The extrinsic coagulation pathway in coronary artery disease and endotoxemia
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Ability of recombinant factor VIIa to reverse the anticoagulant effect of the pentasaccharide fondaparinux in healthy volunteers

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Abstract

Background
The novel anticoagulant fondaparinux proved to be effective and safe in the postoperative prevention of venous thrombosis. Current phase III trials with this synthetic selective factor Xa inhibitor focus on its use in the treatment of patients with venous and arterial thrombosis. As with any anticoagulant therapy, there is a risk of bleeding complications, hence a strategy to reverse the effects of fondaparinux is desirable. The aim of this study was to investigate whether recombinant factor VIIa (rFVIIa) could neutralize the anticoagulant effects of subcutaneous administered fondaparinux.

Methods
In a randomized, placebo-controlled design, 16 healthy male subjects received either a single s.c. dose of fondaparinux (10 mg) and a single i.v. bolus of rFVIIa (90 μg/kg) (n=8), fondaparinux and placebo (n=4), or placebo and rFVIIa (n=4). Fondaparinux (or placebo) was administered 2 hours prior to rFVIIa (or placebo).

Results
Injection of rFVIIa after fondaparinux normalized the prolonged activated partial thromboplastin and prothrombin times and reversed the decrease in prothrombin activation fragments 1+2 (F1+2) as observed with fondaparinux alone. The thrombin generation time and endogenous thrombin potential, which were inhibited by fondaparinux, normalized up to 6 hours after rFVIIa injection.

Conclusions
Recombinant factor VIIa is capable of normalizing coagulation times and thrombin generation during fondaparinux treatment. The duration of this effect ranged from 2 to 6 hours after recombinant factor VIIa injection. These results suggest that rFVIIa may be useful to reverse the anticoagulant effect of fondaparinux in case of serious bleeding complications or need for acute surgery during treatment with fondaparinux.
Introduction

Novel anticoagulant agents aim at improved efficacy and safety by a more selective inhibition of the coagulation cascade, for example targeting thrombin as achieved by hirudin (-analogues), or directed at the factor VIIa-tissue factor complex by recombinant NAPc2 (nematode anticoagulant protein c2) or recombinant TFPI (tissue factor pathway inhibitor). Recently, fondaparinux, a novel selective factor Xa inhibitor has been evaluated for the prevention and treatment of venous and arterial thrombosis. Fondaparinux is a synthetic pentasaccharide, which binds exclusively to the activation site of antithrombin, thereby increasing its activity toward factor Xa inactivation 300-fold. Different from the other antithrombin-dependent anticoagulants, i.e. unfractionated heparin and low-molecular-weight heparin (LMWH), fondaparinux selectively inactivates factor Xa without thrombin inhibition. Fondaparinux was superior to LMWH in the prevention of venous thrombosis after elective major knee surgery, and hip-fracture surgery, reducing the incidence of this complication by an average of 56%. Currently, phase III trials explore the use of fondaparinux in the treatment of venous thromboembolism and acute coronary syndromes.

The potential drawback of any anticoagulant agent is the risk of bleeding complications. Fondaparinux has a biological half-life of approximately 17 hours, and a strategy to reverse the anticoagulant state in case of a life-threatening bleeding or in case of acute surgery, seems desirable.

A candidate for reversal of the anticoagulant effect of fondaparinux is recombinant factor VIIa (rFVIIa), which has the ability to normalize the prothrombin times in subjects taking warfarin, or to restore thrombin generation during inhibition of the tissue factor-factor VIIa complex.

Thus, we investigated, in healthy male volunteers, whether rFVIIa is able to neutralize the anticoagulant effect of 10 mg fondaparinux, which is four times the dose in prophylactic treatment of venous thromboembolism.

Methods

Subjects selection

Healthy male subjects (age 18 to 45 years), with a body mass index between 18 and 30 kg/m² and a maximum weight of 100 kg, were eligible. Subjects with a personal or family history of thrombosis or bleeding disorders were excluded. All subjects gave written informed consent. The study was approved by the Medical Ethics
Committee of the Academic Medical Center, Amsterdam, the Netherlands. The study protocol was conducted in accordance with the International Conference on Harmonization of Good Clinical Practice Guidelines.\textsuperscript{15}

**Study drugs**
Subjects were randomized to one of three treatment strategies: (a) fondaparinux and placebo (n=4); (b) fondaparinux and rFVIIa (n=8); (c) placebo and rFVIIa (n=4). Randomization was single blind (for subjects) for fondaparinux and double blind for rFVIIa. Fondaparinux (10 mg) (Arixtra\textsuperscript{\textregistered}, Organon/Sanofi-Synthelabo, Oss, The Netherlands/Paris, France), or placebo was administered as a single subcutaneous (s.c.) dose of 0.8 ml. Two hours following study drug administration, recombinant factor VIIa (Novo Seven\textsuperscript{\textregistered}, Novo Nordisk, Copenhagen, Denmark), 90 μg/kg or an equal amount of placebo was given as an intravenous (i.v.) bolus injection.

