Effects of a Heparin-Binding Protein on Blood Coagulation and Platelet Function

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ABSTRACT

The objective of this study was to characterize the heparin-binding properties of a protein secreted by mouse myeloma cells. The characterization was performed using clinical assays, such as heparin activity assays and heparin-induced thrombocytopenia (HIT) platelet activation assays. The tests were performed in the presence of heparin, low-molecular-weight heparins (LMWH), or heparinoids and either heparin-binding protein (HBP) or saline to determine whether the HBP affects the activity of heparins. The characterization of the HBP using heparin activity assays showed that the HBP shortened the prolonged clotting times of the activated partial thromboplastin time (aPTT) and thrombin clotting time induced by high concentrations of unfractionated heparin. The chromogenic assays for antithrombin (AT), thrombin inhibition, and factor Xa inhibition demonstrated that this effect is related to heparin concentrations below 0.5 IU/ml. The Heptest assay did not detect these differences. The HBP did not modify the anticoagulant effect of any LMWH or low- or high-sulfated glycosaminoglycans in the aPTT assay. Activation of donor platelets in the presence of unfractionated heparin, platelet factor 4 (PF4), and HIT-serum was not counteracted by the HBP in any of the assays. The characterization of the HBP using a PF4–enzyme-linked immunosorbent assay (ELISA) confirmed the lack of structural identity with PF4. However, the optical density data indicated that the protein structure may be similar to PF4 by binding to a PF4 antibody. These data suggest that the HBP isolated from mouse myeloma cells has a low affinity to heparin and interacts with the secondary binding site to AT and also perhaps to PF4.

KEYWORDS: Heparin-binding protein, heparin, low-molecular-weight heparin (LMWH), heparin-reduced thrombocytopenia (HIT), platelet factor 4

Objectives: Upon completion of this article, the reader should be able to (1) summarize the present knowledge of antibodies to heparins and (2) list some of the properties of the heparin-binding protein obtained from mouse myeloma cells.

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The high biocompatibility and low antigenic potency of heparins is reflected by the lack of heparin antibodies despite their widespread occurrence in tissues and during heparinization of patients. Consequently, only a few reports exist on the detection of antiheparin activity in patients with dysproteinemia and malignant disease. Therefore, only a few reports on the successful production of monoclonal antibodies against heparins and other glycosaminoglycans have been published.

Antibodies have been raised against heparan sulfate,1-2 chondroitin sulfate,3 and keratin sulfate.4-5 The production of heparin antibodies has been reported.6-9 However, further analysis has shown that the antibodies were not directed against native heparin but against heparin conjugates or chemically modified heparins. The reason for the low antigenicity of glycosaminoglycans may be suggested by the nonprotein structure of these compounds.

Pejler et al6 reported on monoclonal antibodies specific for oligosaccharides prepared by partial nitrous acid deamination of heparin. The resulting antibody did not recognize intact heparin but did recognize heparin fragments containing the anhydromannitol residue. Gitel et al7 described polyclonal heparin antibodies produced in rabbits that recognized only heparin covalently adsorbed to a methylated polymer of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and did not react with free heparin. Strauss et al8 reported on a monoclonal antibody of the immunoglobulin M (IgM) class. The antibody was directed against heparin but showed increased sensitivity when heparin was linked noncovalently to surfaces precoated with polylysine compared with free heparin in solution.

A monoclonal HBP secreted by mouse myeloma cells has been characterised by Huhle et al.9 This HBP detected unfractionated heparin and LMWH, in contrast to the antibodies described to date in the literature that detect only modified or surface-attached heparin molecules. The smallest disaccharide unit able to bind to the HBP was the iduronic acid–anhydromannitol. The end point attachment of tyramine to the anhydromannose did not influence the HBP binding, indicating the importance of the iduronic acid for binding to glycosaminoglycans to the HBP.

Formerly, the HBP was referred to as the first monoclonal antibody to heparin. Analyses and applications of the HBP by ELISA methods demonstrated that this compound was not a native antibody and that it could not be detected by any anti-immunoglobulin antibody routinely used for antibody purification. In addition, other routinely used antibody purification techniques failed concerning this substance. Further investigations showed that the compound binding to heparin is not secreted by the hybridoma cell line H1.18, as assumed earlier, but by the mouse myeloma cell line Ag8.

