EFFECT OF FONDAPARINUX ON PLATELET ACTIVATION IN THE PRESENCE OF HEPARIN-DEPENDENT ANTIBODIES.

A BLINDED COMPARATIVE MULTICENTER STUDY WITH UNFRACTIONATED HEPARIN.

Short title: Fondaparinux and heparin-induced thrombocytopenia

P. SAVI,¹ B.H. CHONG,² A. GREINACHER,³ Y. GRUEL,⁴ J.G. KELTON,⁵ T. E. WARKENTIN,⁵ P. EICHLER,³ D. MEULEMAN,⁶ M. PETITOU,¹ J.P. HERAULT,¹ R. CARIOU,¹ J.M. HERBERT¹

¹: Sanofi-Synthélabo Recherche, Toulouse, France
²: Department of Medicine, The St George Hospital, Kogarah, Australia
³: Department of Immunology and Transfusion Medicine, Ernst-Moritz-Arndt-University, Greifswald, Germany
⁴: Service d’Hématologie-Hémostase, Hôpital Trousseau, Tours, France
⁵: Platelet Immunology Laboratory, McMaster University, Hamilton, Ontario, Canada
⁶: NV Organon, Oss, The Netherlands

*The pharmaceutical R & D of fondaparinux (Arixtra®) are being pursued within a partnership agreement between Sanofi-Synthélabo (France) and Organon (the Netherlands). Several authors (PS, DM, MP, JPH, RC, and JMH) are employees of either Sanofi-Synthelabo or Organon.

Correspondence/reprint request: Pierre SAVI
Cardiovascular/Thrombosis Research Department
Sanofi-Synthelabo Recherche
195, route d'Espagne
31036 Toulouse
Tel: (+33. 561.16.24.36) – Fax: (+33. 561.16.22.86)
e-mail: pierre.savi@sanofi-synthelabo.com
Word count (excluding abstract, tables, figure legends, and references): 3753 words (<5000)

Scientific heading: Hemostasis, Thrombosis, and Vascular Biology
ABSTRACT

Heparin-induced thrombocytopenia (HIT) is a complication of heparin therapy caused by antibodies against a complex of platelet factor 4 and heparin. Fondaparinux is a new synthetic selective factor Xa inhibitor. We performed a serological study to determine the cross-reactivity of HIT sera with fondaparinux. Using a prospective, blinded study design, 39 clinically and serologically confirmed sera from patients with HIT and 15 control sera were sent to three different laboratories, each of which specialized in a particular HIT assay. These include the serotonin release assay, heparin-induced platelet agglutination assay, and platelet aggregation assay. Two of 82 assays (2.4%) performed in the presence of control sera were positive, both with unfractionated heparin. In the presence of HIT sera, 75 of 94 (79.8%) evaluable assays were positive with unfractionated heparin: fondaparinux was significantly (p<0.001) less reactive than unfractionated heparin, only three of 91 evaluable assays (3.3%) being positive. Using flow cytometry, unlike unfractionated heparin, fondaparinux did not induce the binding of PAC-1 and anti-CD62 monoclonal antibodies or of annexin V to platelets with HIT sera. Together, this study suggests that fondaparinux is non-reactive to HIT sera and raises the possibility that it may be used for prophylaxis and treatment of thrombosis in patients with a history of HIT.
INTRODUCTION

Heparin-induced thrombocytopenia (HIT) is a rare, but severe complication of heparin therapy.\textsuperscript{1} HIT is caused by heparin-dependent antibodies (typically IgG), which are directed against a molecular complex formed by heparin and the platelet alpha-granule protein, platelet factor 4.\textsuperscript{1} These IgG immune complexes bind to the Fc gamma IIa receptors on the platelet surface, resulting in platelet activation and aggregation, and eventually, to the occurrence of both thrombocytopenia and thrombosis. For patients with HIT, treatment with heparin must be stopped and an alternative antithrombotic agent used. Low-molecular-weight heparins cannot be used since they also cross-react, although to a lesser extent, with HIT-associated antibodies.\textsuperscript{1}

Fondaparinux (Arixtra\textsuperscript{\textregistered}), a new synthetic and selective inhibitor of coagulation factor Xa,\textsuperscript{2,3} has been shown to be an effective and safe antithrombotic agent in a number of thrombotic disorders.\textsuperscript{4-13} No episodes of HIT were reported in the large phase II and III clinical program. This result is consistent with the fact that, in vitro, fondaparinux did not cross react with HIT-associated antibodies.\textsuperscript{14-17} However, these in vitro studies using a limited set of sera and different methodologies of variable sensitivity and specificity, did not permit a definitive conclusion to be made regarding the safety of fondaparinux in terms of its ability to induce platelet activation in the presence of HIT-associated antibodies.

