Half-life extension technologies for haemostatic agents

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Introduction

There have been major advances in the treatment of haemophilia in recent decades. Notably, the development of recombinant technology and a shift from on-demand (when a bleed occurs) to prophylactic (to prevent bleeds) treatment regimens have dramatically improved the prognosis for patients with haemophilia A and B, increasing patient quality of life (QoL) and extending life expectancy (1–4).

Plasma-derived concentrates of coagulation factors VIII (FVIII) and IX (FIX) have been available since the 1970s, and recombinant coagulation factors produced using mammalian cell expression systems since the 1990s for treatment of haemophilia A and B (5). Despite improving the treatment of haemophilia, the short half-life of the clotting factors limits their efficacy and necessitates frequent infusions, making adherence difficult for some patients. The most frequently adopted treatment regimens in countries where prophylaxis is available are two- to three-times-weekly intravenous (i.v.) infusion of FVIII and FIX in patients with haemophilia A and B, respectively. Therefore, current treatment regimens represent a significant burden to patients and healthcare providers (6, 7).

The major drive to improve the treatment of haemophilia, and the focus of this literature review, is the prolongation of coagulation factor half-life, enabling less frequent infusions, with the aim of decreasing bleeding rate and reducing the burden of treatment. The half-life of human FVIII is approximately 10–12 hours (h), approximately 18–34 h for FIX, and that of activated factor VII (FVIIa) is the shortest of the coagulation factors, approximately 2.5 h (8). Several technologies currently in development aim to extend the half-life of FVIIa, FVIII and FIX, including Fc fusion, recombinant albumin fusion and addition of polyethyleneglycol (PEG) (PEGylation). These methods prolong the time in the circulation by reducing degradation and elimination. This review summarises the technologies and products in development and their stages of development, and also discusses their pros and cons.

Keywords
Haemophilia, prolonged half-life, coagulation factors

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terms ‘haemophilia and (CTP or carboxyl-terminal peptide or HE Sylation or XTEN or hyperglycosylation), which retrieved two additional references.

To capture the latest clinical data from the half-life extended molecules still in clinical development, search terms and searches for individual products were also applied to abstracts from the latest international congresses in haemophilia (World Federation of Haemophilia [WFH], American Society of Hematology [ASH] and International Society on Thrombosis and Haemostasis [ISTH]) and www.clinicaltrials.gov to identify ongoing trials for which results are not yet available. Searching the congress abstracts yielded 36 results that were included in this review; six studies were subsequently published as full manuscripts and were included instead of the abstract citations, even though they were published after the original search date. The clinical trials.gov website revealed a further 15 clinical trials either ongoing or with results not publicly available. Therefore, 48 manuscripts and 30 abstracts in total form the basis of this review; the 48 manuscripts are listed in Suppl. Table 1 (available online at www.thrombosis-online.com).

Results

As several half-life extended products are in clinical development for haemophilia A and B, the results section of this review will discuss the different technologies, starting with those at the most advanced state of clinical development. The included studies will compare the half-life of the new coagulation factors with the half-life of non-half-life extended recombinant coagulation factors: rFVIII (Advate®), rFIX (BeneFIX®) and rFVIIa (NovoSeven®) (►Table 1).

Fc fusion

Fc fusion has been selected as a method of prolonging the action of coagulation factors, because the binding of Fc-containing proteins to the neonatal Fc receptor (FcRn) protects them from the lysosomal degradation pathway.

rFVIIIFc: preclinical studies

Biogen Idec/Swedish Orphan Biovitrum (SOBI) developed a HEK cell line-derived recombinant FVIII fusion protein that contains a single molecule of B-domain-deleted FVIII fused by recombinant DNA technology to the dimeric region (Fc) of human immuno-globulin G (rFVIIIFc). As traditional dimeric Fc fusion proteins for FVIII were unsuccessful, a monomeric FVIII fusion protein (rFVIIIFc) was developed, with in vitro assays showing its interaction with the FcRn receptor (11) and von Willebrand factor (VWF) (12). The post-translational modifications of rFVIIIFc and in vitro binding affinity for VWF were similar to those of other rFVIII products (13). Data from haemophilia mice and dogs have suggested that the half-life is prolonged, being two-fold longer than rFVIII, and this correlates with a two-fold increase in duration of efficacy in an acute bleeding model 24 h after dosing (11).

