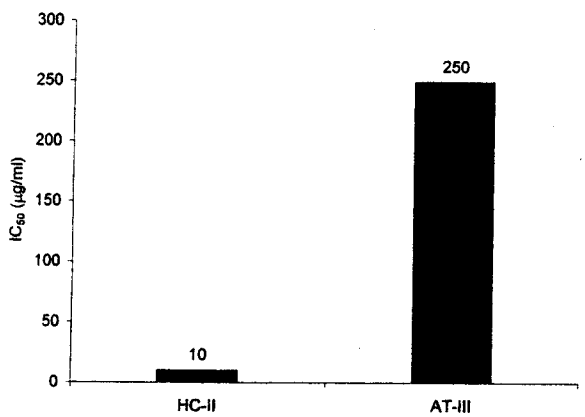


FIG. 8. IC_{50} values of PI-88 on HC-II and ATIII-mediated inhibition of thrombin.

DISCUSSION

Specific antithrombotic drugs represent a group of heterogeneous agents, which produce their pharmacologic effects by inhibiting thrombin directly or through an endogenous mediator known as HC-II. Heparin cofactor-II is a 480 amino acid glycoprotein in plasma that inhibits thrombin and chymotrypsin and is a member of the serpin family of serine protease inhibitors (18). Heparin and synthetic pentasaccharide are capable of interacting with ATIII, which is a major member of the serpin family of serine protease inhibitors, to produce both the anti-Xa and anti-IIa effects. Hence, these drugs are relatively specific for producing antithrombotic effects. In contrast, the other group of agents represents a wide variety of synthetic, semisynthetic, natural, and biotechnology-produced sulfated compounds, which interact with endogenous HC-II and mediate its effects to inhibit thrombin (1). The natural HC-II–mediating drugs include dermatan sulfate, polysynthetic sulfone, and synthetic sulfated oligosaccharides. Through biotechnology, sulfated polysaccharides can be produced to mediate their effects through HC-II. These results indicated that the antithrombotic potency of PI-88 is mainly caused by the activation of HC-II.

From the current experimental considerations, sulfated oligosaccharides simultaneously inhibited the in vitro angiogenesis and heparanase activity. It is speculated that the sulfated oligosaccharides inhibit angiogenesis by interfering with the formation of a ternary complex between cell-surface heparan sulfates and receptors for heparan sulfate-binding angiogenic factors such as β fibroblast growth factor (βFGF) and vascular endothelial growth factor (19). Heparin-derived oligosaccharides between 6 and 10 monosaccharide units in length inhibit βFGF action, whereas longer oligosaccharides and intact heparin either are inactive, or, in some cases, potentiate the activity of heparan sulfate binding growth factors

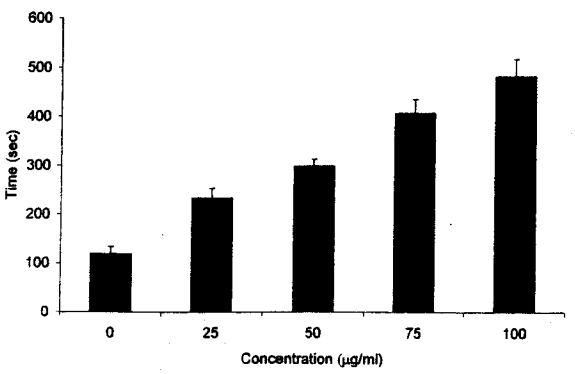


FIG. 9. The anticoagulant effect of PI-88 on activated clotting time. In native whole blood, PI-88 produced potent anticoagulant responses in ACT. At 25, 50, 75, and 100 µg/mL, there is a statistically significant difference between different concentrations and saline control ($P < 0.001$).