Currently, besides immunoenzymoassays that measure antibodies to heparin-platelet factor 4 complex, three functional assays are used in vitro to investigate the effect of HIT-associated antibodies on platelet function, namely serotonin release assay (SRA), considered to be the gold standard,\textsuperscript{18} heparin-induced platelet agglutination assay (HIPA),\textsuperscript{19} and platelet aggregation test.\textsuperscript{20} These biological assays are difficult to perform and to interpret.\textsuperscript{1,21} Additionally, there may be advantages in performing all assays together to further enhance sensitivity and specificity.\textsuperscript{21} Using these three assays, we compared in a blinded manner, the effect of fondaparinux with that of unfractionated heparin on platelet activation and aggregation in the presence of sera collected from
patients with clinically-documented HIT. The effect of fondaparinux on platelet activation in the presence of HIT sera was further assessed using sensitive flow cytometry techniques to measure the expression of specific platelet activation-related markers on the platelet surface.22-24
MATERIALS AND METHODS

Study design

Sera from patients with laboratory-confirmed HIT (HIT sera) and from subjects without HIT (control sera) were collected in three centers (Appendix). The local institutional review boards at the Prince of Wales Hospital, the Universitat Grießwald, and the Hopital Trousseau each approved this study and obtained informed consent according to the Declaration of Helsinki. All the sera were then aliquoted and a sub-sample sent to a predetermined reference laboratory (Appendix) for characterization. This laboratory performed both enzyme immunoassays plus SRA on all of these samples to confirm positive or negative results. These independently verified sera were then sent to the central laboratory. The central laboratory then sent out the positive HIT sera and negative control sera, in a coded and blinded fashion, to the three participating test laboratories (Appendix). All of the three test laboratories were also provided with solutions of buffer, unfractionated heparin and fondaparinux for the serotonin release, the platelet agglutination and the platelet aggregation assays. One specific assay was performed per centre. All investigators were blinded about the type of serum and the type of test solution. All blood donors had given written informed consent.

Sera

A first set of sera was obtained from 45 patients diagnosed with HIT. These patients had HIT defined by a fall in the platelet count to less than 100 x 10^9/L or a 50% decrease in platelet count, five or more days after starting heparin. Other causes of thrombocytopenia (ie: autoimmune thrombocytopenia, secondary immune thrombocytopenia, drug dependent thrombocytopenia and infection dependent thrombocytopenia) were excluded, according to clinical diagnosis. The HIT sera were all positive in the designed reference laboratory. A second set of sera was obtained from 15 healthy volunteers not receiving heparin or other agents known to affect the coagulation system. All sera collected from patients with HIT, positive on SRA and confirmed to have antibodies
against heparin-platelet factor 4 (HIT-IgG) by the reference laboratory, were categorized as “HIT serum”. All sera collected from healthy subjects, negative on SRA and in which the absence of HIT-IgG was confirmed by the reference laboratory, were designated as “control serum”. Before use, the sera were heated at 56°C for 30 minutes to inactivate complement and traces of thrombin. Except for confirmatory testing, the reference laboratory was not part of the laboratory testing.

**Unfractionated heparin and fondaparinux**

Solutions of various concentrations of unfractionated heparin (Calciparine®, Sanofi-Synthelabo, Paris, France) and fondaparinux (Sanofi-Synthelabo, Toulouse, France and Organon, Oss, The Netherlands) were prepared in saline and frozen until use. The final concentrations of unfractionated heparin and fondaparinux used in the different assays on platelet function were 0.1, 0.3, 1.0 and 100 IU/mL, and 0.1, 0.3, 1.0, 3.0 and 100 µg/mL, respectively.