Characterization of HBP Using Heparin Activity Assays

All tests were performed using the HBP, partially purified and albumin free, at a protein concentration of 1 mg/mL. The HBP was coupled with unfractionated heparin (Elkins Sinn, Cherry Hill, New Jersey). These complexes were prepared by diluting heparin 1:10 in HBP to obtain final concentrations of 10, 5, 2.5, 1.25 IU/mL heparin to HBP and were incubated overnight. Test plasma was prepared by diluting the coupled HBP 1:10 in pooled normal human plasma (n = 6 donors).

HEPTEST

The Heptest® (Haemachem, St. Louis, Missouri) assay measures factor Xa and to some extent factor IIa inhibition. Purified bovine factor Xa and test plasma (HBP coupled to heparin and supplemented to human plasma) were incubated for 2 minutes at 37°C. Clotting time was determined on a Fibrometer® (BBL, Cock-
eysville, Maryland) after the addition of 100 μL of prewarmed Recalmix. Clotting times beyond 300 seconds were outside the linear range of the instrument.

**THROMBIN CLOTTING TIME**

The 5-unit thrombin clotting time (TCT) measures the time it takes to convert fibrinogen to fibrin by preformed thrombin. Two hundred microliters of test plasma were equilibrated to 37°C for 3 minutes. The clotting time was measured using a Fibrometer after addition of 100 μL prewarmed Fibrindex® human thrombin (Ortho Diagnostics, Raritan, New Jersey). Clotting times beyond 300 seconds were outside the linear range of the instrument.

**ACTIVATED PARTIAL THROMBOPLASTIN TIME**

The aPTT is a global clotting assay that measures the activity of the coagulation factors in the intrinsic pathway and is commonly used to monitor heparin therapy. One hundred microliters of aPTT reagent (Organon Teknika, Durham, North Carolina) were added to 100 μL of plasma and incubated for 5 minutes at 37°C. Clotting time was measured using a Fibrometer after the addition of 100 μL prewarmed 0.025 M calcium chloride. Clotting times beyond 300 seconds were outside the linear range of the instrument.

**ANTITHROMBIN ASSAY**

The ability of an agent to mediate antiprotease activity via AT was measured using an amidolytic substrate assay run on the ACL-300+ kinetic analyzer (Instrumentation Laboratory [IL], Lexington, Massachusetts). Test plasmas were diluted 1:10 in saline and placed in sample cups. The first reagent consisted of 1.25 IU/ml thrombin in Tris buffer (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH = 8.4 at 25°C). The final concentration of thrombin was 0.0625 IU/mL. The second reagent was 0.5 nM Spectrozyme TH (American Diagnostic, Greenwich, Connecticut). The instrument was programmed such that 100 μL of plasma and 100 μL of reagent 1 were placed into individual compartments in a reaction rotor. These reagents were incubated at 37°C for 1 minute before being mixed together. Then, 50 μL of reagent 2 were added to the reaction rotor, which was spun at 1200 rpm to mix all reagents. Optical density readings at 405 nm for all reactions were made for 60 seconds.

**ANTI–FACTOR XA ASSAY**

Inhibition of factor Xa was measured using an amidolytic assay run on the ACL-300. Anticoagulant present in plasma inhibits factor Xa. Residual factor Xa cleaves a chromogenic substrate to release pNA. The reaction is monitored by measuring the change in the amount of free chromophore pNA detected by the change in optical density at 405 nm. Three hundred seventy-five microliters of buffer (50 nm Tris, 175 nM NaCl, 7.5 nM ethylenediaminetetraacetic acid [EDTA], pH = 8.4 at 25°C) were incubated with 25 μL of test plasma for 2 minutes at 37°C. The instrument was programmed such that 50 μL of Spectrozyme FXa (2.5 nM) (American Diagnostica) were added, and the change in optical density at 405 nm was measured for 1 minute.

