Introduction

Coagulation factor VII (FVII) is a vitamin K-dependent blood coagulation factor with a molecular weight of approximately 50 kDa, synthesized in the liver and secreted as an inactive proenzyme into the bloodstream (1). After forming a complex with tissue factor (TF) and activation, FVIIa/TF initiates the extrinsic coagulation pathway via activation of coagulation factors X and IX. The plasma concentration of FVII is 10 nM, and approximately 1% circulates in the activated form as FVIIa in healthy individuals (2). In 1996, recombinant FVIIa (NovoSeven®) became available as one treatment option for haemophilia A and B inhibitor patients. Since then, recombinant FVIIa has demonstrated efficacy in treating bleeding episodes in haemophilia patients with inhibitors, combined with a good safety profile (3, 4). For treatment of such bleeding episodes, rFVIIa is applied as a bolus injection with a standard dose of approximately 90 μg/kg (= 26 nM) (5), resulting in a 200- to 300-fold increase of the plasma concentration of FVIIa (2). rFVIIa’s balance of efficacy and safety also facilitated the use of rFVIIa in other coagulopathies, promoting it as a general haemostatic agent for cessation of bleeding irrespective of the underlying cause (6). However, the short in-vivo half-life of approximately 2.5 hours (7) often makes multiple injections necessary. For the treatment of joint bleeding episodes in inhibitor patients, at least two injections of NovoSeven® are required (8, 9). During surgical interventions the dosing frequency increases significantly, and NovoSeven is given every 2–3 h for at least two days (10, 11), resulting in median numbers of total injections of 38 (range: 26–67) for minor surgery and 81 (range: 71–128) for major surgical interventions (11).

Thus, improved FVIIa variants with an extended half-life and comparable pro-coagulatory properties to wild-type rFVIIa have a high potential to reduce the number of injections per treatment episode. To date, extension of FVIIa half-life while maintaining the biological activity has not been described. One way to extend the half-life of therapeutic proteins is the genetic fusion to a protein with a longer half-life in circulation, such as IgG (12) or albumin (13). Albumin is the most abundant protein in plasma with an in-vivo half-life ranging from 17 to 19 days (14). For small proteins, it has been demonstrated that genetic fusion to albumin resulted in a significant increase of in-vivo half-life of the fusion protein (15–17). In a study performed in Cynomolgus monkeys, fusion of interferon-α to albumin resulted in an 18-fold longer half-life compared to interferon-α (18). This significant half-life extension was also confirmed in clinical trials in humans (19, 20), and a dosing of once every two weeks was supported by the data compared with a once every two days dosing regimen for native interferon-α.
There has also been an attempt to extend the half-life of coagulation factor IX (FIX) by genetic fusion to albumin (21). However, in this experiment the biological activity of the fusion protein was significantly reduced, and the observed half-life extension of the fusion protein was only moderate. Based on these results, the half-life extension by albumin fusion seemed not to work for complex molecules like FIX. A comparably negative result in terms of half-life extension was expected for a FVII albumin fusion protein as FIX and FVII are highly homologous proteins that both belong to the vitamin K dependent coagulation factor family.

Here we describe the generation of a rFVIIa molecule with an extended half-life based on fusion to human albumin via a flexible glycine-serine linker. The recombinant FVII-albumin fusion protein (rVII-FP) was expressed in mammalian cells and characterized in vitro and in vivo. It was demonstrated that the FVII activity of rVII-FP and rFVIIa-FP was comparable to the wild type rFVII and rFVIIa, respectively. Pharmacokinetic and efficacy studies in rats demonstrated improved properties of the rVIIa-FP. The results of this study demonstrate that it is feasible to develop a half-life extended rFVIIa molecule with very similar haemostatic properties to the wild-type enzyme.

**Materials and methods**

**Cloning and expression of recombinant FVII molecules**

FVII wild-type cDNA, cloned into expression vector pRRESpro3 (Becton Dickinson) was prepared for genetic fusion to mammalian albumin cDNA by introduction of an XhoI restriction site replacing the natural FVII stop codon by site directed mutagenesis using oligonucleotides 5´gagccccatttccctcgagggccgccgcaaggg3´ and 5´ccctcgagcgggggatctggcgggtctggaggctctggagggtcgggaggctct3´ under standard conditions.

