Improved hemostasis with superactive analogues of factor VIIa in a mouse model of hemophilia A

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The authors (MT, KK, CP, RR and EP) are employed by Novo Nordisk whose product was studied in the present work.
ABSTRACT:

It is currently debated whether the mechanism of action of therapeutic doses of recombinant factor VIIa (rFVIIa, NovoSeven®) relies on the tissue factor (TF)-independent activity of the enzyme. The present study was conducted to investigate the in vivo hemostatic effects of rFVIIa and three analogues thereof with superior intrinsic activity (FVIIaIIa, K337A-FVIIaIIa and M298Q-FVIIa) in mice with antibody-induced hemophilia A. A highly significant dose response was observed on the bleeding time and blood loss for each of the rFVIIa variants. The bleeding time and blood loss were normalized after administration of 10 mg/kg rFVIIa, 3 mg/kg K337A-FVIIaIIa and 3 mg/kg M298Q-FVIIa indicating a potency of these FVIIa analogues 3-4 times above that of rFVIIa in FVIII-depleted mice. The different in vivo potencies of the various forms of FVIIa could not be explained by the pharmacokinetics. Histopathological evaluation of kidneys revealed no signs of treatment-related pathological changes even after treatment with the superactive variants. The fact that FVIIa analogues with enhanced intrinsic activity are more efficacious in the murine hemophilia A model strongly suggests that the TF-independent procoagulant activity of FVIIa contributes to its clinical hemostatic effect.

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INTRODUCTION:

Recombinant human coagulation factor VIIa (rFVIIa, NovoSeven®) has proven to be efficacious for the treatment of bleeding episodes in hemophilia patients with inhibitors (1-4). A small fraction of patients may be refractory to rFVIIa treatment (5) and could potentially benefit from genetically modified FVIIa molecules with increased potencies. To this end, FVIIa analogues with increased intrinsic activity have been generated which exhibit superior hemostatic profiles in vitro (6-8). These analogues may also be used as more efficacious hemostatic agents in other indications where efficacy of rFVIIa has been observed, including a number of case reports in thrombocytopenia (9,10) and trauma (11). Recently, an effect of rFVIIa was documented in a randomized placebo-controlled study in patients with a normal coagulation system undergoing retropubic prostatectomy (12).

During the normal hemostatic process FVIIa forms a complex with its cell surface receptor, tissue factor (TF), which becomes exposed to the blood upon vascular injury. This complex formation leads to activation of downstream coagulation processes resulting in the activation of factor X (FX) to factor Xa (FXa) and ultimately the activation of thrombin and fibrin formation. The prevailing rationale for the therapeutic use of rFVIIa is the fact that, at pharmacological doses, rFVIIa binds to the surface of activated platelets and subsequently generates a thrombin burst via activation of FX on the platelets (13). The affinity of rFVIIa for activated platelets is much lower than the affinity for TF, which may explain the high levels of rFVIIa needed to stop bleedings in hemophiliac patients and verify the need for more potent FVIIa analogues or analogues with a higher affinity for the platelet surface. The analogues with improved intrinsic (TF-independent) activity would provide more activity per platelet-bound molecule, potentially improving both efficacy and
convenience. The FVIIa analogues with improved membrane binding would ensure a higher density of the enzyme on the platelet with a maintained activity per molecule. The extent of TF dependency is debated (14-16) and the mechanism of action still needs to be further scrutinized.

The present studies were conducted to compare the \textit{in vivo} haemostatic effects, in a tail-bleeding model in mice with antibody-induced hemophilia A, of native FVIIa and three FVIIa variants with increased TF-independent (intrinsic) enzymatic activity (but unaltered activity when bound to TF) to shed light on the mechanism of action of this clinically useful agent.

\textbf{MATERIALS, ANIMALS, AND METHODS:}

\textbf{Proteins and other reagents}

Antibodies against human factor VIII (FVIII) were raised in goats (Ganløse, Denmark). The anti-FVIII antibody had an inhibitor titer of 2670 mouse Bethesda Units (BU) per ml. Stock solutions were further diluted in 0.9% saline, final concentration 1500 BU/ml. The mice were dosed 3.3 \( \mu \)l/kg (4950 BU/kg). One Bethesda unit is defined as the amount of inhibitor which will neutralize the biological activity of FVIII by 50\%. Recombinant FVIIa was produced in-house, reconstituted in sterile water to the various dosages and kept frozen at -20 °C until use.

The FVIIa analogues are genetically engineered using wild-type FVIIa as the template (6). M298Q-FVIIa has a glutamine substituted for methionine at position 298. FVIIa\textsubscript{IIa} (V158D/E296V/M298Q-FVIIa) contains two additional mutations, valine at position 158 replaced by aspartic acid and glutamic acid at position 296 replaced by valine.
The third analogue K337A-FVIIaIIa (V158D/E296V/M298Q/K337A-FVIIa) also has an alanine residue substitution for lysine at position 337.