**ANTI–FACTOR IIASSAY**

Inhibition of factor IIa was measured using an amidolytic assay run on the ACL-300. Anticoagulant present in plasma inhibits factor IIa. Residual factor IIa cleaves a chromogenic substrate to release pNA. The reaction is monitored by measuring the change in the amount of free chromophore pNA detected by the change in optical density at 405 nm. Four hundred microliters of buffer (50 nm Tris, 175 nM NaCl, 7.5 nM EDTA, pH = 8.4 at 25°C) and 25 μL of plasma were prewarmed for 1 minute at 37°C. Then 25 μL of 10 IU/ml thrombin (Ortho) were added. After a 1-minute incubation, 50 μL of 1-mM Spectrozyme TH (American Diagnostica) were added, and the change in optical density at 405 nm was measured for 1 minute.

**APTT FOR ANALYSIS OF DIFFERENT ANTICOAGULANTS WITH HPB**

The aPTT reagent was used as described previously to test the following substances for binding to the HBP. This reagent was chosen because it had shown a relevant effect of HBP on heparin activity (see later) and because at adequate high concentrations of the compounds mentioned later, an interaction with the HBP was assumed. The LMWHs ardeparin (Wyeth, Cincinnati, Ohio), dalteparin (Pharmacia, Kalamazoo, Michigan), enoxaparin (Rhone-Poulenc Rorer, Bridgewater, New Jersey), and nadroparin (Sanoﬁ-Synthelabo, Paris, France); the polysulfated apicosolate (Luitpold, Munich, Germany); a hypersulfated LMWH (Recordati, Milan, Italy); and the heparinoids ateroid (Crinos, Milan, Italy) and danaparoid (Organon, Oss, The Netherlands) were tested. The substances were used in final concentrations equivalent to the activity of heparin at 1.0, 0.5, 0.25, 0.125, and 0 IU/mL. They were diluted in either saline or 1 mg/mL HBP. The complexes were incubated overnight. The time to clot of the aPTT by the agents incubated with HBP or saline was compared.

**Characterization of the HBP Using HIT Platelet Activation Assays**

Blood from healthy individuals was drawn using a double-syringe technique. The assays were performed with serum from patients diagnosed with HIT as the source of antieparin and PF4 antibodies. These antibodies form complexes with heparin and activate platelets. Heparin was diluted in saline or 1 mg/mL HBP. ___ Is ___ Le ___ Il
FLOW CYTOMETRY ASSAY

One hundred sixty microliters HIT serum; 50 μL heparin in saline or HBP at concentrations of 0, 1, 5, and 1000 IU/ml; and 290 μL nonanticoagulated whole blood were incubated for 10 minutes at 37°C, with stirring. Immediately after blood collection, 50 μL of whole blood were fixed in 1 mL of fixing solution (Sigma) for the nonactivated control. The fixing solution consisted of 2% paraformaldehyde diluted 1:2 in Tyrode’s buffer. For each test, 50 μL of whole blood was fixed after a 10-minute reaction time. After a 30-minute fixation time at 4°C, the samples were centrifuged at 320g for 10 minutes. The supernatant was removed carefully to avoid disturbing the cells. After the addition of 400 μL Tyrode’s buffer, 100 μL of the diluted cells were placed into test tubes containing 100 μL each of CD61-FITC and CD62-PE antibodies (Becton Dickinson, San Jose, California) diluted 1:30 in Tyrode’s buffer. The samples were incubated for 30 minutes at room temperature in the dark and then diluted with 400 μL Tyrode’s buffer and analyzed in the flow cytometer (Becton Dickinson). Parameters for platelet activation, platelet aggregation, and expression of P-selectin of the total platelets were tabulated in percent.

AGGREGOMETRY

To obtain platelet-rich plasma (PRP), 30 ml citrated whole blood was collected and centrifuged at 160g for 15 minutes. The PRP was removed. The remaining blood was centrifuged at 600g for 10 minutes to obtain platelet-poor plasma (PPP). To calibrate the aggregometer baseline, 500 μL of PPP were placed in a cuvette. Three hundred microliters of HIT serum or normal serum (NHS) and 200 μL of PRP were incubated for 3 minutes at 37°C. Fifty microliters of 0, 1, 5, and 100 IU/ml heparin in saline or HBP were added, and the aggregation response recorded for 20 minutes.