A glycine-serine encoding linker was generated by annealing oligonucleotides 5´gtctcagcgggggatctggcgggtctggaggctctggagggtcgggaggctct3´ and 5´caacctccagctccagctccagccacggccagctccagctccagcaccctc3´ in equimolar concentrations (10 μM) under standard PCR conditions and subsequent amplification using a PCR protocol of a 2 minutes (min) initial denaturation at 94°C followed by seven 15-second (s) cycles of denaturation at 94°C, 15 s of annealing at 55°C, and 15 s of elongation at 72°C, and finally by an extension step of 5 min at 72°C. The resulting linker fragment was digested with restriction endonucleases XhoI and NotI and ligated into the above described plasmid opened with XhoI and NotI (vector linker). Subsequently a BamH1 fragment containing the cDNA of mature human albumin was inserted into the BamH1 site of the linker sequence. This fragment had been generated by PCR on an albumin cDNA sequence using primers 5´gtgctcgagcgggggatctggcgggtctggaggctctggagggtcgggaggctct3´ and 5´caacctccagctccagctccagccacggccagctccagctccagcaccctc3´ under standard conditions.

Plasmids were grown in E.coli TOP10 (Invitrogen) and purified using standard protocols (Qiagen). HEK-293 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) and grown in serum-free medium (Invitrogen 293 Express) in the presence of 50 ng/ml vitamin K and 4 μg/ml Puromycin. Transfected cell populations were spread into T-flasks into roller bottles and small scale fermenters from which supernatant was harvested for purification. For CHO expression, FVII coding sequences were inserted into plasmid pCDNA3.1 (Invitrogen). CHO-S cells (Invitrogen) were transfected as above. High expressing clones were selected and grown in serum-free medium (Invitrogen CD-CHO) in the presence of 50 ng/ml vitamin K and 500 μg/ml G418.

**Purification of recombinant FVII proteins**

Recombinant FVII proteins were purified either by Q-Sepharose anion exchange or TF affinity chromatography. Briefly, cell culture harvest containing rFVII or rVII-FP was applied to a Q-Sepharose FF column previously equilibrated with 20 mM HEPES buffer pH 7.4. After washing the column with 10 column volumes (CV) of HEPES buffer, bound FVII molecules were eluted by a linear gradient from 0 to 1.0 M NaCl in 20 mM HEPES buffer. Alternatively, TF (Dade Behring) was coupled to commercially available BrCN activated Sepharose (GE) using the coupling procedure described by the vendor. The column was equilibrated in 20 mM HEPES buffer pH 7.2 containing 0.14 M NaCl and 10 mM CaCl2 before application of cell culture supernatant. The column was then washed with a 20 mM HEPES buffer pH 7.2 containing 0.14 M NaCl and 1 mM CaCl2.Bound FVII was eluted with a 100 mM sodium citrate buffer pH 7.2 containing 0.14 M NaCl.

**Activation of rFVII and rVII-FP**

Two protocols were used for the activation of FVII proteins. rFVII-FP was autoactivated in batch-mode by incubating 75 IU/ml (as determined by the chromogenic assay) in 20 mM Tris, 100 mM NaCl, 0.5 mM CaCl2, pH 8.5 and containing 60 μl Q-Sepharose FF /ml (iHealthcare) at ambient room temperature for about 20 h. Autoactivation was stopped and the protein desorbed from the resin by addition of 2 M NaCl to a final molar concentration of >0.5 M. The resin was separated by centrifugation (approx. 2000 rpm). For autoactivation of rVII-FP on a column, about 600 IU were applied to a 1 ml Hitrap Q-Sepharose FF column (GE Healthcare) at ambient room temperature for about 20 h. After autoactivation, the column was washed with 10 column volumes (CV) of HEPES buffer, bound FVII molecules were eluted by a step gradient to 1 M NaCl. The column was washed at a low flow rate (3 cm/h) with 20 CV of the same buffer solution. Elution of the activated forms was achieved by a step gradient to 1 M NaCl in the same buffer at an increased flow rate (27 cm/h).

**SDS-PAGE/Western blot**

SDS-PAGE analysis was performed on 8–16% Tris-glycine gradient gels using SeeBlue Plus 2 molecular size marker (Invitrogen). Anti-human FVII polyclonal antibody from sheep (Hematologic Technologies Inc.) and a monoclonal anti-human albumin (Ro3 (Becton Dickinson) was prepared for genetic fusion to albumin by introduction of an XhoI restriction site replacing the natural FVII stop codon by site directed mutagenesis using oligonucleotides 5´gtgctcgagcgggggatctggcgggtctggaggctctggagggtcgggaggctct3´ and 5´caacctccagctccagctccagccacggccagctccagctccagcaccctc3´ under standard conditions.