Activated partial thromboplastin time (APTT) and prothrombin time (PT) reagents were obtained from Instrumentation Laboratory (Milan, Italy).

**Mice**

NMRI male mice, bodyweight approximately 30 g, were obtained from M&B, Ry, Denmark. The animals were kept in a barriered facility, group-housed and acclimatized for at least 7 days. Room temperature was kept at 18-22 °C, humidity 30-70% and the animals were subjected to a 12 hours light : 12 hours dark cycle controlled automatically. A pelleted standard rodent diet, Altromin 1324, was offered ad libitum as well as fresh water from a local domestic supply. Experiments were performed according to guidelines from The Danish Animal Experiments Council, the Danish Ministry of Justice.

**Pharmacokinetic study**

Recombinant FVIIa was administered as a single dose intravenously in the tail vein to NMRI mice at a dose level of 1, 3, 6, and 10 mg/kg. The analogues FVIIaIIa, K337A-FVIIaIIa and M298Q-FVIIa were administered at dose levels of 1 and 3 mg/kg. One-hundred mice were enrolled in the study, ten in each group. The test substances were separately dissolved in glycyl-glycine buffered saline. Blood samples were drawn from three animals/time point at predose (time 0), 2, 5, 10, 20, 40 min, 1, 2, 3 and 4 hours. Plasma samples were analyzed for rFVIIa, FVIIaIIa, K337A-FVIIaIIa and M298Q-FVIIa, respectively using an FVIIa ELISA. Pharmacokinetic calculations were performed using mean concentration values at each time point and the following...
parameters were estimated: terminal plasma elimination half-life (t½), based on the natural logarithm of two divided by the slope of the log-linear regression of the concentration versus time (λz). The total body clearance (CL) was determined as dose divided by area under the plasma concentration curve (AUC) from zero to infinity. The volume of distribution based on the terminal phase (Vz) was estimated by dose divided by AUC times λz. WinNonlin Professional Version 3.1 (Pharsight Inc., Mountain View, CA, USA) was used for calculation of pharmacokinetic parameters.

**Induction of hemophilia A**

The body weights of the mice were on average 36.4 g (SD 2.7 g). There were no differences between the groups. During the experiments the body temperature of the mice were kept constant by an automatic heating blanket connected to a thermometer (CMA/Microdialysis AB, Stockholm, Sweden). The mice were anaesthetized by 1% pentobarbital sodium (Nomeco, Copenhagen, Denmark) in sterile water dosed intraperitoneally. Xylocain, 10 mg/ml (Astra Zeneca, Denmark), was injected in the neck and a catheter was placed in the carotid artery for administration of test substance, measurement of blood pressure (Goulds P 23 XL transducer, Kipp & Zonen BD 9 recorder) and blood sampling. The catheter was kept open by slow infusion of 0.9% saline, 0.4 ml/hour. A hemophilia A-like condition was induced by injection of anti-FVIII antibodies 30 minutes before induction of the bleeding as previously described for rabbits (17, 18). The FVIII activity was determined by a chromogen assay (Coatest®, Chromogenix, Mölndal, Sweden) as previously described (19). The cross reactivity of the anti-FVIII antibodies to other relevant coagulation proteins (FV, FX and FII) was tested in a standard assay. Samples were measured using an ACL 9000 Research coagulometer (Instrumentation Laboratory,
Milan, Italy) following the manufacturer’s instructions. Briefly, mouse plasma and antibodies/buffer were incubated for 30 min at 37°C prior to test. The intrinsic pathway was tested using 40 µl sample, 40 µl FVIII-deficient plasma, 40 µl cephalin (APTT, Organon Teknika) and 40 µl CaCl₂. The extrinsic pathway (FX, FV and FII) was investigated testing 40 µl FX-, FV- or FII-deficient plasma, 40 µl sample and 80 µl CaCl₂/TF (Innovin, Dade). Standard curves were generated from a pool of normal mouse plasma.