**Blood sampling**
Blood samples were collected before fondaparinux or placebo administration (t=0) and 1.5, 2 (just before rFVIIa administration), 2.5, 3, 3.5, 4, 5, 6, 8, and 24 hours thereafter. At each sampling, the first 5 ml blood was discarded, after which 9 ml was collected in tubes containing 1 ml citrate (final concentration 0.32%) and 5 ml was collected in K3 EDTA Vacutainer tubes. Blood was centrifuged at 2200g for 20 minutes at 18 °C. Plasma was separated, pooled and filled out in cryocups and frozen at -80 °C until analysis was performed. These procedures were completed within one hour after blood sampling.

**Assays**
The thrombin generation time (TGT) was measured spectrophotometrically using the fibrin polymerization method. Thrombin generation was initiated by adding calcium and recombinant tissue factor (5000 x diluted prothrombin time concentration) and results were expressed as T ½ (time to reach the midpoint of clear to maximal turbid density). The endogenous thrombin potential (ETP) was performed as previously described.\textsuperscript{16} In short, thrombin potential was determined amidolytically at 37 °C in defibrinated plasma containing phospholipids, tissue factor, and calcium. Results were expressed as a percentage of standard pooled plasma. Plasma concentration of prothrombin fragment 1+2 (F1+2) was measured by sandwich-type ELISA assay (Dade-Behring, Marburg, Germany). Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined according to standard
methods. Plasma levels of factor VII antigen were determined using the Asserachrom VII:Ag assay (Diagnostica Stago, Asnieres-sur-Seine, France). Fondaparinux plasma concentrations were measured by an amidolytic photometric assay method based on the anti-Xa activity of the antithrombin-fondaparinux complex. During the sample preparation, factor Xa and the chromogenic substrate S-2222 were added to the samples after which the amount of hydrolyzed substrate was measured by a spectrophotometer.

Statistical analysis
Differences between treatment groups were compared with an analysis of variance with a covariate (ANCOVA) on the log-transformed area under the curve (AUC) measured between the time points 2 and 8 hours after fondaparinux (or placebo) administration, with the log-transformed baseline values as covariate. Additionally, pairwise comparisons using ANOVA and post-hoc Scheffé adjustment were performed to identify differences between the groups per time point. To detect differences in mean parameter values within a group, paired t-tests were used. A p-value of <0.05 was considered statistically significant. Figures present mean values plus standard deviations per group for each parameter.

Results
Thrombin generation and thrombin activity
Fondaparinux doubled the thrombin generation time, which remained elevated up to 8 hours after administration (Figure 1). Rapid normalization occurred after administration of rFVIIa, persisting at least 6 hours after injection. This marked reduction was reflected in the significant lower AUC between time points 2 and 8 in the fondaparinux + rFVIIa group compared to the fondaparinux alone group (p<0.001). Recombinant FVIIa alone resulted in a similar proportional decrease of the thrombin generation time as observed in the fondaparinux + rFVIIa group. The endogenous thrombin potential decreased by 24% after fondaparinux administration (p=0.001) (Figure 2). This reduction persisted up to 8 hours after administration. A rapid increase, although not to baseline levels, was obtained by rFVIIa injection. The ETP between time points 2 and 8 hours was 9% higher in the fondaparinux + rFVIIa group compared with the fondaparinux alone group (p=0.056). Injection of rFVIIa alone resulted in a temporary increase with a maximum of 10% at 0.5 and 1 hour after injection, and returning to baseline values 2 hours post-injection. Plasma levels of prothrombin activation peptides F$_{1-2}$ (which indicate prothrombin
Figure 1. Effect of fondaparinux and/or recombinant VIIa (rFVIIa) administration on thrombin generation time (TGT). S.c. administration of fondaparinux or placebo (at t=0). After 2 hours i.v. injection of rFVIIa or placebo. Significant (Post-hoc Scheffé) differences (p<0.05) per time point between fondaparinux + rFVIIa vs. fondaparinux alone are marked with *, between fondaparinux + rFVIIa vs. rFVIIa alone marked with #.