SDS-PAGE/Western blot analysis. Blots were stained using the horseradish peroxidase system Vector® VIP from Vector Laboratories.

**N-terminal sequencing**

rFVII and rVII-FP preparations were separated by SDS-PAGE (8–16%) and subsequently electroblotted to a PVDF membrane. The bands of interest were cut out and put on a purified Biobrene treated glass fiber filter. The N-terminal sequence analysis was carried out by Edman degradation using a pulsed-labeled protein sequencer (Procise 492, Applied Biosystems) equipped with a 140 C HPLC microgradient system.

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**Determination of FVII activity and antigen**
rFVII and rVII-FP activity were determined using a commercially available chromogenic test kit (Chromogenix Coaset FVII) using standard human plasma (Dade Behring) as reference. FVII antigen (FVII:Ag) was determined by an ELISA using a sheep anti-human FVII IgG (Cedarlane) as capture and a POD-labelled sheep anti-human FVII IgG (Cedarlane) as detection antibody. Concentrations of test samples were calculated using standard human plasma as reference. For pharmacokinetic analyses the respective FVII or FVIIa preparations were used as references. To perform the activated partial thromboplastin time assay (aPTT) FVIIa deficient plasma (Dade Behring) was substituted with rVIIa-FP or NovoSeven (Novo Nordisk) to final concentrations of 100, 20, 4 and 0.8 nM rFVIIa per liter plasma. The molarities were calculated from FVII:Ag values determined by the FVII ELISA. A 25 mM Na-citrate buffer pH 6.0 supplemented with 100 mM NaCl and 0.1% human albumin was used as a control. The aPTT was conducted with substituted deficient plasma in a “Schnitger & Gros” coagulometer using the Pathromtin SL reagent (Dade Behring). For calibration, FVII-deficient plasma was substituted with 0%, 5%, 20% and 100% of standard human plasma.

**Thrombelastography**
To prepare FVIII deficient blood, citrated blood from a single donor was incubated for 2 h at 37°C with an inhibitory polyclonal anti-human FVIII antibody from rabbit (in-house preparation) to a final concentration of 10 Bethesda Units per ml (= inhibitor blood). Inhibitor blood was substituted with rVIIa-FP or NovoSeven to final concentrations of 10, 2, and 0.4 nM. The molarities were calculated from FVII:Ag values determined by a FVII ELISA. A 25 mM Na-citrate buffer pH 6.0 supplemented with 100 mM NaCl and 0.1% human albumin was used as a control. To generate a calibration curve, inhibitor blood was substituted with 0%, 5%, 20% and 100% normal blood. Thrombelastography was carried out in a ROTEM® analyzer using the star-TEM reagents (Pentapharm) according to the manufacturer’s instructions. Clotting time (CT), clot formation time (CFT), maximal clot firmness (MCF) and angle alpha were measured.

**Surface plasmon resonance (SPR) analysis**
SPR analysis was performed on a Biacore 3000 instrument (GE Healthcare). Recombinant TF was coupled to a CM5 chip via aminocoupling. Dilutions of rFVIIa (NovoSeven) and rVIIa-FP preparations (20, 10, 5, and 2.5 μg/ml) in SDB (100 mM HEPES, 150 mM sodium chloride, 0.005% Polysorbate 20, 10 mM calcium chloride, pH 7.4) were injected for 12 min followed by 10 min of SDB and 3 min of regeneration buffer (100 mM sodium citrate). Evaluation was performed using 1:1 Langmuir curve fitting for association and dissociation.

**Pharmacokinetic study**
rFVIIa, rVIIa-FP and NovoSeven were administered intravenously to CD rats (six rats per substance) with a dose of 100 μg/kg body weight. For all in-vivo experiments, the amount of the respective protein was determined on the basis of FVII activity measured with the chromogenic test. For each FVII test substance, the injected amount was calculated by setting 1,000 IU FVII activity equal to 500 μg FVII protein. Plasma-derived human serum albumin (CSL Behring GmbH) was administered intravenously at a dose of 500 mg/kg. Blood samples were drawn retroorbitally using an alternating sampling scheme to minimize interference of the sampling procedure with the plasma levels to be quantified: from three rats at 5 and 30 min, and 1, 2 and 8 h, and from the remaining three rats at 15 and 45 min, and 1.5, 4 and 24 h after application. Plasma was prepared immediately after sampling and stored at –20°C until analysis. FVII and albumin concentrations were quantified in a pooled fashion (the three samples per group and time point were pooled) by the FVII or human albumin specific ELISA. Half-lives were calculated according to the formula $t_{1/2} = \ln 2 / k$, whereby $k$ represents the slope of the regression line of the semi-logarithmic concentration versus time data. The area under the curve (AUC) was calculated using the linear trapezoidal rule (22).