Tail bleeding model

One-hundred and forty one mice were randomized to receive in the carotid artery either saline, (normal control (n=16) or hemophiliac control (n=16)), rFVIIa (1 mg/kg (n=8), 3 mg/kg (n=16), 6 mg/kg (n=8) or 10 mg/kg (n=15)), FVIIa IIa (1 mg/kg (n=14) or 3 mg/kg (n=8)), K337A-FVIIa (1 mg/kg (n=6) or 3 mg/kg (n=5)) or M298Q-FVIIa (1 mg/kg (n=15) or 3 mg/kg (n=14)), see Table 1. The bleeding model was performed as a tail bleeding model, developed in rats by Dejana et al. (20). The bleeding was initiated 30 minutes after injection of anti-FVIII antibodies (saline for the control group) by cutting the tip of the tail, 2 mm, approximately 2.5 mg with a scarp scissor. The tail was placed in saline at 37 °C 10 minutes before the cut. Immediately after cutting the tail it was placed in saline, and 5 minutes later test substance or vehicle was injected in the carotid artery, the tail was replaced in a new container with saline and the bleeding was monitored for 30 minutes. The total bleeding time is defined as the sum of the duration of all the bleeding episodes from injection of drug till termination of the study. Blood loss was determined by measuring the accumulated amount of hemoglobin in the saline from the time of injection of drug. The hemoglobin measurement was performed by addition of
hemoglobin reagent (JT Baker, 3073, Bie & Berntsen A/S), thereby converting hemoglobin into cyanmethemoglobin, and measured spectrophotometrically on a SpectraMAX™ (Molecular Devices Corp., Sunnyvale, CA). Human hemoglobin standards (JT Baker, 3074, Bie & Berntsen A/S) were used for the standard curve. Animals were euthanized at the end of the bleeding observation period and the left kidney was excised for histopathology.

**Blood sampling**

In the pharmacokinetic study, mice were briefly anaesthetized in isoflouran/O₂/N₂O and blood was collected from the orbital vein complex. 45 µl blood was transferred to tubes containing 5 µl trisodium citrate (0.13 M), and 30 µl was transferred to 270 µl FVII ELISA dilution buffer (DAKO A/S, Glostrup, Denmark) and centrifuged at 4000 x g for 5 minutes. The supernatant was transferred to a fresh tube and stored at -80°C until analysis.

In the tail bleeding study, blood samples of 1.0 ml were collected in trisodium citrate (0.13 M) at the termination of the study. Plasma was prepared from whole blood centrifuged at 4000 x g for 5 minutes. The plasma was transferred to new plastic tubes and stored at -80°C until analysis.

**Plasma analyses**

Plasma concentrations of FVIIa antigen were determined by an ELISA assay (Factor VII EIA Kit, DAKO A/S, Glostrup, Denmark) as previously described (21). The APTT and PT were measured using an ACL 9000 Research coagulometer (Instrumentation Laboratory, Milan, Italy) following the manufacturer’s instructions. A pool of normal mouse plasma was used as control. Complete blood counts were
determined from EDTA stabilized whole blood using a Medonic CA 620 (Boule Nordic, Kastrup, Denmark).

**Histopathology**

The left kidney was excised from each animal and fixed in 10% buffered formalin. The kidneys from normal controls (n=8), hemophilia controls (n=8), and animals that had received rFVIIa 10 mg/kg (n=8), FVIIaIIa 3 mg/kg (n=8), K337A-FVIIaIIa 3 mg/kg (n=5) and M298Q-FVIIa 3 mg/kg (n=8), respectively, were trimmed by a mid section transversely so that the kidney was cut in two halves. Both halves were dehydrated in grading ethanol and embedded in paraffin. Two pair of serial sections were cut at a nominal thickness of 5 µm and stained with haematoxylin-eosin (HE) and phosphotungsten acid haematoxylin (PTAH), respectively. The HE stain was meant to give an overview of the general morphology, and the PTAH stain was used for evaluation of any presence of fibrin in the tissue. The histology of each kidney was evaluated blindly using light microscopy (22).

**Design and statistics**

The pharmacokinetic study was carried out as an open randomized experiment. The tail bleeding studies were carried out as blinded randomized experiments. The bleeding time, PT and APTT were tested using non-parametric test, the Kruskal-Wallis test, in case of overall p < 0.05 it was followed by Dunns post test comparing the normal controls (A) and the hemophilia controls (B), as well as the treated groups (C–L) to the hemophilia control group (B).

The body weight, and hematological parameters (hemoglobin and platelet counts) as well as blood loss were tested using ANOVA followed by Bonferronni´s multiple
comparison test. In order to evaluate the dose response of rFVIIa, a non-parametric
correlation, the Spearman test, was performed testing the groups B – F for the
bleeding time and blood loss.