**Immunoenzymoassay for antibodies to heparin-platelet factor 4 complex**

The presence of antibodies to heparin-platelet factor 4 complex (HIT-IgG) was investigated using an immunoenzymoassay described previously.²⁵ Purified platelet factor 4 (20 µg/mL, R&D Systems, Lille, France) and unfractionated heparin (1 IU/mL, Hepalean, Organon Teknika, ON, Canada) were incubated overnight at 4°C in carbonated buffer (50 mmol/L NaHCO₃/Na₂CO₃, pH 9.6) on a 96-well microtiter plate. The plate was then saturated with 10% normal goat serum (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in phosphate buffered saline (PBS, pH 7.2) for two hours at room temperature. Test sera diluted in 2% normal goat serum in PBS were added, and the microtiter plate was incubated at room temperature for one hour. After washing, alkaline-phosphatase conjugated, goat Fc chain-specific anti-human IgG antibody (Jackson Immunoresearch, West Grove, PA) was added to each well. After one hour of incubation and washing, p-nitrophenyl phosphate (1 mg/mL in diethanolamine buffer, 1 mol/L, pH 9.6) was added,
and the absorbance was read at 405 nm. The result was considered as positive when the OD was greater than 0.45.

**Platelet activation assays for HIT-associated antibodies**

**Serotonin Release Assay**

SRA was performed as previously described. Platelet-rich plasma, obtained by centrifugation (165g, 15 minutes) of whole blood collected in acid-citrate-dextrose (ACD, 1/6, v/v, pH 4.5) from healthy individuals who had not taken aspirin within the seven days preceding blood collection, was incubated with $^{14}$C-serotonin (0.1 µCi/mL of platelet-rich plasma, Amersham, Oakville, ON, Canada or Amersham, Little Chalfront, Buckinghamshire, UK) for 30 minutes at 37°C. Sera and solutions of buffer, unfractionated heparin or fondaparinux were added to washed, radiolabeled platelets; the mixture was incubated with constant stirring or under gentle agitation at room temperature for one hour. The reaction was stopped by adding EDTA (0.5%)/PBS buffer, and the amount of $^{14}$C-serotonin released into the supernatant was measured using a scintillation counter after centrifugation (1800g, 5 minutes). The result was considered as “positive” with unfractionated heparin when the release of $^{14}$C-serotonin was more than 20% at 0.1, 0.3 or 1 IU/mL unfractionated heparin and less than 20% at 100 IU/mL unfractionated heparin. For fondaparinux, a result was considered as “positive” when the release was more than 20% at 0.1, 0.3, 1.0 or 3.0µg/mL fondaparinux and less than 20% at 100 µg/mL fondaparinux. If the release was greater than 20% in the presence of buffer, 100 IU/mL unfractionated heparin, or 100µg/mL fondaparinux, the result was considered as “indeterminate”. In all other cases, the result was considered as “negative”. The “positive” results obtained with SRA at 0.1 IU/mL and 0.3 IU/mL of unfractionated heparin were only considered true positive if it could be confirmed by suppression with anti-Fc gamma IIa receptor antibodies (10 µg/mL, ATCC, Rockville, MD).

**Heparin-Induced Platelet Agglutination Assay**
HIPA was performed as previously described. Platelet-rich plasma was prepared by centrifugation (120g, 20 minutes) from citrated blood (ACD, 1.6/8.4, v/v) from healthy donors who had not taken any medication within the previous 10 days. Apyrase (grade IV, 5 µL at 1000 U/mL, Sigma, Munich, Germany) was added, and platelets were washed and resuspended in tyrode buffer (pH 6.3) containing 2.5 U/mL apyrase and 1 U/mL hirudin (Pentapharm, Basel, Switzerland). After incubation in sealed tubes at 37°C for 15 minutes, platelets were centrifuged (650g, 7 minutes), resuspended to obtain a platelet concentration of 300-400 x 10^9/L in 1 mL of suspension buffer (tyrode buffer, 0.212 mol/L MgCl₂, 0.196 mol/L CaCl₂, pH 7.2), and incubated in a sealed tube at 37°C for 45 minutes before use. Sera (20 µL), solutions of buffer, unfractionated heparin or fondaparinux (10 µL), and platelet suspension (75 µL) were dispensed into a microtiter plate that contained two steel spheres, and further incubated at room temperature on a magnetic stirrer for 45 minutes. The transparency of the solution was assessed every five minutes using an indirect light source. Duplicate experiments were performed for each platelet suspension. Each serum was tested with platelets from four different platelet donors. For unfractionated heparin, the result was considered as “positive” if the suspension became transparent due to platelet agglutination with 0.1, 0.3 or 1 IU/mL unfractionated heparin, but not or only weakly and with a significant delay with buffer and 100 IU/mL unfractionated heparin, for at least two of the four platelet donors. For fondaparinux, the result was “positive” if the suspension became transparent with 0.1, 0.3, 1.0 or 3.0 µg/mL fondaparinux, for at least two of the four platelet donors. If platelet agglutination occurred in the presence of 100 IU/mL unfractionated heparin for at least two of the four platelet donors, the result was considered as “indeterminate”. In all other cases, the result was considered as “negative”.