**FVII depletion model**
In order to assess in-vivo activity, the rats were treated once per os with 2.5 mg/kg phenprocoumon (Roche). In a first experiment, a dose of 50 μg (FVII activity)/kg body weight HEK-derived rVIIa-FP and NovoSeven were injected intravenously 15.75 h following this treatment. Blood samples were drawn retroorbitally 15 min later, anticoagulated by citrate, and evaluated for thrombelastography (ex-TEM®) parameters on a ROTEM® device (Pentapharm, Germany). In a second experiment, CHO-derived rVIIa-FP and NovoSeven (100 μg/kg body weight) were injected i.v. using the same design as described above, but immediately following the phenprocoumon administration. Thrombelastography was performed 16 hours after phenprocoumon treatment.

**Results**

**Expression, purification, activation, characterization**
By genetic engineering human albumin cDNA was fused via a linker sequence encoding a 31 amino acid glycine/serine linker to the 3’-end of human FVII cDNA. The length of the linker was designed to separate albumin and FVII in the fusion protein minimizing disturbing interactions between the two protein moieties. The corresponding rVII-FP construct was transfected into either CHO or HEK-293 cells. As a control, a human FVII cDNA was transfected. Expression in HEK-293 and CHO cells of the rVII-FP cDNA gave in general comparable results with regard to FVII activity and other protein characteristics. However, one major difference was that the expression of rVII-FP in HEK-293 cells resulted in the uncleaved rVII-FP, whereas the corresponding rVII-FP from CHO cell supernatant was always a mixture of rVII-FP zymogen and activated rFVII-FP. Thus, the characterization of the rVII-FP zymogen was limited to material expressed in HEK-293 cells.

Purification of the rVII and rVII-FP was accomplished through immobilized TF resulting in a homogenous protein preparation of high purity. On SDS-PAGE (Fig. 1A) the rVII-FP preparation from HEK-293 cells migrated as a single band with an apparent molecular weight of approximately 120 kDa, and the corresponding molecular weight of the FVII control was approximately 59 kDa. Both proteins reacted with antibodies spe-
specific for FVII on immunoblots (Fig. 1B). The rVII-FP also reacted with antibodies specific for albumin (Fig. 1C). The identity of both recombinant proteins was further verified by N-terminal sequencing (data not shown).

For functional characterization the FVII coagulation activity was determined (Table 1). Both recombinant proteins displayed strong FVII activity. Due to the high purity of the protein preparation, the protein concentration was determined by OD280 measurement using the extinction coefficients of human FVII and albumin. The specific FVII activity was calculated from these data, and a specific activity of 2,874 IU/mg was found for rFVII versus 771 IU/mg for rVII-FP, both from HEK-293 cells. In order to facilitate the direct comparison of the two proteins, the molar specific activity was calculated. The molar specific activity of rFVII was 143.7 IU/nM versus 82.7 IU/nM for rVII-FP. Thus, rFVII is approximately 1.7-fold more potent in this assay than rVII-FP.

