Recombinant factor VIIa partially reverses the inhibitory effect of fondaparinux on thrombin generation after tissue factor activation in platelet rich plasma and whole blood

Grigoris T. Gerotziafas[1,2], François Depasse[2], Tahar Chakroun[1], Meyer M. Samama[1,2], Ismail Elalamy[1]

[1]Service d'Hématologie Biologique, Hôpital Hôtel-Dieu de Paris, France
[2]LCL, Ivry sur Seine, France

Summary

Fondaparinux (Arixtra®), a specific AT-dependent FXa inhibitor, is effective and safe in the prevention and treatment of venous thromboembolism, but some major hemorrhagic events may occur. No specific antidote to fondaparinux has been proposed. Recombinant FVIIa (Novoseven®) could be used as an haemostatic treatment, but this option has not been well documented. We studied the effect of rFVIIa (1 μg/ml) on the inhibition of thrombin generation induced by fondaparinux (0.1μg/ml to 1 μg/ml). Coagulation was triggered in platelet rich plasma (PRP) or in whole blood by recalcification in the presence of diluted thromboplastin. In PRP thrombin generation was assessed using the thrombinoscope assay. In whole blood, prothrombin activation was assessed by measuring the kinetics of F_{1+2} formation using an ELISA assay. Fondaparinux at concentrations equal or greater than 0.5 μg/ml prolonged the initiation phase of thrombin generation, and reduced the velocity of prothrombin activation. It also decreased by 60% the endogenous thrombin potential. In the presence of fondaparinux (0.5 μg/ml to 1 μg/ml) rFVIIa accelerated the initiation phase of thrombin generation, but it did not significantly increase the endogenous thrombin potential. However, rFVIIa did not completely reverse the inhibitory effect of fondaparinux on the parameters of thrombin generation and prothrombin activation. This study shows that rFVIIa accelerates thrombin generation, but does not completely reverse the inhibitory effect of fondaparinux on thrombin generation. The potential clinical use of rFVIIa as haemostatic treatment of major bleedings related to fondaparinux has to be evaluated.

Keywords

Pentasaccharide, fondaparinux, thrombin generation, novoseven, tissue factor

Thromb Haemost 2004; 91: 531–7

Introduction

Synthetic pentasaccharide (fondaparinux-Arixtra®), a methoxy form of the natural pentasaccharide sequence is a selective AT-dependent factor Xa inhibitor, but has no effect on thrombin inhibition (1, 2). However, fondaparinux significantly inhibits thrombin generation and prothrombin activation (3-5).

Recent phase III clinical trials for the prevention of venous thromboembolism in major orthopaedic surgery and a meta-analysis, showed that one daily subcutaneous injection of 2.5 mg of fondaparinux is superior to 40 mg of enoxaparin administered subcutaneously once daily, resulting in a 55.2% reduction of the incidence of asymptomatic DVT. Overall, major bleedings occurred significantly more often in patients treated with fondaparinux (2.7%) as compared to enoxaparin (1.7%; p=0.0085), (6-10). In addition, the Matisse trial showed that fondaparinux is as effective and safe as unfractionated heparin in the treatment of pulmonary embolism. In the Matisse

Correspondence to:
Grigoris T. Gerotziafas, MD, PhD
Service d'Hématologie Biologique
Hôpital Hôtel-Dieu de Paris
1 Place du Parvis Notre Dame
75181, Paris, Cedex 04, France
E-mail: gerotziafasgrigoris@hotmail.com

Received July 24, 2003
Accepted after revision December 5, 2003

Prepublished online February 4, 2004 DOI: 10.1160/TH03-07-0483

Parts of this study have been presented as plenary lecture during the 17th International Congress on Thrombosis (Bologna, 26-30/10/2002) and as a poster presentation during the 44th American Society of Hematology Annual Meeting 2002.

pulmonary embolism trial, the frequency of major, or life threatening bleedings was similar between fondaparinux and unfractionated heparin treated patients. It is worthy of note that some rare severe bleeding episodes occurred in both groups (11). Moreover, in the Pentalyse trial, rare major bleeding complications occurred while fondaparinux or unfractionated heparin were administered in connection with fibrinolytic drugs (12). These clinical trials proved that fondaparinux is an effective antithrombotic agent, which also induces a non-negligible bleeding risk. For fondaparinux, as for almost all of the emerging new antithrombotic agents, no specific antidote is available. An attractive haemostatic treatment could be the use of recombinant factor VIIa (rFVIIa, Novoseven®). Recombinant FVIIa has been shown to be an effective and safe haemostatic agent in a variety of clinical conditions (13). The potential use of rFVIIa as a haemostatic treatment of major hemorrhages, which occur during anticoagulant treatment, has been studied in three recent trials (14-16).