14C-SEROTONIN RELEASE ASSAY

The serotonin release assay (SRA) tests for the presence of HIT antibodies by incubating patient serum and heparin with radiolabeled donor platelets. Platelet activation caused by the antiheparin-PF4 antibody was measured by quantitating 14C-serotonin released from the platelets. Radiolabeling of platelets was performed as follows. Acid-citrate-dextrose (ACD) anticoagulated blood was centrifuged at 160g for 15 minutes to obtain PRP; the ACD-PRP was incubated with 0.1 μCi/ml 14C-serotonin for 40 minutes and then centrifuged at 400g for 10 minutes. The supernatant was discarded, and the sediment with the platelets was washed in 10 ml of calcium and albumin-free Tyrode’s (AFT) buffer containing 0.15 mg of apyrase. The platelet count was adjusted to 300 ± 50 × 109/L. The assay was performed in the following manner: 30 μL of AFT buffer and 70 μL of labeled platelets were placed in the appropriate microtiter well as the background sample. Previously tested HIT-positive serum was run as a positive control. Added to the appropriate microtiter well were 10 μL of 0, 1, and 1000 IU/ml heparin in saline or in HBP; 20 μL of the positive control; and 70 μL of the labeled platelets. Final heparin concentrations were 1:10 of the preceding concentrations. The microtiter plate was covered and placed on a shaker at 120g for 60 minutes at room temperature. To stop the reaction, 100 μL of 4.0% EDTA were added. The contents of each well were transferred to a plastic vial and centrifuged at 1500g for 2.5 minutes to pellet the platelets. The supernatant was then fixed after a 10-minute reaction time. After a 30-minute fixation time at 4°C, the samples were centrifuged at 320g for 10 minutes. The supernatant was removed carefully to avoid disturbing the cells. After the addition of 400 μL Tyrode’s buffer, 100 μL of the diluted cells were placed into test tubes containing 100 μL each of CD61-FITC and CD62-PE antibodies (Becton Dickinson, San Jose, California) diluted 1:30 in Tyrode’s buffer. The samples were incubated for 30 minutes at room temperature in the dark and then diluted with 400 μL Tyrode’s buffer and analyzed in the flow cytometer (Becton Dickinson). Parameters for platelet activation, platelet aggregation, and expression of P-selectin of the total platelets were tabulated in percent.

RESULTS

Inhibition of factors Xa and IIa by AT is accelerated by heparin. The amount of factor Xa or factor IIa activity neutralized during a specific time is directly proportional to the concentration of heparin in the reaction mixture. Heparin was diluted 1:10 in normal human plasma for a calibration curve, the final concentrations were 1.0, 0.5, 0.25, 0.125, and 0 IU/ml. The Heptest assay determines mainly factor Xa inhibition and to some extent factor IIa inhibition. The heparin concentration detected in the test plasma, which consisted of HBP-heparin complexes, was similar to the heparin concentration detected without HBP in the Heptest assay (Fig. 1). Therefore, the HBP did not affect the factor Xa and factor IIa inhibiting activity of heparin in this assay.
The TCT is prolonged in the presence of heparin because of the inhibition of factor IIa by the heparin-AT complex. The HBP substantially shortened the prolongation of the coagulation times caused by heparin. This indicates binding of long saccharide chains of the heparin molecule by the HBP (Fig. 2). The longer saccharide units of heparin are responsible for the inhibition of thrombin. This agrees with the finding in the Heptest assay, which is prolonged mainly by LMWH and thus is more sensitive toward factor Xa than it is to factor IIa inhibition.

The HBP affected the prolongation of the aPTT by heparin in a similar pattern, as observed in the timed TCT (Fig. 3). The shortening of the aPTT by HBP-heparin complexes compared with that of heparin alone may be somewhat less pronounced compared with the thrombin inhibition assay because aPTT is sensitive to both longer and shorter heparin chains. Therefore, the aPTT assay was chosen for the interaction studies of the HBP and the other glycosaminoglycans.

The chromogenic AT, anti-factor Xa, and anti-factor IIa assays measured the interaction between heparin and the HBP in more detail. At concentrations below 0.5 IU/ml, the AT-mediated antiprotease activity of heparin was inhibited from 30 to 50% by the HBP. However, the results were similar at 1 IU/ml heparin in the presence of either saline or HBP (Figs. 4 to 6). These results indicate that the HBP is not capable of affecting the heparin activity on thrombin and factor Xa if the concentration of heparin is more than 0.5 IU/ml in these assays.