The purified rVII-FP and rFVII proteins, both from HEK-293 cells, were converted to the corresponding activated forms by autoactivation on an anion exchange resin (23, 24). This procedure also completed the conversion to rVIIa-FP of the rVII-FP/ rVIIa-FP mixture isolated from CHO cells. After this step the rVIIa-FP proteins from HEK-293 and CHO cells were indistinguishable. During activation FVII is cleaved at R152 and the resulting heavy and light chains are held together by a single disulfide bridge. In order to evaluate the generation of rFVIIa and rVIIa-FP, the proteins from HEK-293 cells were compared by SDS-PAGE under reducing conditions (Fig. 1A). The heavy and light chains of rFVIIa were separated and migrated as well-separated bands at approximate molecular weights of 30 and 25 kDa. The rVIIa-FP migrated under reducing conditions as a 100 kDa and a 25 kDa band. Both proteins reacted with antibodies specific for FVII on immunoblots (Fig. 1B). The 100 kDa band of the rVIIa-FP also reacted with antibodies specific for albumin, indicating that this band represents the FVII heavy chain fused to albumin (Fig. 1C). The rVIIa-FP from CHO cells gave identical results and N-terminal sequencing of rVIIa-FP from either CHO or HEK-293 material verified the N-termini of light and heavy chains (data not shown). After activation, both protein preparations contained some amounts of non-activated material migrating on SDS-PAGE at the apparent molecular weight of the non-cleaved form (Fig. 1, lanes 2 and 3). In addition, the rVIIa-FP preparation contained one prominent additional band migrating at approximately 86 kDa on SDS-PAGE. This band also reacted with antibodies specific for FVII and albumin (Fig. 1B, C) and was identified by N-terminal sequencing as fragments starting at G291 and K316 in the serine protease domain of FVII.

### Table 1: Comparison of specific activity data.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein [mg/ml]</th>
<th>FVII activity [IU/mg]</th>
<th>Specific activity [IU/mg]</th>
<th>Specific molar activity [IU/nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFVII wt</td>
<td>HEK-293</td>
<td>0.118</td>
<td>244 ± 20</td>
<td>2874</td>
</tr>
<tr>
<td>rVII-FP</td>
<td>HEK-293</td>
<td>0.083</td>
<td>64 ± 1</td>
<td>771</td>
</tr>
<tr>
<td>rFVIIa wt</td>
<td>HEK-293</td>
<td>0.122</td>
<td>184 ± 9</td>
<td>2069</td>
</tr>
<tr>
<td>rVIIa-FP</td>
<td>HEK-293</td>
<td>0.165</td>
<td>112 ± 4</td>
<td>626</td>
</tr>
<tr>
<td>rVIIa-FP</td>
<td>CHO</td>
<td>0.658</td>
<td>459 ± 2</td>
<td>620</td>
</tr>
</tbody>
</table>

1calculated via OD280; 2based on the chromogenic FVII assay.
activity was calculated (Table 1). A specific FVII activity of 2,069 IU/mg was found for rFVIIa versus 626 IU/mg for rVIIa-FP (HEK-293) and 620 IU/mg for rVIIa-FP (CHO), and the molar specific activities were 104.8 IU/nM (rFVIIa) and 72.1 to 72.8 IU/nM for the rVIIa-FPs from CHO and HEK-293 cells. Thus, after conversion to the corresponding activated forms, rFVIIa was approximately 1.4-fold more potent in this assay than rVIIa-FP.

In summary, it was possible to generate a recombinant FVII albumin fusion protein with properties similar to the wild-type rFVII, which also could be activated like wild type rFVII.

Pharmacokinetic studies in rats

The pharmacokinetics of rVIIa-FP was compared in rats with human albumin, rFVIIa, and NovoSeven. The time course of the plasma levels for all three FVII preparations and human albumin is shown in Figure 2. To analyze the data quantitatively, half-life and area under the curve (AUC) were calculated for each protein (Table 2). Comparison of these clearance parameters revealed that the half-life of rVIIa-FP was prolonged 5.8 and 6.7 times compared to recombinant FVIIa and NovoSeven, respectively. Compared with human albumin, the half-life of rVIIa-FP was shorter, indicating that the fusion of FVII to albumin partially conveys the pharmacokinetic properties of albumin to the fusion protein. The recovery of the fusion protein was also found to be superior to rVIIa. Five min after injection of a fixed dose of the respective proteins, only 19.5% and 34.8% of the injected amount of NovoSeven and rFVIIa, respectively, were detectable in the plasma samples, whereas a recovery of 47.1% was demonstrated in the case of rVIIa-FP. The combination of increased half-life and improved recovery of rVIIa-FP resulted in a 9.5-(rFVIIa) and 14.5-fold (NovoSeven) increase of the AUC.

These results demonstrate that the in-vivo half-life, the recovery, and the AUC of rVIIa-FP are significantly higher than those of rFVIIa and NovoSeven.