RESULTS

Effects of FVIIa and FVIIa analogues in murine tail bleeding model of
hemophilia A

The FVIII activity was evaluated in control mice and was on average 0.8 units/ml. In
mice receiving anti-FVIII antibodies the activity decreased below the detection limit
(< 0.005 units/ml). The cross reactivity of the anti-FVIII antibodies was evaluated
against FVIII, factor V (FV), prothrombin (FII) or FX and revealed no effects on FV,
FII and FX while FVIII activity was efficiently neutralized (Table 2). The bleeding
time increased in a highly significant way in the hemophilia control group (median
1800 seconds (equals the entire observation period)) as compared to the normal
control group (median 45 seconds) (p<0.001, Table 3) thereby verifying the
hemophilic phenotype. The bleeding time was decreased after administration of
FVIIa_{IIIa} at 3 mg/kg and significantly decreased by 10 mg/kg rFVIIa (median 120
seconds, p<0.001), 3 mg/kg K337-FVIIa_{IIIa} (median 35 seconds, p<0.05) and 3 mg
M298Q-FVIIa (median 76 seconds, p<0.001) compared to the hemophilia control
group (Table 3). The dose response for each of the rFVIIa variants on the bleeding
time was tested and a highly significant dose response was observed for all
compounds, rFVIIa (p<0.0001), FVIIa_{IIIa} (p=0.0015), K337A- FVIIa_{IIIa} (p=0.0018) and
M298Q-FVIIa (p<0.0001). The effect of the FVIIa variants was even more
pronounced when evaluating the blood loss (Table 3 and Fig. 1). The blood loss was
significantly increased in the hemophilia controls compared to the normal controls (p < 0.001). The blood loss was significantly decreased after administration of 3 (p<0.001), 6 (p<0.001) and 10 (p<0.001) mg/kg rFVIIa, 1 (p<0.01) and 3 (p<0.001) mg/kg FVIIa_{IIIa}, 1 (p<0.05) and 3 (p<0.01) mg/kg K337A-FVIIa_{IIIa}, and 1 (p<0.05) and 3 (p<0.001) mg/kg M298Q-FVIIa compared to the hemophilia control group. As for the bleeding time, the observed effects on the blood loss verified a highly significant dose response for all FVIIa variants; rFVIIa (p<0.0001), FVIIa_{IIIa} (p=0.0044), K337A-FVIIa_{IIIa} (p=0.0017) and M298Q-FVIIa (p<0.0001).

Hemoglobin concentration and platelet count were measured and showed no differences between the groups, normal versus hemophilia control and between the hemophilia control and the groups treated with rFVIIa or one of the analogues (data not shown). The average hemoglobin level was 9.0 mmol/l (SD 1.7 mmol/l) and the average platelet count was 597x10^9/litre (SD 165x10^9/l).

**Plasma analysis**

Due to the limited blood volume, only one blood sample was taken from each mouse for analysis at termination of the study.

The average PT at termination of the study was 6-8 seconds in all, whereas in the group treated with 10 mg/kg rFVIIa the PT was shortened significantly (p < 0.01) (Table 4). The mean APTT values were 30 seconds in the normal controls, the hemophilic controls and the group dosed 1 mg/kg rFVIIa. In all the other groups the APTT as expected was shortened and even significantly after administration of 3 mg/kg rFVIIa (p<0.001), 10 mg/kg rFVIIa (p<0.05), 1 and 3 mg/kg FVIIa_{IIIa} (p<0.05), 3 mg/kg K337A-FVIIa_{IIIa} (p<0.001), 1 mg/kg M298Q-FVIIa (p<0.001) and 3 mg/kg M298Q-FVIIa (p<0.0001) (Table 4).
Pharmacokinetics of FVIIa and FVIIa analogues in NMRI mice

Following intravenous administration of rFVIIa and the analogues FVIIaIIa, K337A-FVIIaIIa and M298Q-FVIIa, the half-life was in the range of 1.0-1.4 h for both rFVIIa and the analogues as seen in Table 5. The clearance (CL) and volume of distribution ($V_z$) was higher for the analogues than for rFVIIa, increasing in the order FVIIa < M298Q-FVIIa < K337A-FVIIaIIa < FVIIaIIa. A comparison of these parameters at the same dose of rFVIIa and rFVIIa analogues reveals approximately a 2-3 fold increase of the values obtained for the analogues. All mice treated with either rFVIIa or one of the three analogues were exposed to the drug up to 4 hours post dosage.

Renal histopathology

Histopathological evaluation of kidneys from normal controls, hemophilia controls, and the four high-dose groups (rFVIIa 10 mg/kg, FVIIaIIa 3 mg/kg, K337A-FVIIaIIa 3 mg/kg and M298Q-FVIIa 3 mg/kg) was conducted and revealed no evidence of treatment-related pathological changes, in particular no deposition of fibrin in glomeruli.

DISCUSSION

In the present study, the hemostatic potential of rFVIIa and three FVIIa analogues in a tail bleeding model in mice with hemophilia A (induced by injection of anti-FVIII antibodies) was investigated, as well as the pharmacokinetics in the mouse. The antibody-induced hemophilia A phenotype was confirmed in vivo by demonstrating the depletion of FVIII activity in mice receiving anti-FVIII antibodies and in an in vitro setting by testing the specificity of the antiserum. No crossreactivity with FV,
FII or FX could be demonstrated, indicating that the model mimics the FVIII deficiency associated with hemophilia A without any interference with the functions of other coagulation factors.