TABLE 1. *The effects of PI-88 on thromboelastographic parameters*

Drug	Final concentration, μg/mL	R time, mm	K time, mm	Rk time, mm	Maximum amplitude, mm	Angle, °
PI-88	25	132 ± 41.5	55 ± 12.5	165 ± 35.4	18 ± 6	8 ± 5
PI-88	12.5	93.4 ± 71	34.2 ± 13.2	127.6 ± 75.3	46.8 ± 15	17.4 ± 8.6
PI-88	6.2	37 ± 16.9	16.3 ± 6.1	53.3 ± 21.9	61.6 ± 8.4	37.3 ± 11.7
Saline	Control	28 ± 5.6	12 ± 5.4	40 ± 10.2	56.8 ± 6.4	42.5 ± 12.8

PI-88, heparinomimetic mannopentoase phosphate sulfate; R, reaction; K, clotting; Rk, total clotting.

(9,20). More extensive studies with PI-88 revealed that PI-88 could reduce tumor vascularity and support the in vitro angiogenesis-inhibition data obtained with this agent (9). It is speculated that PI-88 can interfere with the VEGF-heparin interaction and FGF-heparan sulfate binding.

Because of this dual nature of the pharmacological action of PI-88, it represents an attractive pharmacological agent for the control of thrombotic and proliferative disorders. This agent is currently under several clinical trials for the management of proliferative disorders. Based on the data of global anticoagulant effects, anticoagulant effects in normal human plasma and antiprotease activity of PI-88 indicated that it could be a potent anticoagulant agent. The potency of PI-88 is comparable with the potency of enoxaparin. In addition, when the effects of PI-88 are compared with other HC-II–mediated drugs like dermatan sulfate and aprosulate, PI-88 is stronger than these compounds when tested via a global coagulation test such as APTT (Fig. 15). Synthetic pentasaccharide, unlike PI-88, mostly exhibits ATIII-mediated anti-Xa activity. However, PI-88 does not have anti-Xa activity, and its antithrombin activity is caused mainly by HC-II activation.

As known, TFPI is characterized as a kunitz-type protease inhibitor that inhibits both FVIIa-TF complex and FXa (21). The TFPI is an important molecule to prevent arterial and venous thrombosis. Drugs like heparin and LMWHs cause an increase in plasma TFPI levels (22). In addition, heparin and related glycosaminoglycans are capable of binding to TFPI, which promotes a synergistic anticoagulant action (23). Heparinomimetic mannopentaose phosphate sulfate (PI-88) markedly results in the release of TFPI in both nonhuman primates and in vitro cell culture systems. These results indicate that TFPI may contribute significantly to the antithrombotic actions of PI-88, besides the HC-II–mediated inhibition of thrombin. It is reported that synthetic pentasaccharide did not induce the release of TFPI (5). It is interesting to note that the number of sulfate groups per saccharide unit in PI-88 is markedly higher than pentasaccharide (3.1 vs 1.8, respectively), and, thus, the number of sulfate groups may be responsible for the observed effect of TFPI release in this study.

In vitro neutralization studies of PI-88 were performed via PS, heparinase I, and PF4. Protamine sulfate is a traditional agent used to reverse the anticoagulant effect of UFH. However, in this study, PS did not completely reverse the effects of PI-88, as did the UFHs. Heparinase is an eliminase isolated from *Flavobacterium heparinum*, and cleaves heparin at α-glycosidic linkages in its major repeating units (14). Preliminary studies suggest that

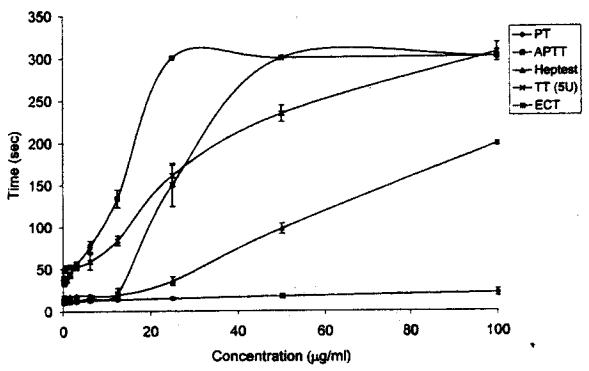


FIG. 10. Anticoagulant effect of PI-88 on normal pooled human plasma as measured by various global clotting tests of coagulation. In human plasma, PI-88 produced concentration-dependent anticoagulant effects in PT, APTT, Heptest, TT (5 unit), and ECT. When these three assays were compared, ECT produced the strongest effect on prolongation of clotting time (ECT, $r^2 = 0.94$; TT, $r^2 = 0.84$; APTT, $r^2 = 0.69$).