In the present study we investigated the effect of rFVIIa on the inhibition of thrombin generation and prothrombin activation induced by fondaparinux at concentrations ranging from 0.1 μg/ml to 1 μg/ml, which are achieved in prophylaxis and treatment of venous thromboembolism (2). In platelet rich plasma, we studied the effect of rFVIIa on fondaparinux-induced inhibition of thrombin generation after tissue factor pathway activation by employing the endogenous thrombin potential (ETP) assay (17). We also used a method, that we developed in our laboratory, to investigate the effect of fondaparinux on the kinetics of prothrombin activation during clotting of normal whole blood in the presence of minimal amounts of tissue factor (TF).

Materials and methods

Reagents and normal human plasma

Synthetic pentasaccharide (Fondaparinux – Arixtra®) was kindly offered by the Greek branch of Sanofi-Synthelabo (Athens, Greece). Recombinant factor VIIa (Novoseven®) was from Novo Nordisk (Bagsvaerd, Denmark). Bovine serum albumin (BSA), EDTA, Tris-HCl were obtained from Sigma laboratories (St. Louis U.S.A.). Relipidated recombinant tissue factor (Hemoliance® RecombiPlasTin) was from Instrumentation Laboratory (Milan, Italy). The Hemoliance® RecombiPlasTin reagent was reconstituted by addition of 5 ml of NaCl 0.9% (the concentration of TF is unknown), and subsequently diluted either in NaCl 0.9% for the experiments of prothrombin activation in whole blood, or in Hepes buffer (containing 20 mM Hepes, 140 mM NaCl and 5 mg/ml BSA; pH 7.35) for the experiments with the Thrombogram-Thrombinoscope™ assay (Synapse b.v., Maastricht, The Netherlands). The ELISA assay for the measurement of F_{1+2}, (Enzygnost F_{1+2} micro) was obtained from Dade Behring (Marburg, Germany). The fluorogenic substrate Z-Gly-Gly-Arg-AMC was obtained from Bachem (Bubendorf, Switzerland).

Venous blood was obtained from 14 healthy volunteers, who work in our laboratory, and were not taking any medication. Blood was collected with atraumatic antecubital veinipuncture, into siliconized vacutainer tubes (Becton Dickinson, Meylan, France) containing buffered trisodium citrate (0.13 mol/l, nine parts of blood and one part of citrate solution). The kinetics of F_{1+2} formation was studied in fresh normal citrated whole blood from 7 volunteers. Thrombogram-Thrombinoscope™ assay was performed in platelet rich plasma (PRP) from 7 other volunteers. The platelet rich plasma was prepared after centrifugation of citrated whole blood for 10 min at 150 g at room temperature. The supernatant PRP was removed and the platelet count was adjusted to $3x10^5/\mu l$ by dilution with autologous PPP obtained after a further centrifugation of the remaining blood for 15 min at 2,000 g. Fondaparinux and rFVIIa were added *in vitro* to the PRP or whole blood.

Thrombin generation test (TGT): Thrombogram-Thrombinoscope™ assay

Thrombin generation (TG) was studied according to the assay described by Hemker et al (18). In each well of a micro-plate, 80 μl of the studied PRP supplemented with saline (control) or with the indicated concentrations of the studied compounds were mixed with 20 μl of diluted Hemoliance® RecombiPlasTin. The employed dilution of thromboplastin (1:1000 final dilution in plasma) was selected because it induced about a 3-minute lag-time of thrombin generation in normal PRP. Thrombin generation was initiated by adding the triggering solution containing $CaCl_2$ (16.7 mM final concentration) and the fluorogenic substrate (417 μM final concentration). A plate reader fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) and the appropriate software (Synapse, Maastricht, The Netherlands) were used for the assessment of thrombin generation. The total amount of thrombin activity was assessed as the area under the curve (i.e. the endogenous thrombin potential) (17). The following parameters were analyzed and the intra-assay and inter-assay coefficients of variation for each parameter are given in parenthesis: a) the lag time of thrombin generation (12% and 13%), b) the time to reach the maximum concentration (Tmax) of thrombin (12% and 11%), c) the maximum concentration (Cmax) of thrombin (5% and 10%) and d) the endogenous thrombin potential (ETP; 10% and 12%).