Platelet Aggregation

Platelet aggregation was performed as previously described. Platelet-rich plasma, obtained by centrifugation (150g, 10 minutes) of whole blood collected in citrate (0.129 mol/L, 1/9, v/v)
from healthy individuals who had not taken aspirin within the seven days preceding blood collection, was adjusted to 300 x 10^9/L. Platelet aggregation was measured at 37°C using an aggregometer (Platelet Aggregation Profiler, BioData, Horsham, PA). The ability of donor platelets to aggregate was first tested in the presence of collagen (70 µg/mL, Stago, Asnières, France). Then, platelet-rich plasma was incubated with sera and solutions of buffer, unfractionated heparin or fondaparinux. Aggregation was considered as positive when it exhibited a sharp initial slope (>20%/minute) and a maximum level of aggregation of 50% or more within 20 minutes. For unfractionated heparin, the result was considered as “positive” when platelet aggregation occurred with 0.1, 0.3 or 1 IU/mL unfractionated heparin, but not with 100 IU/mL unfractionated heparin. For fondaparinux, the result was “positive” when platelet aggregation occurred at 0.1, 0.3, 1.0 or 3.0µg/mL fondaparinux, but not with 100 µg/mL fondaparinux. If platelet aggregation occurred in the presence of buffer, 100 IU/mL unfractionated heparin, or 100µg/mL fondaparinux, the result was considered as “indeterminate”. In all other cases, the result was considered as “negative”, but before concluding the result was negative, platelet-rich plasma from at least two different donors were tested.

**Flow Cytometry Analysis**

Platelet-rich plasma was prepared by centrifugation (100g, 5 minutes) from citrated blood (citrate 0.129 mol/L, 1/9, v/v) from healthy individuals to which was added 180 µL acidifying buffer per 1 mL of PRP. Acidifying buffer contained 75 mmol/L trisodium citrate dehydrate, 38 mmol/L citric acid monohydrate, 140 mmol/L dextrose and prostacyclin (12 ng/mL final, Sigma). Platelets were washed and resuspended in HEPES buffer containing 134 mmol/L NaCl, 2.9 mmol/L KCl, 12 mmol/L NaHCO₃, 0.34 mmol/L Na₂HPO₄, 1 mmol/L MgCl₂, 5 mmol/L glucose, 5 mmol/L HEPES and 0.35% bovine serum albumin (pH 7.4). Platelet suspension samples (140 µL) were incubated for 30 minutes at 28°C with solutions of buffer, unfractionated heparin or fondaparinux (20 µL) and HIT sera (40 µL). Ten µL of these samples were added into tubes containing HEPES
buffer (90 µL) and FITC-PAC-1 monoclonal antibody or PE-anti-CD62 monoclonal antibody or FITC-annexin V, (Becton Dickinson, San Jose, CA). The binding of PAC-1 monoclonal antibody, anti-CD62 monoclonal antibody and annexin V reflects the activation of glycoprotein IIb-IIIa complex, the release of dense granules and the expression of negatively-charged phospholipids, respectively.22-24 After a 15 minute incubation at room temperature and in the dark, the samples were diluted in 400 µL of fixative solution (CellFIX, Becton Dickinson) and analyzed with a flow cytometer (Becton Dickinson FacsCalibur). Analyses were performed on 5000 events with the CellQuest program. A threshold of positive events was set up in order to measure less than 2% of positive events in the blank sample. Results were expressed as the percentage of positive events, defined as events above the threshold. A threshold for positive activity was determined for each individual data. The model was fitted using the Generalized Linear Model function of the S-Plus 2000 software package in the PC/Windows 98 environment.28 Given the expression of the model and the estimates of α, β₁ and β₂, it was possible to estimate the threshold level of stimulation with a 99% probability of successful stimulation.

