Extending the pharmacokinetic half-life of coagulation factors by fusion to recombinant albumin

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Summary
The prophylactic treatment of haemophilia B and the management of haemophilia A or B with inhibitors demand frequent administrations of coagulation factors due to the suboptimal half-lives of the products commercially available and currently in use, e.g. recombinant factor IX (rFIX) and recombinant factor VIIa (rFVIIa), respectively. The extension of the half-lives of rFIX and rFVIIa could allow for longer intervals between infusions and could thereby improve adherence and clinical outcomes and may improve quality of life. Albumin fusion is one of a number of different techniques currently being examined to prolong the half-life of rFIX and rFVIIa. Results from a phase I clinical trial demonstrated that the recombinant fusion protein linking FIX to albumin (rIX-FP) has a five-times longer half-life than rFIX, and preclinical studies with the recombinant fusion protein linking FVIIa to albumin (rVIIa-FP) suggest that rVIIa-FP possesses a significantly extended half-life versus rFVIIa. In this review, we describe albumin fusion technology and examine the recent progress in the development of rIX-FP and rVIIa-FP.

Key words
Coagulation factor IX, coagulation factor VIIa, half-life extension, albumin fusion

Introduction
Clinical care for haemophilia has improved dramatically over recent decades, with both plasma-derived and recombinant factor VIII (FVIII) and factor IX (FIX) concentrates proving to be highly effective at treating and preventing bleeding in haemophilia A and B, respectively (1). Regular, long-term prophylaxis with factor concentrates prevents recurrent haemorrhagic episodes and the resulting development of haemophilic arthropathy, with associated improvement in clinical outcomes, quality of life and life expectancy in patients with haemophilia (2, 3). For haemophilia B, the relatively short half-life of FIX of approximately 17-20 hours (h) (4–6) means that it must be infused every 3–4 days for effective prophylaxis (7), which can be a significant burden for some patients. FIX concentrates with longer half-lives could improve prophylaxis for haemophilia B by decreasing the frequency of infusions required; this, in turn, may improve adherence to treatment, clinical outcomes and quality of life.

Although the development of plasma-derived and recombinant concentrates for haemophilia has greatly improved patients’ quality of life (1), almost one-third of people with haemophilia A develop antibodies (inhibitors) to FVIII. Similarly, inhibitors against FIX replacement concentrates can also develop in 1.5 to 3.0% of people with haemophilia B (8, 9). The presence of inhibitors reduces the efficacy of replacement therapy, as these antibodies inhibit the activity and may enhance the clearance of injected coagulation factors. This necessitates the use of alternative factors known as “bypassing agents” to provide haemostatic support for acute bleeding (10). Activated prothrombin complex concentrates (aPCC) and recombinant activated factor VII (rFVIIa) are commonly used as bypassing agents in both haemophilia A and B with inhibitors. In the case of rFVIIa, sufficient amounts are infused to provide supraphysiological levels of FVIIa to promote effective coagulation via an alternative coagulation pathway (11, 12). One limitation of the available rFVIIa concentrate is the short half-life of the protein (approximately 2.5 h) (6), which results in the need for frequent infusions in cases of acute bleeding or in prophylactic regimens (4, 5, 13). Extending the half-life of FVIIa could facilitate less frequent dosing and potentially enable successful single-dose treatment of acute bleeds. In addition, extending the half-life of rFVIIa could ultimately allow successful prophylaxis of haemophilia in individuals with inhibitors, offering further improvements in quality of life for these patients.

In order to address the above issues, a number of techniques to prolong the half-lives of clotting factors are currently under investigation, including the covalent attachment of polyethylene glycol polymers (PEGylation) or the fusion of the Fc subunit of immunoglobulin G (IgG) to the therapeutic protein; these procedures...
have been described elsewhere for recombinant factor IX (rFIX) and factor VIIa (FVIIa) (14–18). Here, we consider the application of albumin fusion technology to extend the half-lives of rFIX and rFVIIa. This technique involves the creation of single recombinant molecules linking recombinant human albumin to the recombinant therapeutic proteins of choice. The present review will highlight the recent progress in the development of FIX and FVIIa albumin fusion proteins.