Kinetics of prothrombin activation

To study the kinetics of prothrombin activation after tissue factor pathway activation in whole blood, we developed the following method: 100 μl normal citrated whole blood, supplemented with 10 μl of saline (control) or with the indicated concentrations of the studied compounds, were mixed with 25 μl of diluted Hemoliance® RecombiPlasTin. The final concentrations of fondaparinux and rFVIIa were corrected according to the hematocrit, and are expressed as μg/ml or ng/ml in

plasma, respectively. The final dilution of thromboplastin in plasma was 1:3,200. In preliminary experiments, a variety of thromboplastin dilutions was examined (data not shown). The employed dilution of thromboplastin was selected, because it induced clotting of whole blood within about 3 minutes. After the addition of thromboplastin, 25 μl of $CaCl_2$ (0.1M) were added to each sample. The procedure was performed in plastic (polystyrene) tubes at 37°C. For each studied interval, a separate tube was used. Before (t0) and at various intervals after $CaCl_2$ addition (1 min, 2 min, 3 min, 5 min, 7 min, 10 min, 15 min and 20 min) 50 μl of serum were mixed with 200 μl of buffer containing 0.1 M NaCl, 0.05 M Tris, 1% (w/v) BSA and 100 mM EDTA (pH 7.4). After quenching, samples were centrifuged for 15 min at 2,500 g and the serum was frozen at –70°C until analysis. Prothrombin F_{1+2} were measured in sera using a commercial ELISA assay (Enzygnost F_{1+2} micro, Dade Behring Marburg, Germany). The intra-assay and inter-assay coefficients of variation for prothrombin F_{1+2} measurements were 3% and 7%, respectively. The following parameters were analyzed: a) the lag-time for prothrombin F_{1+2} formation corresponding to the time point after $CaCl_2$ addition, of a 10% increase of F_{1+2} levels in serum as compared to the baseline (in one experiment the lag-time of prothrombin activation was estimated with a curve fit and calculation of the time derivative), b) the maximal velocity of prothrombin F_{1+2} formation (ΔnM/min) and c) the maximum levels of prothrombin F_{1+2} (nM) in the serum.

Statistical analysis

Paired Student's t-test was used for the comparison of the variables, before and after the addition of each studied concentration of fondaparinux. Paired Student's t-test was also used for the comparison between the values obtained before and after the addition of rFVIIa in the presence of the indicated concentrations of fondaparinux. $P<0.05$ was considered as statistically significant. The Statistical Package for Social Sciences (SPSS) software was used for statistical analysis.

Results

Effect of fondaparinux on thrombin generation

After TF pathway activation in PRP, the lag-time and the Tmax of thrombin generation were 3.3±1.7 min and 9.9±3.3 min, respectively. Each studied concentration of fondaparinux significantly prolonged the lag-time and the Tmax of thrombin generation as compared to the control. Fondaparinux at the lower studied concentration (0.1 μg/ml) increased the lag-time to 4.8±2 min and the Tmax to11±2 min. In the presence of fondaparinux 0.8 μg/ml and 1 μg/ml the lag-time was