TF interaction and in-vitro efficacy of rVIIa-FP

The physiological ligand of FVII/ FVIIa is TF and binding of FVII to TF is paramount for the initiation of coagulation (26, 27). Due to the fusion of albumin to FVIIa, this interaction could theoretically be influenced. The interaction with TF was investigated by surface plasmon resonance analysis using immobilized TF and NovoSeven as control protein. TF and Q Sepharose FF (QSFF) purified rVIIa-FPs were used. Both rVIIa-FPs and NovoSeven displayed comparable affinities to TF, as indicated by a

Table 2: Calculated parameters of the rat pharmacokinetic study. rFVIIa wt and rVIIa-FP proteins were expressed in CHO cells, purified and activated by Q Sepharose FF chromatography. The PK study was conducted as described in Materials and methods.

<table>
<thead>
<tr>
<th></th>
<th>t1/2 [min]</th>
<th>Recovery [% of initial dose]</th>
<th>AUC [%·min]</th>
<th>Time points considered</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFVIIa wt</td>
<td>39.5</td>
<td>34.8</td>
<td>1811</td>
<td>5 min – 4 h</td>
</tr>
<tr>
<td>NovoSeven</td>
<td>45.6</td>
<td>19.5</td>
<td>1194</td>
<td>5 min – 4 h</td>
</tr>
<tr>
<td>rVIIa-FP</td>
<td>262.7</td>
<td>47.1</td>
<td>17267</td>
<td>5 min – 24 h</td>
</tr>
<tr>
<td>Albumin</td>
<td>621.2</td>
<td>106.4</td>
<td>67774</td>
<td>5 min – 24 h</td>
</tr>
<tr>
<td>Ratio rVIIa-FP/NovoSeven</td>
<td>5.8</td>
<td>2.4</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>Ratio rVIIa-FP/rFVIIa wt</td>
<td>6.7</td>
<td>1.4</td>
<td>9.5</td>
<td></td>
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</tbody>
</table>
Weimer et al. rFVIIa albumin fusion

similar affinity constant (K_{aff}) (Table 3). The association rate constants (k_{ass}) of the rVIIa-FPs were approximately 2- to 3-fold higher, indicating that the rVIIa-FPs displayed a faster binding to TF than NovoSeven. In addition, it was found that the dissociation rate constants (k_{diss}) of the rVIIa-FPs were up to 2-fold higher compared with NovoSeven. Based on the results of dissociation and association rate constants, the affinity constants for both fusion proteins and NovoSeven were calculated and found to be comparable.

**In-vitro efficacy**

NovoSeven is used therapeutically for the treatment of haemophilia A and B patients who have developed an inhibitory antibody response against the replaced coagulation factors VIII and IX, respectively. In order to compare the efficacy of the rVIIa-FP with NovoSeven in this indication, an in-vitro evaluation was performed in a human whole blood test system using ROTEM for analysis. Human blood was collected and the FVIII activity inhibited by the addition of a specific polyclonal antibody preparation, and, as a result, the clot formation time after recalcification was prolonged (Fig. 3). In this system, rVIIa-FP isolated from both HEK-293 and CHO cells and NovoSeven were compared at three different concentrations. All proteins were able to reduce the clotting time significantly. The two rVIIa-FP preparations were comparable. The dose response of rVIIa-FP and rFVIIa in this assay was also similar.

These results indicate that while augmenting the desired pharmacokinetic attributes of rFVIIa the albumin fusion retains the haemostatic properties of FVIIa in this in-vitro model and that the FVIIa moiety in the fusion protein has haemostatic properties similar to NovoSeven.

**In-vivo efficacy**

In order to evaluate the in-vivo activity of the rVIIa-FP, rats were treated with phenprocoumon to inhibit the vitamin-K-dependent gamma-carboxylation of the FVII gla domain. Due to its short half-life, FVII is depleted faster than the other vitamin K-dependent coagulation factors. It has been described that FVII activity was almost completely depleted 16 h after phenprocoumon application. Up to that time point, externally added FVIIa was able to correct the clotting time in rats (28). In order to compare NovoSeven and rVIIa-FP, equal doses of both proteins were injected into rats 15.75 h post phenprocoumon treatment. As shown in Figure 4A, the clotting time of rat blood was corrected to normal values by either rVIIa-FP or NovoSeven. Thus, both proteins display a comparable effect in this model.