Statistical Analysis

The statistical analysis was performed using a logit model, assuming the binomial distribution of the response, including the three assays (SRA, HIPA, and platelet aggregation) and treatments (unfractionated heparin and fondaparinux) as fix factors.
RESULTS

Characterization of the sera

Sera were collected from 15 healthy subjects without HIT and 45 patients with documented HIT. The reference laboratory reported that SRA was negative in all 15 sera obtained from healthy subjects, and none contained antibodies to heparin-platelet 4 complex. SRA was negative in 6 of the 45 sera collected from patients with HIT. All the 39 remaining HIT sera gave positive results.

Control Sera

Using SRA at another reference laboratory, one control serum (6.7%) was positive with 0.1 IU/mL of unfractionated heparin; all other sera were negative, regardless of the compound tested (Table 1). Using HIPA, all control sera were negative with unfractionated heparin or fondaparinux. Platelet aggregation results were indeterminate with 4 sera (26.7%), platelets aggregating with 100 IU/mL unfractionated heparin. Out of 11 remaining sera, platelet aggregation was positive with one control serum (9.1%) in the presence of 0.3 IU/mL unfractionated heparin.

HIT Sera

The results of the three types of platelet assays for heparin-dependent anti-platelet antibodies on the sera obtained from 39 patients with HIT in the presence of buffer or various concentrations of unfractionated heparin or fondaparinux are shown in Tables 1 and 2.

Serotonin Release Assay

SRA results were indeterminate for 5 HIT sera, leaving 34 evaluable sera. SRA was positive in 26 of these sera (76.5%) with unfractionated heparin, but only in 2 sera with fondaparinux (5.9%). Interestingly, one serum testing positive with fondaparinux (serum 24) was negative with unfractionated heparin. In addition, the mean percentage of maximum serotonin release obtained
with unfractionated heparin was 65.7% (n=26, range: 25-100%) as compared to 33.5% with fondaparinux (n=2, 39% and 28%).

**Heparin-Induced Platelet Agglutination Assay**

HIPA results were indeterminate for 3 HIT sera, leaving 36 evaluable sera. Of those 33 sera (91.7%) were positive with unfractionated heparin at low but not at high concentrations (100 IU/mL).

Of these 33 sera, 16 were negative with buffer and 17 reacted weakly with platelets without addition of heparin or fondaparinux. This reaction was strongly enhanced by heparin but not by fondaparinux with respect to the concentrations tested. However, the very high concentration of fondaparinux (100 µg/mL) inhibited the heparin independent platelet activating effect of HIT antibodies in 9 of 17 sera. These weak reactions in the absence of heparin might be due to unknown antibodies or platelet activating agents in patient sera, not related to HIT. Alternatively they may be HIT antibodies giving a weak positive reaction because of contamination with traces of heparin. The effect of fondaparinux occurs only at high concentration and does not seem to be relevant for its anti Xa activity. Of the 16 sera not showing any platelet activating effect in the presence of buffer, 15 did not cause platelet activation in the presence of any of the fondaparinux concentrations tested. One serum showed a weak platelet activating effect in the presence of fondaparinux.

**Platelet Aggregation**

Platelet aggregation was not done in 6 samples due to insufficient amount of serum. The result was indeterminate with 9 HIT sera, leaving 24 evaluable sera. The test was positive in 16 sera (66.7%) with unfractionated heparin and in none with fondaparinux.

**Statistical Analysis**
With fondaparinux, the overall level of positive activity (without distinction of the methods) in the presence of HIT sera (3.3%, 3/91) was not different from that in the presence of control sera (0.0%, 0/41, p=0.91). In contrast, with unfractionated heparin, the overall level of positive activity was significantly (p<0.001) greater in the presence of HIT sera (79.8%, 75/94) than in the presence of control sera (2/41, 4.9%). In addition, in the presence of HIT sera, the level of positive activity was significantly (p<0.001) greater with unfractionated heparin than with fondaparinux (79.8% versus 3.3%, respectively).