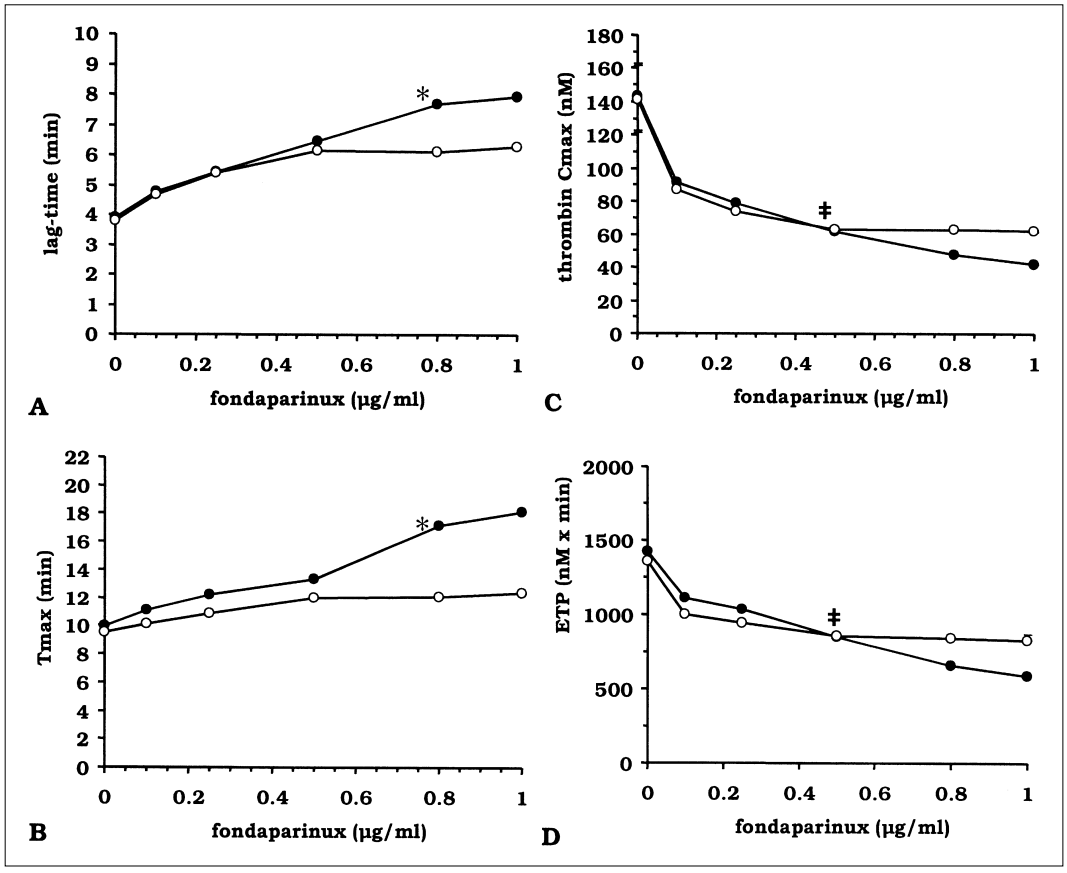

Figure 1: Effect of increasing concentrations of fondaparinux (black cycles) on the parameters of thrombin generation in PRP and influence of rFVIIa (1 μg/ml) on the effect of fondaparinux (open cycles). Values are means of 7 experiments; the standard deviations are given in the Results section. *p<0.05 as compared to the respective values in the presence of rFVIIa. ‡p<0.05 as compared to the control experiment.

7.2±3 min and 7.7±3 min and the Tmax was 12±4 min and 12.3±4 min respectively (Fig. 1 frames A and B).

In the control experiment the Cmax of thrombin and the ETP were 142±50 nM and 1426±323 nM x min respectively. Fondaparinux concentrations achieved in prophylaxis (0.1 μg/ml and 0.25 μg/ml) reduced the Cmax of thrombin and the ETP by about 30% and 27%, respectively, but this reduction did not reach statistical significance as compared to the control ($p>0.05$). In the presence of fondaparinux 0.5 μg/ml, the Cmax and the ETP were 61±19 nM and 848±300 nM x min, respectively; being significantly reduced as compared to the control experiment (142±50 nM and 1426±323 nM x min respectively; $p<0.05$). In the presence of fondaparinux 0.8 μg/ml and 1 μg/ml, the Cmax was 47±15 nM and 41±15 nM, respectively; being significantly reduced as compared to the Cmax observed in the presence of fondaparinux 0.5 μg/ml (61±19 nM; $p<0.05$). The ETP in the presence of fondaparinux 0.8 μg/ml and 1 μg/ml was 657±330 nM x min and 580±288 nM x min respectively; being significantly reduced as compared to ETP observed in the presence of fondaparinux 0.5 μg/ml (848±300 nM x min; $p<0.05$). The maximum reduction of throm-bin Cmax and ETP was 66% and 59%, respectively, and it was observed in the presence of fondaparinux 0.8μg/ml. A further increase in fondaparinux concentration did not result in an additional reduction of thrombin generation (Fig. 1 frames C and D).

Effect of rFVIIa on thrombin generation

After TF pathway activation in PRP in the presence of rFVIIa, the lag-time and the Tmax of thrombin generation were 3.7±2 min and 9.4±4min, being similar to those observed in the control experiment. Moreover, rFVIIa did not significantly modify the Cmax (141±37 nM) and the ETP (1426±323 nM x min) as compared to the control (Fig. 1).

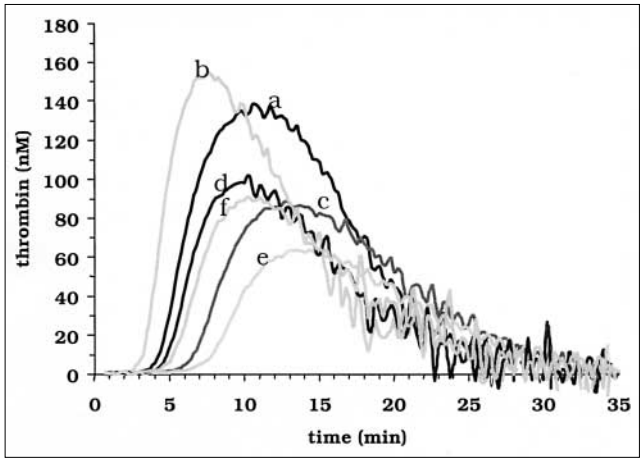

Figure 2: Effect of rFVIIa and fondaparinux on thrombin generation in PRP. Representative curves of thrombin generation from one out of 7 experiments: a: control, b: control + rFVIIa (1 μg/ml), c: fondaparinux (0.5 μg/ml), d: fondaparinux (0.5 μg/ml) + rFVIIa (1 μg/ml), e: fondaparinux (1 μg/ml), f: fondaparinux (1 μg/ml) + rFVIIa (1 μg/ml).