Figure 2. Effect of fondaparinux and/or recombinant VIIa (rFVIIa) administration on endogenous thrombin potential (ETP). S.c. administration of fondaparinux or placebo (at t=0). After 2 hours i.v. injection of rFVIIa or placebo. No significant (Post-hoc Scheffé) differences (p<0.05) per time point were observed between the groups.
Recombinant factor VIIa to reverse fondaparinux

Figure 3. Effect of fondaparinux and/or recombinant VIIa (rFVIIa) administration on prothrombin fragment 1+2 (F1+2). S.c. administration of fondaparinux or placebo (at t=0). After 2 hours i.v. injection of rFVIIa or placebo. Significant (Post-hoc Scheffé) differences (p<0.05) per time point between fondaparinux + rFVIIa vs. rFVIIa alone are marked with #.

to thrombin conversion) were reduced after the administration of fondaparinux (from 0.80 ± 0.14 nmol/L at baseline to a minimum of 0.54 ± 0.07 nmol/L at 24 hours; p=0.045; Figure 3). Administration of recombinant factor VIIa prevented this decrease up to 3 hours post-injection. Prothrombin activation between time point 2 and 8 was increased by 34% in the fondaparinux + rFVIIa group compared to the fondaparinux alone group (p=0.022).

Clotting times
Administration of fondaparinux resulted in a mean increase of the aPTT from 33.5 ± 4.1 sec to 38.8 ± 5.3 sec 1.5 hours post-administration (p=0.004). The aPTT remained elevated at least 8 hours after administration (Figure 4a). Immediately after rFVIIa injection, the aPTT decreased to values similar to baseline, whereas the aPTT remained prolonged after placebo injection. After this initial correction, the aPTT gradually increased again and 6 hours after injection, the effect of rFVIIa was no longer detectable. The AUC analysis between time point 2 and 8 hours revealed a significant reduction of the aPTT in the fondaparinux + rFVIIa group compared to the fondaparinux alone group (p=0.015). Administration of rFVIIa alone produced a similar relative decrease and duration of the aPTT as observed in the fondaparinux + rFVIIa group.
Figure 4a and 4b. Effect of fondaparinux and/or recombinant VIIa (rFVIIa) administration on activated partial thromboplastin time (aPTT) and prothrombin time (PT). S.c. administration of fondaparinux or placebo (at t=0). After 2 hours i.v. injection of rFVIIa or placebo. Significant (Post-hoc Scheffe) differences (p<0.05) per time point between fondaparinux + rFVIIa vs. fondaparinux alone are marked with *, between fondaparinux + rFVIIa vs. rFVIIa alone marked with #.
The PT slightly increased after fondaparinux administration, from 13.2 ± 0.6 sec to 14.3 ± 0.9 sec at 1.5 hours (Figure 4b). Subsequent administration of rFVIIa resulted in a marked shortening of the PT to 9.2 ± 0.9 sec (p<0.0001). Between time points 2 and 8 hours, rFVIIa injection resulted in a 26% reduction of the PT after fondaparinux compared to fondaparinux alone (p<0.001). In the group receiving rFVIIa alone, the PT fell to 8.0 ± 0.3 sec, and remained lower up to 24 hours.

**Factor VII and fondaparinux plasma levels**

Injection of rFVIIa resulted in a sharp increase of factor VII plasma levels, rising from 64% to 251% at 30 minutes after injection. Thereafter, factor VII levels decreased with an estimated plasma half-life of 1.25 hour and reached virtually normal levels at 24 hours after injection (data not shown). Subjects treated with fondaparinux and rFVIIa had somewhat lower maximal peak factor VII levels (186% at 30 minutes), with a similar plasma half-life as the rFVIIa alone group. Injection of rFVIIa had no effect on the pharmacokinetic profile of fondaparinux. Maximum plasma levels of fondaparinux were reached at 1.5 to 2.5 hours after administration (±1.1 mg/L), with a half-life of 16 hours, and plasma levels at 24 hours of approximately 0.3 mg/L (data not shown).