**Albumin fusion technology**

**Albumin – a plasma protein with a very long half-life**

Finding the optimal fusion partner to extend the half-life of a therapeutic protein can be difficult, as a number of considerations must be made. The fusion molecule should ideally have a very long half-life. It should not cause an immune response or possess other properties that could potentially affect the biological properties of the therapeutic protein. Based on these criteria, albumin was identified as a suitable fusion partner for coagulation FIX and FVIIa. Albumin is a large, globular transport protein that is present in very high concentrations in plasma. This abundance is a consequence of albumin’s extraordinary long half-life of 19 days (19), which is in part due to having a molecular weight large enough to prevent renal excretion (20), and also to the neonatal Fc-receptor-dependent recycling of albumin. During this latter process, endocytosed albumin is rescued from degradation in lysosomes via binding to the neonatal Fc receptor (FcRn) within the slightly acidic pH environment of the endosome. The FcRn binds albumin and IgG at acidic pH but not at physiological pH. Functional albumin bound to the FcRn is then eventually recycled via vesicles from the sorting endosomes and released at physiological pH into the plasma by exocytosis (21) (Figure 1).

While the long half-life of albumin alone would make it an attractive fusion partner for FVIIa and FIX, albumin possesses additional beneficial qualities. Firstly, it does not exhibit enzymatic activity nor is it involved in any immune system-related interactions, which implies that it does not add any unwanted properties to the fusion protein. Secondly, it is unlikely to be recognised as a foreign molecule and thereby induce an immune reaction when administered as part of a fusion protein since it is already highly abundant in plasma. Lastly, it is a particularly stable protein that remains structurally intact even at elevated temperatures and, therefore, does not pose any additional hurdles to the development of stable formulations.

**Albumin fusion technology**

Albumin fusion proteins are produced using vectors containing the complementary DNA (cDNA) of the target molecule in frame with the cDNA of albumin; these vectors are expressed within eukaryotic cells. In the case of coagulation factors, expression in mammalian cells is required to ensure that certain post-translational protein modifications such as γ-carboxylation, phosphorylation and N- and O-glycosylation take place. The eukaryotic cells produce and secrete albumin fusion proteins as discrete polypeptides; such a ‘linker’ can prevent or minimize possible steric hindrance of interactions between the therapeutic polypeptide and its cofactors or substrates, while retaining the benefit from albumin’s half-life-extending properties.

**Albumin fusion proteins in development**

A number of therapeutic albumin fusion proteins are in development and some are currently being examined in phase III trials. For example, an albumin-linked version of the gut hormone GLP-1, known as albiglutide, is being investigated as a treatment...
for type 2 diabetes (22, 24). Albumin fusion was shown to extend the half-life of GLP-1 from 5 minutes (min) to 5 days, and albiglutide was reported to be well-tolerated and efficacious in phase II trials (25). The development of albiglutide may, in future, reduce the number of GLP-1 infusions required, and improve treatment adherence and quality of life, in patients with type 2 diabetes (22). Promising results were also observed with another albumin fusion protein known as neugranin, comprising granulocyte colony stimulating factor (G-CSF) linked to albumin (26). Recombinant G-CSF is used to treat patients with neutropenia. Pre-clinical experiments in mice and monkeys showed that neugranin could have a similar therapeutic effect to the currently available recombinant G-CSF, even when dosed less frequently than the latter, at similar concentrations (26). Phase II and phase III studies analysing the safety and efficacy of neugranin in breast cancer patients who are receiving chemotherapy - and are frequently neutropenic, have been performed (27, 28).

The results above highlight the promise of albumin fusion as a safe and effective technique for extending the half-life of therapeutic proteins, and emphasise the potential of this method for prolonging the half-life of coagulation factors FVIIa and FIX.

Extending the half-life of FIX via albumin fusion

Currently, prophylaxis of haemophilia B with FIX concentrates requires the administration of about 15–30 IU/kg body weight every 3–4 days (7). Due to these frequent dosing requirements, suboptimal adherence may be a concern in some patients (29) and compliance with prophylactic haemophilia B therapy has been shown to influence clinical outcomes (30). Extending the half-life of rFIX may decrease the frequency of infusions and so facilitate improved adherence to such a regimen when compared to current procedures. The fusion of FIX to albumin is currently being examined as a novel method to achieve this. To form a recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP), a specifically designed, innovative linker containing part of the FIX activation peptide sequence was inserted between rFIX and recombinant albumin (31–33). This cleavable linker containing a section of one of the FIX activation sites allows the release of FIX from albumin after activation by FXIa or FVIIa/tissue factor and thus avoids albumin conferring steric hindrance between rFIXa and its binding partners. This concept extends the half-life while retaining a high coagulation factor activity of rFIX. In fact, although the molar specific activity of rIX or plasma-derived FIX was not completely achieved by the fusion protein, the addition of a cleavable linking amino acid sequence provided a 10– to 30-fold higher molar specific activity in a one-stage clotting assay when compared with recombinant FIX-albumin fusion proteins containing non-cleavable linkers (31). Furthermore, the cleavable linker ensures that the half-life of the activated factor is not extended (34).