**Flow Cytometry Analysis**

The results of the binding of PAC1 and anti-CD62 monoclonal antibodies or annexin V to the platelets in the presence of 14 HIT sera and various concentrations of unfractionated heparin and fondaparinux are shown in Table 3.

PAC1 is a monoclonal antibody that recognizes the active conformation of human GpIIb-IIIa present at the platelet surface. The activation of GpIIb-IIIa is clearly correlated to the ability of the complex to bind soluble fibrinogen and result in platelet aggregation. 29

CD62 is an intra-platelet protein that is not present on the surface of resting platelets. It is stored in the membrane of the α-granules and is exposed when these granules fuse with the external membrane. It can therefore be considered as a marker of platelet release. 30

Annexin V is a positively-charged protein, which binds to electronegative phospholipids such as phosphatidyl-serine. It is currently used as a probe for measuring the exposure of electronegative phospholipids allowing the platelet surface to trigger thrombin generation. 31

Using unfractionated heparin, the mean binding of PAC1 and anti-CD62 monoclonal antibodies or annexin V increased according to a bell shape dose-effect curve: the binding was maximum with 0.3 and 1 IU/mL, and negligible with 100 IU/mL. Platelet activation, according to a threshold of positive activity threshold set for each individual HIT serum, in the presence of unfractionated heparin concentrations of 0.1, 0.3 or 1 IU/mL was observed with annexin V, PAC1 monoclonal
antibody and anti-CD62 monoclonal antibody in 14 (100%), 9 (64.3%, i.e. all sera except no. 4, 18, 32, 33, 39) and 13 (92.9%, i.e. all sera except no.4) HIT sera, respectively. In contrast, in the presence of fondaparinux, the mean binding of annexin V, PAC1 monoclonal antibody and anti-CD62 monoclonal antibody did not change, regardless of the fondaparinux concentration; none of the 14 HIT sera tested positive with fondaparinux, regardless of the fondaparinux concentration and the platelet activation membrane marker.
DISCUSSION

Using three functional assays, our study shows that, in most cases, fondaparinux does not activate platelets in the presence of sera obtained from patients with HIT: the platelet aggregation test was negative with all evaluable sera, the HIPA test was positive with only one serum (3.0%) and the SRA was positive with only 5.9% of the HIT sera. In contrast, with unfractionated heparin, the results were positive with the three tests in approximately 67% to 92% of HIT sera. We suggest that our results are reliable for several reasons. First, the sera were carefully selected and characterized. Second, a combination of three reference complementary functional tests was used. Third, all control and HIT sera were investigated in a blinded manner using a large range of fondaparinux and unfractionated heparin concentrations. Furthermore, the inability of fondaparinux to activate platelets in the presence of HIT sera was confirmed by the flow cytometry analysis of glycoprotein IIb-IIIa activation, dense granule release and expression of negatively-charged phospholipids on the platelet membrane, three highly sensitive markers of platelet activation.

There is variability among different biological assays in detecting the presence of heparin-dependent platelet antibodies. For this reason, we studied the ability of fondaparinux and unfractionated heparin to induce platelet activation in the presence of HIT sera using three reference functional assays. Compared to platelet aggregation performed in platelet-rich plasma, tests using washed platelets, e.g. HIPA and SRA, are generally considered to be the more sensitive for detecting heparin-dependent platelet antibodies.

The generation of HIT-related antigen has been shown to be dependent on the molecular weight of polysaccharides, the optimal chain length being sixteen saccharides.\textsuperscript{14} Fondaparinux, a pentasaccharide, may therefore be considered to be too small to induce the expression of such antibodies. Indeed, the present study confirms that fondaparinux does not appear to interact with HIT-related antibodies to induce platelet activation and aggregation. This result differentiates fondaparinux from low-molecular-weight heparins, which show complete cross-reactivity with the antibodies that cause HIT and which are known to exacerbate HIT in those patients with preformed
antibodies.\textsuperscript{21} Overall, our results are consistent with the report that no episodes of HIT were reported in the phase II and phase III program of fondaparinux,\textsuperscript{4-13} notably in major orthopedic surgery of the lower limbs, a setting at particularly high risk for developing HIT and thrombosis,\textsuperscript{32} in which fondaparinux was given to approximately 5,000 patients.\textsuperscript{4-9} Moreover, some cases have been reported of successful treatment with fondaparinux of patients hypersensitive to heparin and low-molecular-weight heparin.\textsuperscript{33,34} In contrast, orthopedic patients treated with unfractionated heparin have about a 5\% risk of HIT with at least half of the affected patients developing HIT-associated thrombosis; the HIT risk with low-molecular-weight heparins in this setting has been estimated as 0.75\%.\textsuperscript{1}