rFVIIIFc: clinical studies

The first human single-dosing study in 16 previously treated patients showed that the elimination of rFVIIIFc was prolonged by 1.54- to 1.7-fold vs a full-length rFVIII (14). A total of 44 adverse events were reported by 11 patients, with just one event thought to be related to the investigational product (dysgeusia), compared with six adverse events in five patients treated with rFVIII (14).

The 52-week, open-label, phase III (A-LONG) study conducted in 165 previously treated patients with haemophilia A showed that the terminal half-life of rFVIIIFc was 19 h vs 12.4 h for rFVIII (15). rFVIIIFc resulted in an annualised bleeding rate (ABR) of 1.6 in those treated with rFVIIIFc prophylactically every 3–5 days (median weekly dose 77.7 IU/kg), 3.6 with weekly prophylaxis (fixed weekly dose of 65 IU/kg) and 33.6 for episodic treatment (10–50 IU/kg) (15). In this study, 87.3% of all bleeding episodes were resolved with one injection and 97.8% with ≤2 injections, and 45.3% of patients experienced no bleeding episodes during the study (15). Nine major surgeries were performed on patients enrolled in the trial, with a single injection required to maintain haemostasis during surgery at a median dose of 51.4 IU/kg (16).

Ex vivo thrombin generation and rotation thromboelastometry (ROTEM) assays confirmed the prolonged efficacy of FVIIIIFCs vs rFVIII (17, 18). Additionally, a trial in paediatric patients is ongoing (NCT01458106). In May 2013, the US Food and Drug Administration (FDA) accepted Biogen Idec’s Biologics License Application (BLA) for the marketing approval of rFVIIIFc. It has been launched under the brand name ELOCTATE®.

rFIXFc: preclinical studies

Biogen Idec/SOBI is also developing a FIX fusion protein (rFIXFc). In vivo studies investigating the pharmacokinetic (PK) properties of rFIXFc demonstrated a half-life of 34.8 and 46.2 h in rats and haemophilia mice, respectively, compared with 5.8 and 13.2 h for rFIX (19). In cynomolgus monkeys, dose proportionality was demonstrated and the half-life was approximately 47 h (19). The coagulant activity of blood collected from rFIXFc-treated FIX-deficient dogs showed prolonged coagulant activity vs rFIX (19).

rFIXFc: clinical studies

A phase I/II clinical study showed that rFIXFc was well tolerated with a three-fold longer half-life than rFIX, with dose-proportional increases in activity (20). The B-LONG phase III, non-randomised, open-label trial assessed the efficacy and safety of rFIXFc in 123 patients with severe haemophilia B (21). The mean half-life of rFIXFc was 82.1 h compared with 33.8 h for rFIX (2.43-fold extension), with a mean ABR in the weekly prophylaxis arm (starting dose 50 IU/kg) of 2.95 vs 1.38 in the individualised prophylaxis arm (starting with doses of 100 IU/kg every 10 days) and 17.69 in the on-demand arm (21). Serious adverse events were reported by 10.9% of patients, with one (an obstructive clot in the urinary collecting system) thought to be related to the product. Treatment of on-demand bleeding was resolved with a single in-
Injection in 90.4% of episodes and ≤2 injections were required in 97.3% of cases, with a median dose of 46.1 IU/kg to resolve bleeding (22). Fourteen major surgeries were performed during the study, with haemostasis maintained by a single injection in 87.5% of cases; however, a post-operative blood transfusion was required in two patients (23). PK modelling has suggested that weekly (50 IU/kg) or 10-day or fortnightly (100 IU/kg) dosing could maintain factor levels > 1% in the majority of patients (24). A BLA was submitted to the FDA for rFIXFc fusion protein in January 2013 under the name ALPROLIX™ and the product was launched in 2014. An extension trial is enrolling (NCT01425723) and a paediatric trial is currently recruiting (NCT01440946).