For all the FVIIa variants tested, a highly significant dose response was demonstrated for the bleeding time with a concomitant reduction in blood loss. The bleeding time and blood loss were normalized after administration of 10 mg/kg rFVIIa, 3 mg/kg K337A-FVIIaIIa and 3 mg/kg M298Q-FVIIa. Though the bleeding time was not quite normalized after administration of 3 mg/kg FVIIaIIa, the blood loss was. These data provides proof-of-concept and dose response of rFVIIa in a mouse tail bleeding model in antibody-induced hemophilia A. Furthermore, the efficacy and increased potency of three FVIIa analogues are confirmed and to our knowledge these are the first in vivo data on these compounds. Controversy exists regarding the involvement of TF in the pharmacological effects of rFVIIa (14-16). However, the infusion of test substance 5 minutes after injury gives endogenous murine FVIIa time to initiate hemostasis without competition with human FVIIa or variants thereof. Moreover, it is known that human FVIIa binds murine TF with an affinity at least two orders of magnitude lower than that for human TF (23). Thus, the hemostatic effect of the FVIIa variants in our model is most likely to a large extent TF-independent. The activity of the three FVIIa analogues when bound to TF is not significantly higher than that of FVIIa (6). Thus, the increased in vivo potency of the three FVIIa analogues, with documented increased intrinsic (TF-independent) activity (6) as compared with wild-type FVIIa strongly indicates that the TF-independent activity of FVIIa is of physiological relevance in a mouse model of hemophilia A. A significant TF-independent contribution is supported by the observation that FVIIa shows procoagulant effects under conditions where TF is blocked (24), and these effects are
most likely much more pronounced at a site of injury with an abundance of activated 
platelets. Though the effective dose of rFVIIa in this model is 100 times higher than 
the doses normally used in patients and the species differences between mice and 
humans have to be considered, it is most likely that this also applies to the human 
setting and infers a potential beneficial effect in the treatment of hemophilic patients. 
The mere 3- to 4-fold increase in potency of the FVIIa analogues, much less than the 
enhancements of human FX activation in vitro and of procoagulant activity in TF-free 
clotting of human plasma (6), is presumably to some extent explained by reduced 
compatibility with murine FX or FIX (unpublished observation). All three FVIIa 
analogues exhibit a higher increase in proteolytic than in amidolytic activity, the full 
additional enhancement of proteolytic activity perhaps requiring human FX as the 
substrate. Alternatively, other factors contribute to a diminished net effect of the 
compounds which reduce the degree of superiority of the FVIIa variants with 
increased intrinsic activity. The plasma analyses support the findings of reduced 
bleeding time and blood loss, with a statistically significant reduction in the APTT in 
all high-dose groups. 
The pharmacokinetics revealed systemic exposure of rFVIIa and all three analogues 
for up to 4 hours. The fact that the total body clearance (CL) and the volume of 
distribution based on the terminal phase (Vz) was higher for the analogues than for 
rFVIIa as well as the fact that the plasma elimination half-life (t1/2) was within the 
same range (1.0-1.4 hours) support that the increased in vivo potency of the analogues 
is due to their higher intrinsic activity rather than due to an increased exposure. It 
should be noted that the more rapid inhibition of the three superactive FVIIa 
analogues, for instance by antithrombin (6), infers a shorter half-life of the biological 
activity as compared with that of wild-type FVIIa. This is one mode by which the
relative net potency of the superactive FVIIa analogues might be reduced. Nevertheless, these preclinical data encourage continued investigations and support the potential use of FVIIa analogues with increased activity in various clinical settings. The increased potency and TF-independent mechanism of action of the new FVIIa variants might improve the cessation of bleedings in a clinical setting and the hemostatic response in the 10% of the hemophilia patients with inhibitors currently treated with rFVIIa without satisfactory effect (5). Finally, histopathological evaluation of kidneys from the high-dose groups was performed and revealed no evidence of treatment-related pathological changes, such as deposition of fibrin in the glomeruli. However, further studies are needed to fully explore the safety of the drugs tested.