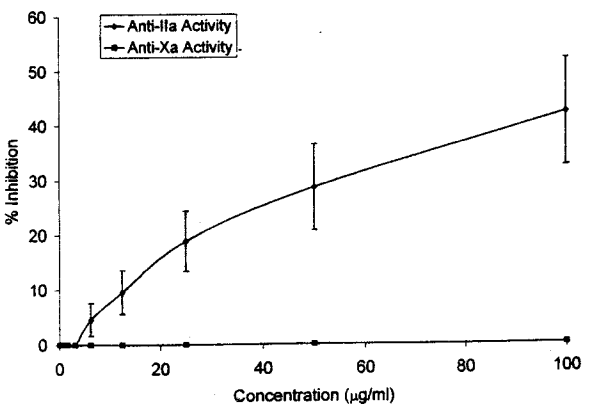


FIG. 11. Anti-Xa and Anti-IIa activities of PI-88 as measured by amydolytic assay. Heparinomimetic mannopentaose phosphate sulfate (PI-88) did not show any anti-Xa activity. However, it produced a concentration-dependent anti-IIa activity. This anti-IIa activity mainly is due to the HC-II mediated thrombin inhibition.

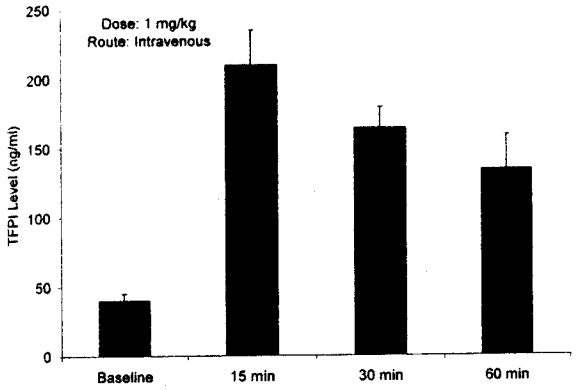


FIG. 12. Effect of intravenously administered of PI-88 on the TFPI release in nonhuman primates. Heparinomimetic mannopentaose phosphate sulfate (PI-88) produced a significant increase (> fivefold) in the TFPI levels as early as 15 minutes after administration.

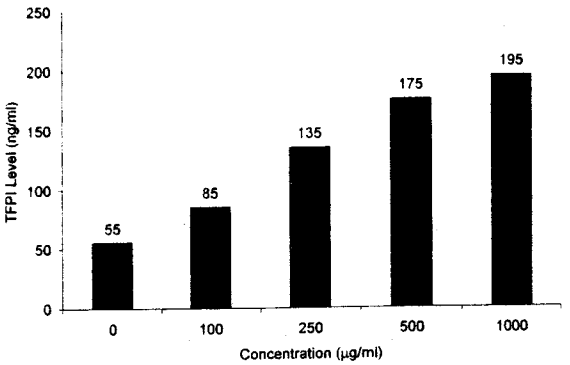


FIG. 14. Effect of PI-88 on the release of the TFPI in HUVEC culture. The TFPI level was increased in a concentration-dependent manner by PI-88 in this culture system.

heparinase may be equally effective as protamine sulfate for reversing the anticoagulant effects of heparin, and may not cause significant hemodynamic changes. Heparinase also partially neutralized the effects of UFH, LMWHs, and some heparinomimetics (20). However, PI-88 was resistant to degradation with heparinase I. Platelet factor 4 treatment produced partial assay dependent neutralization. Thus, a pharmacological antagonist of PI-88 may still be needed.