Pharmacokinetic (PK) studies with rIX-FP

The PK properties of rIX-FP were analysed following intravenous infusion of 50–200 IU/kg of rIX-FP into rats (31) (Figure 2). In this study, a 4.7-fold increase in terminal half-life was observed with rIX-FP compared with rFIX (BenefIX®) (31). In addition, recovery improved 1.7-fold with rIX-FP when compared with rFIX (Table 1) (31). rIX-FP also exhibited a 3.96-times longer half-life, and a 1.57-fold higher recovery than rFIX in rabbits during PK studies (Table 1) (31).

In vitro and in vivo efficacy studies with rIX-FP

The efficacy of rIX-FP was also tested in vitro via a thrombin generation assay using surface reagents as initiators of coagulation. Here, the addition of rIX-FP allowed a similar rate of thrombin generation in human FIX-depleted plasma when compared to rFIX (31). In addition, the in vivo efficacy of rIX-FP was determined by a tail-tip bleeding model in FIX knockout mice, in which rIX-FP was seen to be as efficacious as rFIX at correcting bleeding time (Figure 3) (31). Improvement in bleeding time was dose...
dependent, and FIX-deficient mice, when given 100 IU/kg of rFIX or rIX-FP, exhibited a bleeding time similar to that of healthy control mice (31).

Experiments performed in cynomolgus monkeys have demonstrated that rIX-FP had a terminal half-life 3.5-fold longer than rFIX when infused intravenously at doses of 50–500 IU/kg, with individual rIX-FP half-life values measured as high as 83.4 h (35). Further supporting evidence for rIX-FP comes from analyses conducted in haemophilia B dogs. Here, animals were administered 100 IU/kg of either rIX-FP or rFIX, and a human FIX antigen-specific immunoassay was then used to detect levels of rIX-FP or rFIX. The time until the levels of human FIX antigen were below 0.05 IU/ml was more than three-times longer with rIX-FP than with rFIX (7.3 days vs 2.3 days, respectively) (Figure 4A) (35). In addition, activated partial thromboplastin time (aPTT) was measured in plasma samples of both the rIX-FP and the rFIX group (Figure 4B). With rIX-FP, aPTT remained at the lower level of 60 seconds (s) for approximately four times longer than with rFIX (5.9 days vs 1.5 days, respectively; Figure 4B), suggesting that the ability to promote clotting is much more prolonged with rIX-FP than with rFIX (35).

Taken together, these very promising results have warranted further investigation, and phase I results have recently been published (36): In a first-in-human dose-escalation trial, the safety and PK of three doses of rIX-FP (25, 50 and 75 IU/kg) were assessed in 25 previously treated patients with haemophilia B. In those subjects administered 50 IU/kg, the mean half-life of rIX-FP was calculated to be 92 h, about five-times longer than the half-lives of the recombinant or plasma-derived FIX products that had previously been used by the patients (Figure 5). In addition, the incremental recovery of rIX-FP was higher than that of both recombinant and plasma-derived FIX (1.4 vs 0.95 and 1.1 IU/dl per IU/kg, respectively) (36). Due to these encouraging phase I outcomes, a phase II/III clinical study of rIX-FP is currently underway (37). For a summary of the published pre-clinical and clinical trial results concerning rIX-FP see Table 2.

Extending the half-life of FVIIa via albumin fusion

As rFVIIa has a very short half-life of approximately 2.5 h, acute bleeds often require 2–3 infusions at 2–3 h intervals to achieve haemostasis (38). Extended pharmacodynamic action due to a longer half-life of rFVIIa may facilitate the resolution of acute bleeds with a single infusion, e.g. in haemophilic patients with inhibitors, reducing the treatment time needed and potentially providing greater patient satisfaction. It is even possible that a rFVIIa

<table>
<thead>
<tr>
<th>Rats</th>
<th>Terminal t½ [h]</th>
<th>Recovery [% of initially expected level]</th>
<th>Rats</th>
<th>Terminal t½ [h]</th>
<th>Recovery [% of initially expected level]</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFIX (BeneFIX®)</td>
<td>5.13</td>
<td>27.6</td>
<td>rIX-FP</td>
<td>4.70</td>
<td>1.71</td>
</tr>
<tr>
<td>pdFIX (Mononine®)</td>
<td>4.43</td>
<td>41.7</td>
<td>rIX-FP</td>
<td>4.70</td>
<td>1.71</td>
</tr>
<tr>
<td>rIX-FP</td>
<td>24.1</td>
<td>47.1</td>
<td>rIX-FP/rFIX (BeneFIX®)</td>
<td>4.70</td>
<td>1.71</td>
</tr>
<tr>
<td>Recovery [% of initially expected level]</td>
<td>27.6</td>
<td>41.7</td>
<td>47.1</td>
<td>1.71</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Pharmacokinetic properties of rFIX (BeneFIX®), plasma-derived FIX (pdFIX) and rIX-FP in rats and rabbits (31).
product with an extended half-life could be used for prophylactic management and improve quality of life for haemophilia patients with inhibitors.