Influence of rFVIIa on the inhibitory effect of fondaparinux on thrombin generation

Recombinant FVIIa did not modify the inhibitory effect of fondaparinux at the concentrations of 0.1 μg/ml and 0.25 μg/ml on the lag-time and the Tmax. In the presence of fondaparinux 0.5 μg/ml rFVIIa reduced by 1 min both the lag-time and the Tmax. In contrast, when clotting of PRP was triggered in the presence of 0.8 μg/ml or 1 μg/ml of fondaparinux, the addition of rFVIIa decreased the lag time by 3±1.5 min and the Tmax of thrombin generation decreased by 4±2 min. The decrease of the lag-time and the Tmax induced by rFVIIa was significant as compared to the respective values observed in the presence of the same concentrations of fondaparinux without addition of rFVIIa. However, in the presence of rFVIIa and fondaparinux both the lag-time and the Tmax remained significantly prolonged as compared to the control (Fig. 1, frames A and B).

The addition of rFVIIa in PRP supplemented with fondaparinux concentrations equal to or lower than 0.5 μg/ml did not modify the Cmax and the ETP as compared to the values observed in the presence of the same concentrations of fondaparinux alone (Fig. 1 frames B and C). Interestingly, in the presence of rFVIIa and fondaparinux 0.8 μg/ml the Cmax (63±15nM) as well as the ETP (840±330 nM x min) were slightly, but not significantly, increased as compared to the Cmax, and ETP obtained when clotting was triggered in the presence of fondaparinux alone (47±15 nM and 657±330 nM x min; $p>0.05$). Similarly, in the presence of fondaparinux 1 μg/ml, rFVIIa slightly but not significantly increased the Cmax (62±15 nM) and the ETP (825±296 nM x min) as compared to the Cmax and ETP values obtained when clotting was triggered in the presence of fondaparinux alone (41±15 nM and 580±288 nM x min; $p>0.05$, Fig. 1, frames C and D). A representative experiment of the effect of rFVIIa on the fondaparinux induced inhibition of thrombin generation is shown in Figure 2.

Effect of fondaparinux on prothrombin activation during clotting of whole blood

An intermediate concentration of fondaparinux (0.5 μg/ml) significantly prolonged the lag-time of prothrombin activation (5 min) as compared to the control (3 min) and reduced the velocity of F_{1+2} formation (60±10 nM/min) versus the control (160±40 nM; $p<0.05$). In addition, fondaparinux reduced by 35% the maximal concentration of F_{1+2} as compared to the control (500 ± 50 nM versus 760±60 respectively; $p<0.05$, Fig. 3).

Effect of rFVIIa on prothrombin activation during clotting of whole blood

After TF-pathway activation in whole blood in the presence of rFVIIa (1 μg/ml) the lag time (3 min), and the velocity of

prothrombin activation (113±20 nM/min), as well as the maximal concentration of F_{1+2} (666±60) were similar with those observed in the control experiment (Fig. 3).

Influence of rFVIIa on the inhibitory effect of fondaparinux on prothrombin activation during clotting of whole blood

As shown above, the lag-time of prothrombin activation was 5 min in the presence of fondaparinux 0.5 μg/ml. At this time-point the concentration of prothrombin F_{1+2} was 2.8±0.5 nM. Five minutes after clotting activation in whole blood, spiked with both rFVIIa (1 μg/ml) and fondaparinux (0.5 μg/ml), the levels of F_{1+2} were 40.4±3 nM, but they were significantly lower as compared to those observed in the control experiment (130±20 nM) in the same time-point (Fig. 3). Thus, when both rFVIIa and fondaparinux were present, the lag-time of prothrombin activation was calculated to be about 4 min. In the presence of rFVIIa and fondaparinux the velocity of F_{1+2} formation was 70±8 nM/min, being slightly but not significantly increased as compared to that observed in the presence of fondaparinux alone (60±10 nM/min $p>0.05$). The maximal concentration of F_{1+2} in the presence of rFVIIa and fondaparinux was similar to that observed in the presence of fondaparinux alone (554±50 nM versus 500±50 nM respectively; $p>0.05$).