The aPTT reagent was chosen to analyze the interaction of the HBP with the different glycosaminoglycans. Some of the available LMWH compounds, as well as the low-sulfated (danaparoid) and over-sulfated compounds, were studied. The concentrations of the compounds were high to substantially prolong the aPTT in the presence of saline. After incubation with 1 mg/ml HBP, no dose dependent inhibition of the activity in the assay was observed. The single difference found was less than 30% of inhibition, and it occurred at

![Figure 1](image1.png)
**Figure 1** The concentration of heparin (IU/ml) is plotted against the coagulation time (seconds) in the presence of saline (control, open symbols) and 1 mg/mL HBP (closed symbols) using the Heptest assay.

![Figure 2](image2.png)
**Figure 2** The concentration of heparin (IU/mL) is plotted against the coagulation time (seconds) in the presence of saline (control, open symbols) and 1 mg/mL HBP (closed symbols) using the TCT assay.

![Figure 3](image3.png)
**Figure 3** The concentration of heparin (IU/ml) is plotted against the coagulation time (seconds) in the presence of saline (control, open symbols) and 1 mg/mL HBP (closed symbols) using the aPTT assay.
only one and not at the same concentration of the compounds. The activity of the LMWHs did not differ in the presence of saline and HBP up to 1 IU/mL. The degree of sulfation did not seem to play a major role in the interaction with HBP in this assay. Therefore, the HBP did not affect the aPTT, factor IIa, and factor Xa inhibiting activity of any glycosaminoglycans other than unfractionated heparin.

The flow cytometry was performed with HIT serum and showed a platelet aggregation of 7.4% without heparin and 4.5, 3.2, and 7.9% in the presence of 0.1, 0.5, and 100 IU/ml heparin in saline, respectively. The expression of P-selectin was 25.3, 39.4, and 16.1% in the presence of the different heparin concentrations in saline, respectively. In the presence of HBP, heparin caused platelet aggregation of 9, 2.4, and 8.3% and P-selectin expression of 12.2, 35.9, and 11.1%, respectively. These data demonstrate that at less than 0.5 IU/mL, heparin expression of P-selectin is counteracted by HBP in the presence of HIT serum, heparin, and donor platelets using flow cytometry analysis. This agrees with the results obtained in the blood coagulation studies.

Platelet aggregometry was performed using two different HIT sera (A and B) to activate platelets. Normal human serum, run as negative control led to no activation of the platelets, and HIT serum A caused a 20% activation without adding heparin. After the addition of heparin diluted in saline in the concentrations of 1, 5, and 100 IU/ml, the final platelet activation was 88, 86, and 86%, respectively. The time to 50% of final activation was 0, 140, 125, and 310 seconds for 0, 1, 5, and 100 IU/ml. HIT serum B caused an activation of 13% without heparin; 1, 5, and 100 IU/ml heparin in saline activated platelets to 75, 70, and 16%, respectively. The time to 50% of the final activation was 0, 105, 190, and 0 seconds for the respective concentrations of heparins.

The HBP had the following effects on heparin in the presence of HIT serum and donor platelets in the
aggregometry studies. HIT serum B caused an activation of 17% without heparin. For 1, 5, and 100 IU/mL heparin in saline, platelets were activated to 69, 66, and 22%, respectively. The time to 50% of final activation was 0, 375, 330, and 0 seconds, respectively. In the presence of HBP no differences were detected with regard to the final platelet activation or the time to 50% of final activation. Therefore, the HBP does not influence the heparin-PF4 antibody-mediated platelet activation.

HIT serum gave a positive response in the presence of heparin in the SRA. In the absence of heparin no serotonin was released from donor platelets in the presence of HIT serum. The addition of 0.1 and 100 IU/mL heparin in saline or HBP caused a release of serotonin of 50% and 1% compared with 71% and 4%, respectively. Thus, the serotonin release caused by heparin-HIT serum was not altered by the HBP, indicating a lower affinity of the HBP to heparin compared with PF4, which in this assay is required to induce the neoantigen and the activation of donor platelets in the presence of HIT serum.

The standard PF4 concentration of 12.5 IU/mL in the PF4-ELISA showed an optical density (OD) of 0.481. The negative control with 5% albumin and 12.5 IU PF4 showed an OD of 0.450. The HBP at concentrations of 1, 0.5, and 0.25 mg/l showed ODs of 0.147, 0.019, and 0.004, respectively. Dilution buffer set the background. The HBP showed a concentration-dependent OD higher than that of the background but lower than the nonspecific binding of the PF4 antibody to albumin. Therefore, the positive result must be interpreted as a nonspecific binding of the PF4-antibody to the HBP.