To create a recombinant fusion protein linking coagulation factor VIIa to albumin (rVIIa-FP), the amino acid sequences of albumin and FVII were connected using a specifically designed, flexible, 31-amin acid glycine-serine linker (39, 40). The fusion proteins used for characterization purposes were produced in human embryonic kidney 293 (HEK-293) or in Chinese hamster ovary (CHO) cells and were activated during the manufacturing process (39). Wild-type rFVIIa prepared in CHO cells (wt rFVIIa) and commercially available rFVIIa (Novoseven®) were used as control preparations.

**PK studies with rVIIa-FP**

The PK properties of rVIIa-FP were analysed by infusing 100 μg/kg of this recombinant albumin fusion protein intravenously into rats; PK profiles were compared with those generated by equivalent doses of wt rFVIIa and rFVIIa (NovoSeven®) (39, 40). Using FVIIa antigen levels of rVIIa-FP, wt rFVIIa and rFVIIa to calculate the PK properties, the half-life of rVIIa-FP was found to be 5.8- and 6.7-times longer than that of wt rFVIIa and rFVIIa, respectively (▶Table 3 and ▶Figure 6) (39). Additionally, the *in vivo* recovery 5 min after infusion was increased 1.4- and 2.4-fold for rVIIa-FP over wt rFVIIa and rFVIIa, respectively (▶Table 3) (39). Together, the longer *in vivo* half-life and improved recovery observed with rVIIa-FP resulted in a 9.5- and 14.5-fold increase in the area under the curve (AUC) (i.e. bioavailability) when compared with wt rFVIIa and rFVIIa, respectively (▶Table 3) (39).

Similarly, in FVIII knockout (FVIII⁻/⁻) mice, a single, intravenous infusion of 100 μg/kg of rVIIa-FP exhibited a 4-fold increased half-life and an 11-fold decreased clearance rate when compared with the same concentrations of rFVIIa or plasma-derived FVIIa (41).
rVIIa-FP also exhibited a longer half-life than rFVIIa when administered at 100 μg/kg in rabbits; in this study the half-life of rVIIa-FP was 8.1-times longer than rFVIIa (Table 3 and Figure 7) (41, 42). In addition, the clearance rate decreased 14-fold from 49 (ml/kg)/h for rFVIIa to 3.6 (ml/kg)/h with rVIIa-FP (Table 3) (41, 42), suggesting that rVIIa-FP remains much longer in the circulation than rFVIIa.

Table 2: Main outcomes of pre-clinical and clinical trials with rIX-FP.

Table 3: Pharmacokinetic properties of wt rFVIIa, rFVIIa (NovoSeven®) and rVIIa-FP in rats and rabbits as determined by FVII antigen concentration.

Figure 6: Pharmacokinetic profiles of rVIIa-FP, wild-type (wt) rFVIIa and rFVIIa (NovoSeven®) in rats infused with equimolar concentrations of each product (39). *p<0.005 for rVIIa-FP versus wt rFVIIa and rFVIIa. Adapted with permission from Weimer T, Wormsbächer W, Kronthaler U, et al. Prolonged in-vivo half-life of factor VIIa by fusion to albumin. Thromb Haemost 2008; 99: 659–667. ©2009 Schattauer GmbH, Stuttgart.

In vitro and in vivo efficacy studies with rVIIa-FP

The activity of rVIIa-FP was examined in vitro by measuring the clot formation time (CFT) of human blood from healthy individuals in which FVIII activity was inhibited (39). CFT decreased upon the addition of rVIIa-FP, in a dose-dependent manner, suggesting that blood clotting was accelerated (39).