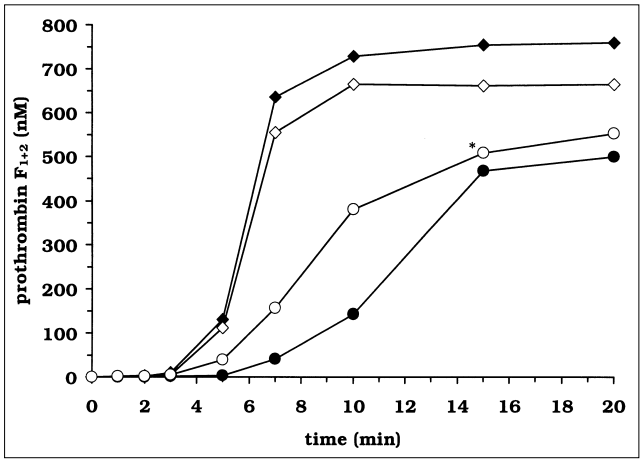

Figure 3: The effect of rFVIIa (1 μg/ml) on the inhibition of prothrombin activation by fondaparinux (0.5 μg/ml) after TF pathway activation in whole blood as described in Materials and Methods. Black diamonds: control, open diamonds: control + rFVIIa, black cycles: fondaparinux (0.5 μg/ml), open cycles: fondaparinux (0.5 μg/ml) + rFVIIa (1 μg/ml). Values are means of 7 experiments. The standard deviations of the velocity of prothrombin F_{1+2} formation and the maximal concentration of prothrombin F_{1+2} are given in the Results section.
* $p<0.05$ as compared to the respective control.

Discussion

Fondaparinux, at concentrations achieved in prophylaxis (equal to or lower than 0.5 μg/ml), significantly prolonged the initiation phase and reduced the velocity of the propagation phase of thrombin generation, but it did not significantly reduce either the maximum amount of generated thrombin, nor the endogenous thrombin potential. Fondaparinux concentrations higher than 0.5 μg/ml were required to decrease the Cmax of thrombin and the ETP. It is noteworthy, that fondaparinux, even at the highest studied concentration (1 μg/ml) as well as in supratherapeutic concentrations (5 μg/ml; data not shown), did not induce more than a 60% reduction in the ETP. Our observations are in accordance with a study conducted by Walenga et al in animal models, which showed that supratherapeutic concentrations of fondaparinux completely abrogated *in vivo* thrombus formation, but did not suppress the *ex vivo* generation of thrombin by more than 60% (19). In addition, in a study conducted in healthy volunteers, Lormeau et al showed that fondaparinux concentrations as high as 5 μg/ml did not reduce the *ex vivo* thrombin generation by more than 60% (3). According to the pharmacokinetics profile of fondaparinux, the highest plasma concentration obtained after the third day of prophylaxis in subjects with normal renal function is about 0.5 μg/ml (20).

Recent clinical trials showed that fondaparinux is effective and safe in the prophylaxis and treatment of venous thromboembolism and as adjunctive drug in thrombolysis, but it is potentially associated with a non negligible risk of major hemorrhage (6-12). Therefore, an effective and safe haemostatic treatment for potential major bleedings is necessary. It has already been shown by our group and others, that when haemostasis is defective, rFVIIa in the presence of TF, accelerates the initiation phase of thrombin generation and of prothrombin activation, without any significant increase in the amount of generated thrombin (21-23). These findings prompted the present study of the effect of rFVIIa on the fondaparinux induced inhibition of thrombin generation.

The assessment of thrombin generation after minimal TF pathway activation in PRP was done using the classical thrombinoscope assay. However, this assay cannot be performed in whole blood, which is considered to be a more physiologically relevant experimental system. Prothrombin activation is closely related to thrombin generation. The formation of prothrombin F_{1+2} directly reflects the activation of prothrombin by the prothrombinase complex (FXa/FVa/phosphospholipids) since FXa is the only plasma enzyme known to efficiently convert prothrombin to thrombin and prothrombin F_{1+2} (24, 25). Therefore, we assessed the kinetics of prothrombin activation using a method developed in our laboratory.

The present study shows that a clinically relevant concentration of rFVIIa (1 μg/ml) did not significantly modify the kinetics of thrombin generation, and the ETP as well as the kinetics

of prothrombin activation after TF pathway activation in normal PRP or whole blood. In addition, rFVIIa did not significantly modify the moderate inhibitory effect of low concentrations of fondaparinux on the kinetics of thrombin generation. In contrast, in the presence of fondaparinux concentrations equal to or higher than 0.5 μg/ml, the addition of rFVIIa accelerated the generation of thrombin and the activation of prothrombin. To our knowledge, a clear explanation for this distinct effect of rFVIIa is not yet available. Recent experimental evidence shows that rFVIIa has a minimal effect on platelet function and clot structure after clotting of normal whole blood (26). In the presence of TF, rfVIIa accelerates the initiation and the propagation phase of thrombin generation resulting in thrombin dependent enhancement of platelet activation when the haemostatic balance is significantly disturbed towards hypocoagulation, i.e. in severe thrombocytopenia (submitted). The present study shows that rFVIIa manifests its effect when the thrombin generation process is significantly impaired, i.e. in the presence of fondaparinux concentrations higher than 0.5 μg/ml. More detailed studies on the mechanism of action of rFVIIa have to be done in order to clarify this issue.