**DISCUSSION**

The characterization of the HBP using heparin activity assays showed that the HBP affected the aPTT and TCTs in the presence of high concentrations of unfractionated heparin. The chromogenic assays for AT, thrombin, and factor Xa inhibition demonstrated that this effect is related to heparin concentrations less than 0.5 IU/mL. The Heptest assay did not detect these differences. The aPTT was chosen to perform cross-reactivity tests with different heparins and heparin-like substances because of the most significant effect of the HBP in this test. The HBP did not modify the anticoagulant effect of any LMWH or low- or high-sulfated glycosaminoglycans in this assay. This suggests that the HBP binds to the long saccharide chains of unfractionated heparin, thus reducing their anticoagulant effect in most assays. In contrast, activation of donor platelets in the presence of unfractionated heparin, PF4, and HIT serum was not counteracted by the HBP in any of the assays. It was assumed that PF4 possesses a higher affinity to heparin than the HBP does. For binding of AT to heparin, a high-affinity binding site, that is, the pentasaccharide binding site, and a less characterized secondary binding site have been defined. The characterization of the HBP using a PF4-ELISA showed the lack of structural identity with PF4. However, the OD data indicated that the protein structure may be similar to that of PF4 by binding to a PF4-antibody. We also suggest that the HBP has a low affinity to heparin and interacts with the secondary binding site to AT.

To investigate the type of heparin binding to the HBP, heparins of different sizes, degree of sulfation, and AT affinity were serially diluted in PBS and tested in the competitive radioimmunoassay. The mean molecular weight of the heparins correlated ($r = 0.967$) with the binding to the HBP in vitro. Oversulfation of heparin decreased the affinity to the antibody, whereas desulfation increased the affinity. The only disaccharide unit binding to the HBP in vitro was the iduronic acid–anhydromannose. The importance of the heparins' high-affinity binding sites for AT and binding of heparin to the HBP was analyzed by interaction studies with purified human AT. At a physiological concentration of AT 10 U/mL, only 20% of heparin was detected by the HBP.

The biological relevance of the HBP was demonstrated by the inhibition of the biological activity of heparin toward factor Xa. The HBP was capable of inhibiting about 60% of the anti–factor Xa activity induced by heparin. This is in agreement with the finding that the antibody competes with AT for binding to heparin. The results demonstrated that the HBP detected heparin and LMWH in vitro, in contrast with the antibodies described so far in the literature, which detect only modified or surface-attached heparin molecules.

The results of the biological relevance of the interaction of the HBP with heparins were studied more in detail in the present investigation. The characterization of the HBP using heparin activity assays shows that the HBP shortened the coagulation times of the aPTT and TCT in the presence of unfractionated heparin. The chromogenic assays for AT, thrombin, and factor Xa inhibition demonstrated that this effect is related to heparin concentrations less than 0.5 IU/mL. The Heptest assay did not detect these differences. The aPTT was chosen to perform cross-reactivity tests with different heparins and heparin-like substances because of the most significant effect of the HBP in this test. The HBP did not modify the anticoagulant effect of any LMWH or low- or high-sulfated glycosaminoglycans in this assay. The differences of the in vitro result, and the data in the presence of plasma remain to be investigated.

In addition to earlier studies, the interaction of AQ2, the HBP with heparin was investigated on platelets. For this we used platelets from healthy donors and serum from HIT patients. Activation of donor platelets as it occurs in the presence of unfractionated heparin, PF4, le II
and HIT serum was not counteracted by the HBP in any of the platelet assays. It is assumed that PF4 possesses a higher affinity to heparin than the HBP does. For binding of AT to heparin, a high-affinity binding site, that is, the pentasaccharide binding site, and a less-characterized secondary binding site have been defined. The characterization of the HBP using a PF4-ELISA confirms the suggestion of a lack of structural identity with PF4. However, the OD data indicate that the protein structure may be similar to PF4 by binding to a PF4 antibody. We also suggest that the HBP has a low affinity to heparin and interacts with the secondary binding site to AT.

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