The inability of fondaparinux to induce platelet activation in the presence of HIT sera was demonstrated in previous smaller studies.\textsuperscript{14,15} We confirm this finding over a large range of fondaparinux concentrations using three complementary qualitative assays. Furthermore, using flow cytometry, we show that the addition of fondaparinux to HIT sera and platelets did not result in platelet activation as measured using annexin V, activation of glycoprotein IIb-IIIa or the release of dense granules. Interestingly, among these three markers of platelet activation, annexin V was the most sensitive for detecting heparin-dependent platelet antibodies. The positivity of SRA with fondaparinux in two sera and of HIPA in one serum is of uncertain significance and its biological relevance is uncertain; of note, serum 24 was not positive with unfractionated heparin and serum 33 was negative when the binding of annexin V was studied in the presence of fondaparinux on flow cytometry. A previous study showed that, in contrast to unfractionated heparin, fondaparinux did not increase the binding of HIT-related antibodies to purified platelet factor 4.\textsuperscript{16} The inability of fondaparinux to induce platelet activation in the presence of HIT may be related to the fact that this pentasaccharide does not have the minimal chain length and minimum charge per carbohydrate required for induction of the HIT antigen on platelet factor 4.\textsuperscript{14}

Selection of HIT patients, from whom serum was collected, was on the basis of both clinical and laboratory criteria, as recommended in a recent consensus manuscript.\textsuperscript{21} HIT sera were defined
by the presence of IgG antibodies to heparin-platelet factor 4 complex.\textsuperscript{35} The specificity of assays for heparin-dependent platelet activation was verified since samples were only categorized as positive if the positive results observed in the presence of clinically relevant heparin concentration were reversed in the presence of high concentrations of unfractionated heparin\textsuperscript{1}; in the case of heparin-dependent platelet activation, high concentrations of unfractionated heparin displace the heparin/platelet factor 4 complex from the platelet surface and the test becomes negative.\textsuperscript{22}

In conclusion, the safety of fondaparinux administration to patients in terms of HIT, which has yet to be firmly established clinically, may be an important advantage of fondaparinux over the heparins. Further, our study suggests that fondaparinux which does not enhance the platelet activation effect of HIT sera could be used as a treatment for HIT.
REFERENCES


APPENDIX

Collection of the sera: B.H. Chong (Department of Haematology, Prince of Wales Hospital, Randwick, NSW, Australia), A. Greinacher (Universität Greifswald, Greiswald, Germany), Y. Gruel (Service d’Hématologie-Hémostase, Hôpital Trousseau, Tours, France).

Preparation of sera, and of heparin and fondaparinux solutions: Sanofi-Synthélabo Recherche, Toulouse, France.

Characterization of sera: J.G. Kelton, T.W. Warkentin (Platelet Immunology Laboratory, Hamilton, Ontario, Canada).


Heparin-Induced Platelet Agglutination: A. Greinacher.

Platelet Aggregation: Y. Gruel.


Data Collection: Sanofi-Synthélabo Recherche.

Acknowledgements:

This study was supported by a grant from Sanofi-Synthelabo and NV Organon. Studies performed in Dr Kelton's laboratory were supported by the Heart and Stroke Foundation of Ontario (Canada).
**Table 1.** Results of serotonin release, heparin-induced platelet agglutination and platelet aggregation assays in the presence of control and HIT sera with unfractionated heparin or fondaparinux

<table>
<thead>
<tr>
<th></th>
<th>Control sera</th>
<th>HIT sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfractionated heparin</td>
<td>Fondaparinux</td>
</tr>
<tr>
<td></td>
<td>n/N (%)</td>
<td>n/N (%)</td>
</tr>
<tr>
<td><strong>Serotonin release assay</strong></td>
<td>1/15 (6.7)</td>
<td>0/15 (0.0)</td>
</tr>
<tr>
<td><strong>Heparin-induced platelet agglutination</strong></td>
<td>0/15 (0.0)</td>
<td>0/15 (0.0)</td>
</tr>
<tr>
<td><strong>Platelet aggregation</strong></td>
<td>1/11 (9.1)</td>
<td>0/11 (0.0)</td>
</tr>
</tbody>
</table>