Interestingly, rFVIIa had a partial effect on the reversal of fondaparinux activity since it did not normalize the lag-time and the Tmax of thrombin generation nor the lag time or the velocity of prothrombin activation. In the beginning of the initiation phase of thrombin generation, FVIIa/TF complex activates factor IX and factor X leading to initial generation of picomolar amounts of thrombin, which induce initial platelet activation. The propagation phase of thrombin generation is characterized by further activation of platelets by thrombin, which also activates factors VIII, V and XI. During the propagation phase the formation of the enzymatic complexes (intrinsic tenase and prothrombinase) occurs, resulting in a burst of thrombin generation (27, 28). Fondaparinux inhibits free FXa but not the FXa included into the prothrombinase complex (29-31). During the initiation phase, the activation of FX precedes the generation of FVa, so that picomolar amounts of free FXa are present (32). Thus, free FXa is accessible to the inhibition by AT/pentasaccharide complex (31). We hypothesize that in the presence of tiny amounts of TF, the employed concentration of rFVIIa accelerates, and increases the generation of some free FXa which overcomes the inhibitory effect of fondaparinux. It is probable that, higher concentrations of FXa need to be produced in order to completely overcome the inhibitory effect of fondaparinux, and to normalize thrombin generation. However, this hypothesis has to be controlled in appropriate experiments.

In neither of the two experimental systems did the addition of rFVIIa induce a significant increase of the maximal amount of generated thrombin expressed, either as Cmax of thrombin or as ETP. This finding is consistent with recent studies, which showed that rFVIIa does not induce an increase in the total amount of generated thrombin in patients with severe thrombocytopenia and in a reconstituted thrombocytopenia-like system (21, 23).

Our study supports the concept that rFVIIa could be a safe and effective haemostatic therapy by achieving localised TF dependent haemostasis. Recombinant FVIIa seems to fulfill some requirements of safety in order to be used as antidote to fondaparinux. *In vitro* it partially reverses the attenuation of thrombin generation induced by fondaparinux at the concentrations of 0.5 μg/ml to 1 μg/ml, but it does not increase significantly the total amount of generated thrombin, that could be associate to an hypercoagulable state. Our findings are further supported by a recent study conducted in healthy volunteers receiving fondaparinux in conjunction to a single dose of 90 μg/ml of rFVIIa. In that study, in accordance with our results, the administration of rFVIIa significantly reduced the thrombin generation time *ex vivo* and induced a slight, but not significant increase of the ETP (33). The clinical relevance of these data has to be evaluated in clinical practice. Nevertheless, these results might encourage future research on the potential clinical use of rFVIIa as antidote to fondaparinux if a major or life-threatening bleedings occurs.

Acknowledgements

We thank Prof H.C. Hemker and the Synapse b.v. for lending the fluorometer and the appropriate software for the assessment of thrombin generation.

References

1. Choay J, Petitou M, Lormeau JC, et al. Structure-activity of relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. Biochem Biophys Res Comm 1983; 116:492-9.
2. Samama MM, Gerotziafas GT. Evaluation of the pharmacological properties and clinical results of the synthetic pentasaccharide (Fondaparinux). Thromb Res 2003; 109:1-11.
3. Lormeau JC, Hérault JP. The effect of the synthetic pentasaccharide SR 90107/ORG 31540 on thrombin generation ex vivo is uniquely due to ATIII-mediated neutralization of factor Xa. Thromb Haemost 1995; 74: 1474-7.
4. Lormeau JC, Hérault JP. Comparative inhibition of extrinsic and intrinsic thrombin generation by standard heparin, a low molecular weight heparin and the synthetic AT-III-binding pentasaccharide. Thromb Haemost 1993; 69: 152-6.
5. Beguin S, Choay J, Hemker HC. The action of a synthetic pentasaccharide on thrombin generation in whole plasma. Thromb Haemost 1989; 61: 397-401.
6. Lassen MR, Bauer KA, Eriksson BI, et al. Postoperative fondaparinux versus preoperative enoxaparin for prevention of venous thromboembolism in elective hip-replacement surgery: a randomized double-blind comparison. Lancet 2002; 359: 1715-20.
7. Turpie AG, Bauer KA, Eriksson BI, et al. Postoperative fondaparinux versus postoperative enoxaparin for prevention of venous thromboembolism after elective hip-replace-