In summary, this study reports that PI-88 is capable of producing potent anticoagulant responses that are comparable with heparins at adjusted dosages. Heparinomimetic mannopentaose phosphate sulfate (PI-88) produced strong anticoagulant effects, which are comparable in the APTT assay with enoxaparin. However, it produced stronger antithrombotic effects in the thrombin clotting time assays, which are independent of ATIII. This compound is a specific ligand for HC-II. It did not interact with ATIII, and did not produce anti-Xa effect. Therefore, it is a selective inhibitor of FIIa. In addition, PI-88 therefore can be used in patients who are deficient in ATIII. Heparinomimetic mannopentaose phosphate sulfate (PI-88) may resist digestion by heparinase, but PS and PF4 partially digested the effects of PI-88. However, a pharmacological antagonist of PI-88 still may be needed. Finally, PI-88 resulted in a strong release of TFPI both in nonhuman primates and in the HUVEC culture. The duration of the released TFPI effect was greater than 60 minutes. In the subcutaneous studies, a pronounced increase in the TFPI release also was noted.

CONCLUSIONS

Based on the above properties, it is concluded that PI-88 exhibits a predictable antithrombotic effect and measurable pharmacodynamic modulation of the hemostatic system in animal models. The mechanism of action of this agent is unique and distinguishes itself from other drugs. Although PI-88 is prepared by fermentation, it can be compared with chemically synthesized pentasaccha-

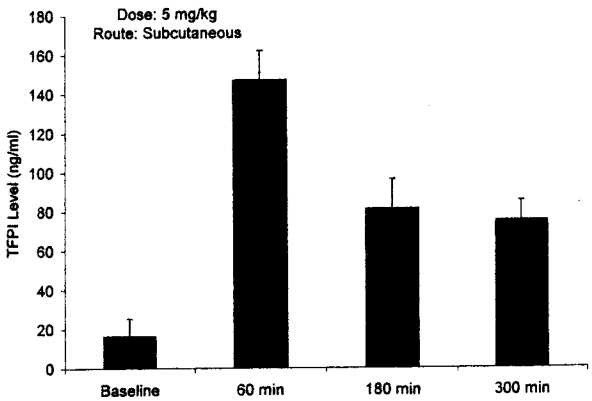


FIG. 13. Effect of subcutaneously administered of PI-88 on the TFPI release in nonhuman primates. This subcutaneous administration resulted in a maximum release of TFPI at 1 hour after administration; however, at 5 hours after administration, TFPI levels remained higher than the baseline (two–fivefold increase).

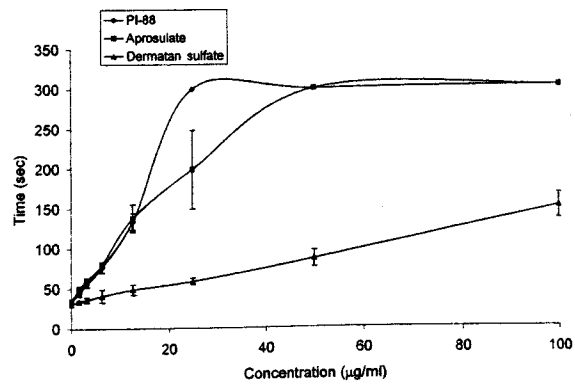


FIG. 15. Comparative effects of PI-88 with other HC-II–mediating drugs such as dermatan sulfate and aprosulate on APTT. Heparinomimetic mannopentaose phosphate sulfate (PI-88) exhibited stronger effects on APTT than did dermatan sulfate.

ride, which is a ligand for ATIII and is a sole FXa inhibitor. On the other hand, PI-88 is a ligand for HC-II but it also releases enormous amounts of TFPI. Together, these actions may be very potent in mediating antithrombotic effects. Additional pharmacological effects of PI-88 at cellular level combined with the observed antithrombotic effects warrant its development, to determine its clinical potential.

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