*n is the number of positive sera and N the number of evaluable sera.
**Table 2**: Serum number from patients with heparin-induced thrombocytopenia positive in the presence of unfractionated heparin or fondaparinux on serotonin release, heparin-induced platelet agglutination or platelet aggregation assays

<table>
<thead>
<tr>
<th>HIT-Serum (No.)</th>
<th>SEROTONIN RELEASE ASSAY</th>
<th>HEPARIN-INDUCED PLATELET AGGLUTINATION ASSAY</th>
<th>PLATELET AGGREGATION*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive result</td>
<td>Heparin 1,2,4,6,9-12,14-22,27,28,30,31,33-35,38,24,33,22,24-32,34-39</td>
<td>Heparin 1-12,15-18,21,22,24-32,34-39,25</td>
<td>Heparin 1,2,4,6,7,9,10,12-14,20-22,29,32,35,25</td>
</tr>
<tr>
<td>Indeterminate result</td>
<td>3,7,23,26,29</td>
<td>20,23,33</td>
<td>3,8,11,15,16,23,26,31,33,</td>
</tr>
</tbody>
</table>

*For sera no. 17, 28, 34, 36, 38, and 39, platelet aggregation was not done due to insufficient amount of serum. The result was negative with all other sera not presented in this Table.
**Table 3**: Effect of unfractionated heparin or fondaparinux on the binding of annexin V, PAC-1 monoclonal antibody and anti-CD62 monoclonal antibody to platelets in the presence of sera* from patients with heparin-induced thrombocytopenia

<table>
<thead>
<tr>
<th>Concentration</th>
<th>n†</th>
<th>Annexin V Mean±SEM</th>
<th>PAC-1 Mean±SEM</th>
<th>anti-CD62 Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>4.30±1.74</td>
<td>0.82±0.33</td>
<td>1.51±0.58</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.1 IU/mL</td>
<td>14</td>
<td>18.30±3.64</td>
<td>3.44±1.00</td>
</tr>
<tr>
<td></td>
<td>0.3 IU/mL</td>
<td>14</td>
<td>35.85±4.78</td>
<td>5.49±1.19</td>
</tr>
<tr>
<td></td>
<td>1.0 IU/mL</td>
<td>10</td>
<td>47.58±4.30</td>
<td>4.76±1.26</td>
</tr>
<tr>
<td></td>
<td>100.0 IU/mL</td>
<td>14</td>
<td>2.64±0.59</td>
<td>0.35±0.07</td>
</tr>
<tr>
<td>Fondaparinux</td>
<td>0.1 µg/mL</td>
<td>14</td>
<td>3.44±0.66</td>
<td>0.61±0.25</td>
</tr>
<tr>
<td></td>
<td>0.3 µg/mL</td>
<td>14</td>
<td>3.24±0.65</td>
<td>0.59±0.24</td>
</tr>
<tr>
<td></td>
<td>1.0 µg/mL</td>
<td>10</td>
<td>2.20±0.48</td>
<td>0.24±0.05</td>
</tr>
<tr>
<td></td>
<td>3.0 µg/mL</td>
<td>10</td>
<td>2.01±0.41</td>
<td>0.19±0.04</td>
</tr>
<tr>
<td></td>
<td>100.0 µg/mL</td>
<td>14</td>
<td>2.47±0.44</td>
<td>0.24±0.05</td>
</tr>
</tbody>
</table>

*Sera no. 2, 4, 6, 10, 11, 14, 18, 19, 22, 31, 32, 33, 35, 39
†n denotes the number of experiments
Effect of fondaparinux on platelet activation in the presence of heparin-dependent antibodies. A blinded comparative multicenter study with unfractionated heparin

Pierre Savi, Beng H Chong, Andreas Greinacher, Yves Gruel, John G Kelton, Theodore E Warkentin, Petra Eichler, Dick Meuleman, Maurice Petitou, Jean-Pascal Herault, Roger Cariou and Jean-Marc Herbert