In a separate experiment, NovoSeven and rVIIa-FP protein were given immediately after phenprocoumon treatment, and coagulation parameter were determined 16 h later (Fig. 4B). NovoSeven was not able to correct the clotting time under these conditions, as expected from its short half-life of 2.5 h. In contrast, the clotting time of animals treated with rVIIa-FP was corrected to values close to the results of healthy controls, indicating that the rVIIa-FP was still present with full biological activity. These data further confirmed the extended half-life of rVIIa-FP and  

![Figure 3: In-vitro evaluation of rVIIa-FP and NovoSeven in human inhibitor whole blood.](image-url)
also verified that the protein was still functionally active 16 h after injection in rats.

**Discussion**

One of the unmet needs in the coagulation field is to improve the relevant pharmacokinetic parameters of coagulation factors, such as half-life, while retaining the biological activity of the base molecule. In this study, a fusion protein of FVIIa and albumin was generated to extend the half-life of FVII after intravenous injection. An rVII-FP was generated in which albumin was linked via a flexible 31 amino acid glycine-serine linker to the C-terminus of FVII (a fusion to the N-terminus of FVII was not considered as either in the presence of the propeptide the fusion protein would be cleaved upon secretion or in the absence of the propeptide the fusion protein would not be gamma carboxylated). The length of the linker was designed to allow optimal separation of the albumin and FVII in the fusion protein, minimizing potential disturbing interactions between the two protein moieties. While several point mutations are described for FVII (29, 30), the fusion to albumin represents a different approach to improve the properties of FVII. Secretion of the fusion protein was readily achieved in either HEK-293 or CHO cells in the presence of vitamin K, assuring efficient gamma carboxylation of the fusion protein. One notable difference between HEK-293 and CHO cell expression of rVII-FP was that the HEK-293 cells did express solely the rVII-FP zymogen, whereas the CHO cell culture supernatant contained a mixture of rVII-FP zymogen and the activated form. The reason for this difference was not identified in this study. Thus, the characterization of the rVII-FP zymogen was limited to material expressed in HEK-293 cells. The rVII-FP from HEK-293 cells displayed a 1.7-fold lower molar specific FVII activity than wild-type rFVII, which indicates that FVII activation or the FVII activity are affected by the fusion to albumin in the chromogenic assay system. This is not surprising considering the fact that a ternary complex has to be formed upon activation of FX and that an even more pronounced reduction in activity of 2- to 3-fold was described for
fusions of murine FIX to albumin (21). After purification, rVII-FP and rFVII were converted to the corresponding activated forms based on published methods (23, 24). This procedure also completed the conversion to rVIIa-FP of the rVII-FP/rVIIa-FP mixture isolated from CHO cells. Taking into account the contribution of albumin to the molecular weight of rVIIa-FP, the molar specific FVII activity was 1.4-fold lower than rFVIIa. This difference in molar specific FVII activity was identical for rVIIa-FP from either CHO or HEK cells and might be attributed to a steric effect, or to the presence of inactive material, which is composed of both non-activated and autodigested FVII molecules, the latter being generated towards the end of the activation procedure (Fig. 1). Such material was present in both preparations but was more prominent in the rVIIa-FP preparation than in the control wild-type rFVIIa preparation. This might also explain the observed loss of specific activity upon activation of the zymogens.

The interaction of rVIIa-FP with TF was investigated by surface plasmon resonance analysis using immobilized TF. The approximately 2- to 3-fold higher association rate constants of the rVIIa-FPs compared to NovoSeven indicating a faster binding to TF (Table 3) were unexpected and might be explained by the enrichment of strong TF binding rFVII-FP forms during TF affinity purification. However, rVIIa-FP purified by a different method without immobilized TF displayed the same affinity. In addition, it was found that the dissociation rate constants of rVIIa-FPs were up to 2-fold higher than that of NovoSeven. Overall, the affinity constants for the fusion proteins and NovoSeven were comparable.

Recombinant FVIIa (NovoSeven) is licensed for the treatment of haemophilia A and B inhibitor patients. For further comparison of rVIIa-FP with NovoSeven, an additional assay was employed with regard to therapeutic relevance. The FVIII activity in human blood was inhibited by a specific polyclonal antibody preparation, and, as a result, the clotting time after recalcification was prolonged (Fig. 3). In this system, rVIIa-FP isolated from both CHO and HEK-293 cells and NovoSeven were compared at three different concentrations. All proteins were able to reduce the clotting time significantly. The dose response of both rVIIa-FP preparations and NovoSeven in this assay was similar, indicating that the efficacy in this in-vitro model is comparable.