In summary, we find that rFVIIa and the three FVIIa analogues investigated significantly shorten the bleeding time and decrease the blood loss in an antibody-induced hemophilia A model in mice without any evidence of adverse effects. The bleeding time and blood loss were normalized after administration of 10 mg/kg rFVIIa, 3 mg/kg K337A-FVIIaIIa or 3 mg/kg M298Q-FVIIa (and almost normalized with 3 mg/kg FVIIaIIa) indicating a potency of these FVIIa analogues roughly 3-4 times that of rFVIIa in FVIII-depleted mice in vivo. These data provide in vivo proof-of-concept for the (increased) hemostatic activity of the superactive FVIIa analogues in hemophilia A mice and indicate the usefulness of improved FVIIa analogues in the currently approved indication for rFVIIa.

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performed by Ingrid Sjögren is gratefully appreciated.
REFERENCES


Table 1. Hemophilia A Mice: Dosages and Injection Times of Test and Control Substances

<table>
<thead>
<tr>
<th>Group</th>
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<td>A. Control</td>
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<tr>
<td>B. Hemophilia Control</td>
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<td>C. Hemophilia / rFVIIa, 1 mg/kg</td>
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<tr>
<td>D. Hemophilia / rFVIIa, 3 mg/kg</td>
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<td>E. Hemophilia / rFVIIa, 6 mg/kg</td>
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<td>F. Hemophilia / rFVIIa, 10 mg/kg</td>
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<tr>
<td>L. Hemophilia / M298Q-FVIIa, 3 mg/kg</td>
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$^1$ 3.3 ml 0.9% (w/v) NaCl/kg bodyweight
$^2$ 10.0 ml 0.9% (w/v) NaCl/kg bodyweight
$^3$ 4950 BU anti-FVIII/kg bodyweight ~ 3.3 ml/kg bodyweight
$^4$ Reconstituted rFVIIa and analogues 10.0 ml/kg bodyweight
Table 2. Cross reactivity of anti-FVIII antibodies.

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<th>Coagulation time (sec.)</th>
<th>FVIII:C (U/ml)</th>
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<td>Mouse plasma + Ab</td>
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<td>Mouse plasma - Ab</td>
<td>11.7 11.8</td>
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<td>19.2 19.3</td>
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<td><strong>FX:C (U/ml)</strong></td>
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Table 3. Bleeding time and blood loss.

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<tr>
<td>B. Hemophilia Control</td>
<td>1800 *** vs. A</td>
<td>0-1800</td>
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<tr>
<td>C. Hemophilia / rFVIIa, 1 mg/kg</td>
<td>1685 ** vs. A</td>
<td>575-1762</td>
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<td>649 ** vs. B</td>
<td>0-1800</td>
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<td>487 ** vs. B</td>
<td>75-1690</td>
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<td>0-1637</td>
</tr>
<tr>
<td>G. Hemophilia / FVIIaIIa, 1 mg/kg</td>
<td>1083 *** vs. B</td>
<td>0-1800</td>
</tr>
<tr>
<td>H. Hemophilia / FVIIaIIa, 3 mg/kg</td>
<td>568 *** vs. B</td>
<td>122-1671</td>
</tr>
<tr>
<td>I. Hemophilia / K337A-FVIIaIIa, 1 mg/kg</td>
<td>862 * vs. B</td>
<td>0-1800</td>
</tr>
<tr>
<td>J. Hemophilia / K337A-FVIIaIIa, 3 mg/kg</td>
<td>35 * vs. B</td>
<td>26-816</td>
</tr>
<tr>
<td>K. Hemophilia / M298Q-FVIIa, 1 mg/kg</td>
<td>1334 * vs. B</td>
<td>0-1800</td>
</tr>
<tr>
<td>L. Hemophilia / M298Q-FVIIa, 3 mg/kg</td>
<td>76 *** vs. B</td>
<td>0-76</td>
</tr>
</tbody>
</table>
Table 4. Hematological parameters.