In addition, the efficacy of rVIIa-FP was tested in vivo in rats treated with phenprocoumon, a derivative of coumarin that inhibits the γ-carboxylation of endogenous vitamin K-dependent factors synthesised in the liver (39). Although inhibition of endogenous FVII γ-carboxylation should be the main contributor to the clotting defect observed in rats 16 h post phenprocoumon application due to FVII's short half-life of 8 h (43), γ-carboxylation of the other vitamin K-dependent factors is also reduced to some extent (the degree of which is dependent on their respective half-lives) and this most probably also make a minor contribution to the overall blood clotting defect seen in this rat model. It was successfully demonstrated that the blood clotting time could be corrected by administration of rVIIa-FP and that, in the case of the albumin fusion protein, this effect was still detectable even when it was applied together with the phenprocoumon due to its extended half-life (39). In FVIII knock-out mice, rVIIa-FP dosed according to activity exhibited a comparable efficacy to rFVIIa (NovoSeven®) when measuring total blood loss in a tail bleeding model (44). Furthermore, the extended pharmacodynamic activity of rVIIa-FP versus wt rFVIIa has also been demonstrated in FVIII knock-out mice using a thrombin generation assay (44).

Together, these results suggest that the fusion of albumin to FVIIa can greatly increase the half-life of this coagulation factor and that rVIIa-FP could represent a potential therapeutic strategy for extending haemostasis compared with current commercially available rFVIIa. A phase I clinical trial in healthy volunteers is currently underway (45). For a summary of the published pre-clinical trial results concerning rVIIa-FP see Table 4.

Extending the half-life of other coagulation factors using albumin fusion

While FIX and FVIIa have successfully been linked to recombinant albumin, albumin fusion technology may be less applicable for a therapeutically relevant half-life extension of FVIII. This is because the natural clearance of FVIII is strongly linked to the clearance of von Willebrand factor (VWF). Therefore, it may be that any half-life extension of recombinant FVIII linked to albu-

Table 4: Summary of pre-clinical trials with rVIIa-FP.

<table>
<thead>
<tr>
<th>Source</th>
<th>Animal model</th>
<th>Terminal half-life</th>
<th>Recovery</th>
<th>Clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weimer et al. Thromb Haemost 2008; 99: 659–667 (39)</td>
<td>Rats</td>
<td>4.4 h</td>
<td>47.1 % of initial dose</td>
<td>–</td>
</tr>
<tr>
<td>Zollner et al. Haemophilia 2010; 16 (Suppl 4): 33 (41)</td>
<td>Mice</td>
<td>3.7 h</td>
<td>–</td>
<td>19 (ml/kg)/h</td>
</tr>
<tr>
<td>Rabbits</td>
<td>13.0 h</td>
<td>–</td>
<td>3.6 (ml/kg)/h</td>
<td></td>
</tr>
<tr>
<td>Kronthaler et al. XXII Congress of the ISTH 2009 [Abstract PP-TH-561] (42)</td>
<td>Rabbits</td>
<td>10-times longer than rFVIIa</td>
<td>Twice as high as rFVIIa</td>
<td>–</td>
</tr>
<tr>
<td>Zollner et al. Haemophilia 2012; 18 (Suppl 3): 97 (43)</td>
<td>Mice</td>
<td>4-times longer than rFVIIa</td>
<td>100% higher than rFVIIa</td>
<td>11-fold lower than rFVIIa</td>
</tr>
</tbody>
</table>
min would be overwhelmed by the natural clearance via VWF and would only have a minor effect on the FVIII half-life under physiological conditions.

More recently, full-length, recombinant VWF has been linked to albumin (rVWF-FP) (46). The biological activity of rVWF-FP was examined via VWF:ristocetin cofactor and VWF:collagen binding activity testing, and was seen to be similar to that of plasma-derived VWF. In addition, PK analysis in rabbits showed that the half-life of rVWF-FP was approximately five-times greater than the half-life of plasma-derived VWF (46). Further PK testing of rVWF-FP is currently underway, and additionally, experiments to examine whether the half-life of FVIII is extended in the presence of rVWF-FP are being conducted (46).

**Conclusion**

In summary, the currently available preclinical data and the encouraging results from the first-in-human study of rIX-FP suggest that the recombinant albumin fusion technology has been effectively applied to the half-life extension of two coagulation factors—FIX and FVIIa. Additional results from the clinical trial programs (PROLONG-9FP and PROLONG-7FP, respectively) currently underway to investigate these promising fusion molecules are also awaited. Should the encouraging results be further confirmed during clinical development, rIX-FP and rVIIa-FP may offer the potential to advance the treatment of patients with haemophilia B, and of patients with haemophilia A or B with inhibitors, respectively.

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**Conflicts of interest**

H. J. Metzner, T. Weimer and S. Schulte are employees of CSL Behring. S. Pipe has received honoraria for participation in symposia and has served as a consultant to CSL Behring.

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