ment surgery: a randomized double-blind trial. Lancet 2002; 359: 1721-6.
8. Bauer KA, Eriksson BI, Lassen MR, et al. Fondaparinux compared with enoxaparin for the prevention of venous thromboembolism after elective major knee surgery. N Engl J Med 2001; 345: 1305-10.
9. Eriksson BI, Bauer KA, Lassen MR, et al. Fondaparinux compared with enoxaparin for the prevention of venous thromboembolism after hip-fracture surgery. N Engl J Med 2001; 345: 1298-304.
10. Turpie AGG, Bauer KA, Eriksson BI, et al. Fondaparinux versus enoxaparin for the prevention of venous thromboembolism in major orhtopedic surgery. A meta-analysis of 4 randomized double-blind studies. Arch Intern Med 2002; 162: 1833-40.
11. The Matisse investigators. Subcutaneous fondaparinux versus intravenous unfractionated heparin in the initial treatment of pulmonary embolism. N Engl J Med 2003; 349: 1695-702.
12. Coussement PK, Bassand JP, Convens C, et al. A synthetic factor-Xa inhibitor (ORG31540/SR9017A) as an adjunct to fibrinolysis in acute myocardial infarction. Eur Heart J 2001; 22: 1716-24.
13. Hedner U. General haemostatic agents – fact or fiction. Pathophysiol Haemost Thromb 2002; 32(suppl): 33-6.
14. Friederich PW, Levi M, Bauer KA, et al. Ability of recombinant factor VIIa to generate thrombin during inhibition of tissue factor in human subjects. Circulation; 2001; 103: 2555-9.
15. Deveras RAE, Kessler CM. Reversal of warfarin induced excessive anticoagulation with recombinant factor VIIa concentrate. Ann Intern Med 2002; 137: 884-8.
16. Sørensen B, Johansen P, Nielsen GL, et al. Reversal of the International Normalized Ratio with recombinant activated factor VII in central nervous system bleeding during warfarin thromboprophylaxis: clinical and biochemical aspects. Blood Coagul Fibrinolysis 2003; 14: 469-77.
17. Hemker HC, Wielders S, Kessels H, et al. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. Thromb Haemost 1993; 70: 617-24.
18. Hemker HC, Giesen PLA, Ramjee M, et al. The thrombogram: monitoring thrombin generation in platelet rich plasma. Thromb Haemost 2000; 83: 589-91.
19. Walenga JM, Petitou M, Lormeau JC, et al. Antithrombotic activity of a synthetic heparin pentasaccharide in a rabbit stasis thrombosis model using different thrombogenic challenges. Thromb Res 1987; 46: 187–98.
20. Boneu B, Necciari J, Cariou R, et al. Pharmacokinetics and tolerance of the natural pentasaccharide (SR90107/ORG31540) with high affinity to antithrombin III in man. Thromb Haemost 1995; 74: 1468– 73.
21. Gerotziafas GT, Zervas K, Arzoglou P, et al. On the mechanism of action of rFVIIa administered to patients with severe thrombocytopenia and life-threatening haemorrhage. Focus on prothrombin activation. Br J Haematol 2002; 117: 705-8.
22. Butenas S, Brummel KE, Branda RF, et al. Mechanism of factor VIIa–dependent coagulation in hemophilia blood. Blood 2002; 99: 923-30.
23. Kjalke M, Ezban M, Monroe DM, et al. High dose factor VIIa increases initial thrombin generation and mediates platelet activation in thrombocytopenia-like conditions in a cell-based model system. Br J Haematol 2001; 114: 114-20.
24. Rosing J, Tans G, Grovers-Riemslag JWP, et al. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem1980; 255: 274-83.
25. Esmon CT, Owen WG, Jackson CM. A plausible mechanism for prothrombin activation by factor Xa, factor Va, phospholipid, and calcium ions. J Biol Chem 1974; 249: 8045-47.
26. Carr ME, Martin EJ, Kuhn JG, et al. Effects of recombinant factor VIIa on platelet function and clot structure in blood with deficient prothrombin conversion. Thromb Haemost 2003; 89: 803-11.
27. Mann KG, Butenas S, Brummel K. The dynamics of thrombin formation. Arterioscler Thromb Vasc Biol 2003; 23: 17-25.
28. Hemker HC, Beguin S. Phenotyping the clotting system. Thromb Haemost 2000; 84: 741-51.
29. Barrowcliffe TW, Havercroft SJ, Kemball-Cook G, et al The effect of $Ca^{2+,}$ phospholipids and factor V on the anti-factor Xa activity of heparin and its oligosaccharides. Biochem J 1987; 243: 31-7.
30. Pieters J, Willemsg G, Hernker HC, et al. Inhibition of factor IXa and factor X, by Antithrombin III/Heparin during factor X activation. J Biol Chem 1988; 263: 15313-18.
31. Rezaie AR. Prothrombin protects factor Xa in the prothrombinase complex from inhibition by the heparin-antithrombin complex. Blood 2001; 97: 2308-13.
32. Hemker HC, Choay J, Beguin S. Free factor Xa is on the main pathway of thrombin generation in clotting plasma. Biochimica et Biophysica Acta 1989; 992: 409-11.
33. Bijsterveld NR, Moons AH, Boekholdt AM, et al. Ability of recombinant factor VIIa to reverse the anticoagulant effect of pentasaccharide fondaparinux in healthy volunteers. Circulation 2002; 106: 2550-4.