<table>
<thead>
<tr>
<th>Group</th>
<th>PT (seconds)</th>
<th></th>
<th>APTT (seconds)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>SD</td>
<td>Average</td>
<td>SD</td>
</tr>
<tr>
<td>A. Control</td>
<td>7.6</td>
<td>1.0</td>
<td>27.9</td>
<td>10.7</td>
</tr>
<tr>
<td>B. Hemophilia Control</td>
<td>8.1</td>
<td>3.2</td>
<td>35.1</td>
<td>9.9</td>
</tr>
<tr>
<td>C. Hemophilia / rFVIIa, 1 mg/kg</td>
<td>6.2</td>
<td>2.5</td>
<td>30.1</td>
<td>7.3</td>
</tr>
<tr>
<td>D. Hemophilia / rFVIIa, 3 mg/kg</td>
<td>5.6</td>
<td>1.5</td>
<td>13.9</td>
<td>4.6</td>
</tr>
<tr>
<td>E. Hemophilia / rFVIIa, 6 mg/kg</td>
<td>6.4</td>
<td>2.3</td>
<td>18.5</td>
<td>9.5</td>
</tr>
<tr>
<td>F. Hemophilia / rFVIIa, 10 mg/kg</td>
<td>4.2 **</td>
<td>vs. B</td>
<td>14.3 *</td>
<td>vs. B</td>
</tr>
<tr>
<td>G. Hemophilia / FVIIa, 1 mg/kg</td>
<td>7.0</td>
<td>0.1</td>
<td>20.3 *</td>
<td>vs. B</td>
</tr>
<tr>
<td>H. Hemophilia / FVIIa, 3 mg/kg</td>
<td>7.0</td>
<td>14.6 *</td>
<td>vs. B</td>
<td>2.1</td>
</tr>
<tr>
<td>I. Hemophilia / K337A-FVIIa, 1 mg/kg</td>
<td>7.4</td>
<td>16.2</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>J. Hemophilia / K337A-FVIIa, 3 mg/kg</td>
<td>7.2</td>
<td>12.6 ***</td>
<td>vs. B</td>
<td>0.6</td>
</tr>
<tr>
<td>K. Hemophilia / M298Q-FVIIa, 1 mg/kg</td>
<td>6.6</td>
<td>18.6 **</td>
<td>vs. B</td>
<td>6.2</td>
</tr>
<tr>
<td>L. Hemophilia / M298Q-FVIIa, 3 mg/kg</td>
<td>6.4</td>
<td>14.1 ***</td>
<td>vs. B</td>
<td>7.8</td>
</tr>
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</table>
Table 5. Pharmacokinetic parameters of rFVIIa and analogues administered as a single dose iv. to NMRI mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>( t_{1/2} ) (h)</th>
<th>CL (ml/h/kg)</th>
<th>( V_z ) (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFVIIa</td>
<td>1</td>
<td>1.0</td>
<td>204</td>
<td>302</td>
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<tr>
<td></td>
<td>3</td>
<td>1.1</td>
<td>121</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.2</td>
<td>128</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.4</td>
<td>117</td>
<td>232</td>
</tr>
<tr>
<td>FVIIa_{IIa}</td>
<td>1</td>
<td>1.1</td>
<td>425</td>
<td>673</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.4</td>
<td>326</td>
<td>644</td>
</tr>
<tr>
<td>K337A-FVIIa_{IIa}</td>
<td>1</td>
<td>1.3</td>
<td>339</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.1</td>
<td>261</td>
<td>430</td>
</tr>
<tr>
<td>M298Q-FVIIa</td>
<td>1</td>
<td>1.3</td>
<td>256</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.3</td>
<td>262</td>
<td>476</td>
</tr>
</tbody>
</table>
Table and figure legends.

Table 1. Illustrating the group sizes, dosages and injection times of test and control substances.
Table 2. Cross reactivity of anti-FVIII was evaluated against FVIII, FV, FII and FX.
The cross reactivity of the anti-FVIII antibodies to other relevant coagulation proteins (FV, FX and FII) was tested in a standard assay and revealed no effects on FV, FII and FX while FVIII activity was efficiently neutralized.

Table 3. Total bleeding time and blood loss.
Bleeding time:
Statistical significance was tested using non-parametric test, the Kruskal-Wallis test, in case of overall p < 0.05 it was followed by Dunns post test comparing all groups to the hemophilia control group.
The total bleeding time is defined as the sum of the duration of all the bleeding episodes from injection of drug till termination of the study. Observation time was 30 minutes.
The bleeding time was increased in a highly significant way in the hemophilia control group compared to the normal control group (** p<0.001). The bleeding time was significantly decreased after administration of 10 mg/kg rFVIIa(p<0.001), 3 mg/kg K337-FVIIa_{M298Q} (p<0.05) and 3 mg M298Q-FVIIa (p<0.001) compared to the hemophilia control group.
The dose response of each of the rFVIIa compounds on the bleeding time was tested and a highly significant dose response was observed for all compounds, rFVIIa (p<0.0001), FVIIa_{M298Q} (p=0.0015), K337A-FVIIa_{M298Q} (p=0.001$) and M298 Q-FVIIa (p<0.0001).

Blood loss:
Statistical significance was analyzed by ANOVA in case of overall p < 0.05 it was followed by Bonferroni’s multiple comparison test, comparing all groups to the hemophilia control group.
The blood loss was significantly decreased after administration of 3 (p<0.001), 6 (p<0.001) and 10 (p<0.001) mg/kg rFVIIa, 1 (** p<0.01) and 3 (p<0.001) mg/kg FVIIa_{M298Q}, 1 (p<0.05) and 3 (p<0.01) mg/kg K337A-FVIIa_{M298Q} and 1 (p<0.05) and 3 (p<0.001) mg/kg M298Q-FVIIa compared to the hemophilia control.
The dose responses of the FVIIa compounds were tested for the blood loss with a highly significant dose response for all compounds, rFVIIa (p<0.0001), FVIIa_{M298Q} (p=0.0044), K337A- FVIIa_{M298Q} (p=0.0017) and M298Q-FVIIa (p<0.0001).