**Discussion**

The central role of factor Xa in coagulation makes this protease a desirable target for antithrombotic therapy. Heparin and low-molecular-weight heparins are inhibitors of factor Xa but their lack of specificity (due to simultaneous inhibition of thrombin and other activated coagulation factors) may contribute to a relatively small therapeutic window and to the risk of bleeding. Pentasaccharides are synthetic agents capable of highly selective factor Xa inhibition. Fondaparinux 2.5 mg s.c. was shown to effectively and safely prevent venous thromboembolism after orthopedic surgery. Fondaparinux has a relatively long elimination half-life and higher dosages are now being evaluated for the treatment of venous and arterial thrombotic disease. For patients who experience bleeding complications or require acute surgical intervention, reversal of the anticoagulant effect of fondaparinux may be desirable. Our results demonstrate that administration of recombinant factor VIIa is able to overcome the inhibition of thrombin generation in healthy subjects treated with 10 mg s.c. fondaparinux. We observed a normalization of the fondaparinux-induced prolongation of aPTT and PT by administration of rFVIIa. Sensitive thrombin generation assays
demonstrated the efficacy of rFVIIa in restoring impaired thrombin formation after fondaparinux administration. These \textit{in vivo} results add to \textit{in vitro} data, showing that rFVIIa not only reverses the anticoagulant effect of fondaparinux but also the profibrinolytic effects of this agent\textsuperscript{17}, probably through activation of thrombin-activatable fibrinolysis inhibitor (TAFI) by rFVIIa\textsuperscript{18}. This effect appears not to be specific for fondaparinux since rFVIIa is able to reverse the effect of other anticoagulants such as the tissue factor inhibitor rNAPC2\textsuperscript{14}, indicating that infusion of high levels of factor VIIa activates sufficient amounts of non-fondaparinux-inhibited factor X to achieve normal thrombin generation.

Fondaparinux administration resulted in both an aPTT and PT increase up to 5.6 seconds and 1.1 seconds, respectively. Although these post-fondaparinux clotting times were significantly higher than baseline, these measurements are difficult to use in a clinical setting on individual patients to determine the anticoagulant effect of fondaparinux, since the effects are relatively small and are not always consistent with changes in anti-Xa levels. Whereas the observed normalization of coagulation \textit{in vivo} strongly suggests clinical efficacy, it is unknown whether these results imply that rFVIIa is effective in the treatment of clinically significant hemorrhages during fondaparinux therapy, even though rFVIIa demonstrated efficacy in other clinical bleeding conditions\textsuperscript{14,19-25}.

The duration of the effect of a single 90 µg/kg i.v. dose of rFVIIa differed between parameters and varied from 2 to 6 hours. Continued activation up to 6 hours as seen with the prothrombin and thrombin generation assays indicated a sustained effect of rFVIIa, overcoming the inhibitory effect of fondaparinux. These data support earlier observations in which rFVIIa was capable of inducing thrombin generation in the absence of tissue factor\textsuperscript{14,26-27}, possibly due to the high supraphysiological plasma levels of factor VIIa obtained (251%). In patients with active bleeding sites, the complex of exposed tissue factor and factor VIIa has an even greater potential of factor X activation compared to rFVIIa alone, and therefore the effect of rFVIIa is likely to be more pronounced in these cases.

The safety of recombinant factor VIIa administration needs consideration. The number of reports of thrombotic complications following rFVIIa therapy is relatively low\textsuperscript{28-30}, while its application is extending rapidly for various indications\textsuperscript{19,25,31-35}. However, the risk of thrombotic complications is higher in patients treated with anticoagulant therapy due to recently diagnosed venous or arterial thrombosis, especially in patients treated for acute coronary syndromes due to increased tissue factor expression at the
culprit coronary lesion. Therefore, until more evidence becomes available, rFVIIa should be used prudently and only if conventional treatments fail. The dosage of rFVIIa used in this study did not result in an overshoot of coagulation, with none of the parameters showing possible procoagulant values.

Current data in over 2000 patients receiving the prophylactic 2.5 mg dose of fondaparinux\textsuperscript{6-8} reveal a low incidence of serious bleeding complications with no bleedings in critical organs, and 0.4% bleedings requiring reoperation. Higher dosages (up to 12 mg), used in the arterial thrombosis trials (unstable angina) had a incidence of major bleeding varying between 0% and 1.8%, without a clear dose-response relationship\textsuperscript{10}, although the combination with a thrombolytic agent (alteplase) increased the incidence of major bleeding between 4.9% and 7.8% (similar to patients receiving alteplase and UFH\textsuperscript{9}. However, clinical experience is limited, and in case of bleeding complications in vital organs (e.g. intracranial) or life-threatening bleeding, the use of an antidote may be desirable.

We conclude that recombinant factor VIIa is capable of normalizing thrombin generation after subcutaneous administration of 10 mg fondaparinux in healthy male subjects, and may be a suitable antidote in case of serious bleeding complications in patients treated with fondaparinux.

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