The fusion to albumin seems to have only a small impact on the coagulation properties of the connected FVIIa moiety. This is most likely accomplished by the 31 amino acid glycine/serine linker, which provides sufficient separation of the two proteins.

Fusion of FVIIa to albumin resulted in a prominent half-life extension of 5.8- to 6.7-fold and an increase in recovery of 1.4- to 2.4-fold compared with rFVIIa and NovoSeven, respectively. Although in comparison with human albumin the half-life of the rVIIa-FP was shorter, indicating that the fusion of FVII to albumin does not fully convey the pharmacokinetic properties of albumin to the fusion protein, the half-life of rVIIa-FP was increased considerably over rFVIIa. The combination of increased half-life and improved recovery of rVIIa-FP resulted in a 9.5- (rFVIIa) and 14.5-fold (NovoSeven) increase of the AUC.

Thus, our data demonstrate that it is possible to extend the half-life of a complex protein by fusion to albumin. Albumin and IgG have a similar half-life in circulation, which is the longest of all known plasma proteins (14, 31). Also, the physiological mechanism for this half-life extension appears to be very similar (32, 33). Basically, both proteins bind to the neonatal Fc receptor at two different sites at low pH. It is assumed that ligation with this receptor salvages albumin and IgG from a lysosomal degradation pathway. Instead, the receptor bound proteins are moved back to the cell surface where the complexes dissociate and albumin and IgG are exocytosed to the extracellular space. This specific salvage pathway for albumin and IgG is considered to be the main reason for the extraordinarily long half-life of both proteins. It is reasonable to assume that fusion of proteins to either albumin or the Fc-part of IgG prolongs the half-life of the fusion protein partner via the same mode of action. However, the observed half-life of rVIIa-FP in rats is lower than that of albumin (Fig. 2, Table 2). An explanation for this difference might be that the rVIIa-FP is not only cleared by an albumin-specific mode of action, but also by a presumably faster FVIIa-dependent pathway. The action of these two different clearance pathways resulted in a clearance curve of the rVIIa-FP inbetween the corresponding curves for FVIIa and albumin.

Our results contradict the results described for a FIX-albumin fusion protein (21) with an observed half-life close to wild-type FIX in different animal models. This is an unexpected result because FVII and FIX are highly homologous proteins belonging to the same protein family (1). Both are serine proteases with a very similar domain structure.

It is possible that the half-life extending effect of albumin relative to the wild-type enzyme is more prominent for FVIIa compared to FIX because of the extraordinarily short half-life of FVIIa. The latter has a half-life in humans of approximately 2.5 h (7) compared with FIX with a half-life of approximately 14.9 to 16.8 h (34). Another possible explanation for the different results is that the mode of action for the clearance of FIX is different from FVII and may not be so strongly influenced by albumin fusion. In a recent publication, Ghosh et al. (35) described binding of FIX/FVIIa to the endothelial protein C receptor, suggesting that this interaction may play a role in FVII/FVIIa endocytosis and clearance. Another difference between FIX and FVII in the circulation is the endothelial binding of FIX (36, 37), which is not the case for FVII. This may result in a different in vivo clearance of FIX. It has been demonstrated by mutations in the corresponding regions of FIX that this endothelial binding does indeed influence the in vivo half-life of FIX (38).

The in-vivo efficacy of the rVIIa-FP was demonstrated in a FVII depletion model in rats and found to be comparable to rFVIIa (NovoSeven). In subsequent experiments this model was then slightly changed, and the results demonstrated that the rVIIa-FP protein was still efficient by the time recombinant FVIIa (NovoSeven) had already lost its efficacy due to its short plasma half-life. The clotting time of animals treated with rVIIa-FP was still very close to untreated animals, indicating that the rVIIa-FP was still present with full haemostatic activity.

The superior pharmacological properties of the rVIIa-FP could facilitate a single dosing regimen of one injection per bleeding event as well as significantly reduce the number of injections needed in this patient population during surgical interventions. Recombinant FVIIa (NovoSeven) has a very good
safety and efficacy profile in inhibitor patients. Our in-vitro test data demonstrate that the FVIIa moiety in the fusion protein displays molecular properties close to wild-type rFVIIa and shows comparable clotting properties. The safety profile with regard to thrombogenicity of the rFVIIa-FP has not been addressed in this study, but is assumed to be similar to wild-type rFVIIa. It is now feasible to test the rFVIIa-FP in further models of thrombosis and haemostasis.

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