Table 4. Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT).
Statistical significance was tested using non-parametric test, the Kruskal-Wallis test, in case of overall p < 0.05 it was followed by Dunns post test comparing all groups to the hemophilia control group.
   a. PT: The PT was significantly decreased (** p < 0.01) after administration of 10 mg/kg rFVIIa at termination of the study. In all other groups the PT was 6-8 seconds with no significant differences between the groups.
   b. APTT: There were no differences in the APTT between the normal control, hemophilia control and the hemophilia group dosed 1 mg/kg rFVIIa. In all other groups APTT was decreased, even significantly compared to the hemophilia control group in the following groups: 3 mg/kg rFVIIa (** p<0.001), 10 mg/kg rFVIIa (* p<0.05), 1 and 3 mg/kg FVIIa_{M298Q} (p<0.05), 3 mg/kg K337A-FVIIa_{M298Q} (p<0.001), 1 mg/kg M298Q-FVIIa (p<0.001) and 3 mg/kg M298Q-FVIIa (p<0.0001).
Table 5. Pharmacokinetics

Blood samples were drawn from three animals/time point at predose (time 0), 2, 5, 10, 20, 40 min, 1, 2, 3 and 4 hours. Plasma samples were analyzed for content of rFVIIa and FVIIaIIa, K337A-FVIIaIIa and M298Q-FVIIa and the following parameters were estimated: terminal plasma elimination half-life ($t_{1/2}$), based on the natural logarithm of two divided by the slope of the log-linear regression of the concentration versus time ($\lambda_z$). The total body clearance (CL) was determined as dose divide by area under the plasma concentration curve (AUC) from zero to infinity. The volume of distribution based on the terminal phase ($V_z$) was estimated by dose divide by AUC times $\lambda_z$. The clearance and volume of distribution ($V_z$) was higher for the analogues than for rFVIIa, increasing in the order FVIIa < M298Q-FVIIa < K337A-FVIIaIIa < FVIIaIIa. A comparison of these parameters at the same dose of rFVIIa and rFVIIa analogues reveals approximately a 2-3 fold increase of the values obtained for the analogues. All mice treated with either rFVIIa or one of the three analogues were exposed to the drug up to 4 hours post dosage.

Figure 1. Blood loss.

The blood loss is expressed as the amount of hemoglobin bleed from the tail into the container with saline as nmol hemoglobin per ml saline.

A. Control; B. Hemophilia Control; C. Hemophilia / rFVIIa, 1 mg/kg; D. Hemophilia / rFVIIa, 3 mg/kg; E. Hemophilia / rFVIIa, 6 mg/kg; F. Hemophilia / rFVIIa, 10 mg/kg; G. Hemophilia / FVIIaIIa, 1 mg/kg; H. Hemophilia / FVIIaIIa, 3 mg/kg; I. Hemophilia / K337A-FVIIaIIa, 1 mg/kg; J. Hemophilia / K337A-FVIIaIIa, 3 mg/kg; K. Hemophilia / M298Q-FVIIa, 1 mg/kg; L. Hemophilia / M298Q-FVIIa, 3 mg/kg.

Data are presented as bars plus standard deviation. Statistical significance was tested, using one-way analysis of variance (ANOVA). In cases of $p < 0.05$ pair-wise comparison were made at the same level and corrected for multiple comparisons (Dunnett) testing all groups to the hemophilia control (B). The blood loss was increased significantly in the hemophilia control (B) compared to the normal control (A) (**$p < 0.001$).

The blood loss was significantly decreased after administration of 3 (p<0.001), 6 (p<0.001) and 10 (p<0.001) mg/kg rFVIIa, 1 (**p<0.01) and 3 (p<0.001) mg/kg FVIIaIIa, 1 (*p<0.05) and 3 (p<0.01) mg/kg K337A-FVIIaIIa, and 1 (p<0.05) and 3 (p<0.001) mg/kg M298Q-FVIIa compared to the hemophilia control.
Figure 1.

Blood loss

![Bar graph showing blood loss in nmol/ml with significance levels indicated by asterisks: *** for p<0.001, ** for p<0.01, * for p<0.05. The graph compares different conditions labeled A to L.]
Improved hemostasis with superactive analogues of factor VIIa in a mouse model of hemophilia A

Mikael Tranholm, Kim Kristensen, Annemarie T Kristensen, Charles Pyke, Rasmus Roejkjaer and Egon Persson