**rFVIIaFc: clinical studies**

No human studies of rFVIIaFc are currently registered on [www.clinicaltrials.gov](http://www.clinicaltrials.gov).

**Albumin fusion**

Albumin has a very long half-life (17–19 days) due to its interaction with the FcRn receptor and subsequent protection from degradation. It is the most abundant plasma protein and has been used to successfully extend the half-life of several other proteins such as interferon-alfa (26).

**rFVIIaFc: preclinical studies**

A similar approach is being applied to develop a long-acting rFVIIa fusion protein (rFVIIaFc, Biogen Idec/SOBI); however, this product is still at an early stage of development. Studies in haemophilia mice indicated comparable efficacy with rFVIIa and an extended half-life (25).

**rIX-FP: preclinical studies**

Initially, fusion of recombinant FIX to recombinant albumin resulted in a significant reduction in the biological activity of FIX (27). Therefore, a novel concept was applied to develop an innovative, fully recombinant FIX albumin fusion protein (rIX-FP, CSL Behring), with a proteolytically cleavable linker, which is cleaved...
during the activation process to release FIXa. The presence of a cleavable linker eliminates the steric hindrances that a permanent linker may have on the activity of the protein, so that rIX-FP benefits from the long circulating half-life of albumin but, once activated, behaves in the same way as endogenous FIXa. In vitro investigations confirmed the activity of rIX-FP and in vivo PK studies in rats, rabbits and haemophilia B dogs showed promising results, demonstrating a prolonged PK and pharmacodynamic (PD) profile, with activity maintained for approximately four-times as long as rFIX (29). The biodistribution of rIX-FP in rats indicated a similar pattern to that of rFIX. However, rIX-FP could be observed in tissues up to 240 h post-dosing, and a more prolonged retention in joints, such as the knee, was apparent vs rFIX (30).

rIX-FP: clinical studies

A phase I dose-escalation study conducted in 25 patients with severe haemophilia A demonstrated a greater than five-fold increase in half-life, a 44% increase in recovery, a seven-fold reduction in clearance compared with rFIX, and a linear-dose response (31). No allergic reactions or inhibitors were developed during this study and all adverse events that were possibly treatment-related were classified as mild using rIX-FP at 25–75 IU/kg (31, 32). Preliminary results from the open-label PROLONG9-FP phase I/II trial, which included an on-demand and a weekly prophylaxis treatment arm, indicated an excellent safety profile based on over 600 exposure days, with no serious adverse events reported (33). After 11 months’ treatment, the 13 patients treated prophylactically had a mean ABR of 1.26, and those switching from pre-trial on-demand therapy to prophylaxis (n=3) had a > 80% reduction in bleeding episodes (34). Furthermore, the efficacy of rIX-FP was confirmed as 100% of bleeding events were treated successfully with ≤2 injections and ~90% treated with a single injection of rIX-FP (34). Patients completing this trial were invited to enrol in an ongoing phase II/III trial (NCT01496274), and two other trials are recruiting subjects (an extension trial: NCT02053792, a paediatric trial: NCT01662531).

rVIIa-FP: preclinical studies

Recombinant albumin has also been fused to rFVIIa via a novel, flexible glycine-serine linker to create a fully recombinant fusion protein (rVIIa-FP, CSL Behring), which remains intact during the activation process, for the treatment of haemophilia A in patients with inhibitors. The length of the linker was designed to allow optimal separation between the two proteins, minimising interactions between the recombinant albumin and recombinant FVIIa moieties (35). PK studies in rodents indicated that the half-life of rVIIa-FP was 5.8-fold longer and recovery was improved by 1.4-fold compared with rFVIIa (36, 37). Further investigation of PK parameters in haemophilia A mice, rats, rabbits and monkeys confirmed the prolonged half-life, enhanced recovery and reduced clearance, with haemostatic efficacy maintained in rVIIa-FP-treated animals 12 h after administration, when it was negligible in rFVIIa-treated animals (38). No safety issues were described in these studies. It has been shown that the biodistribution of rVIIa-FP is similar to rFVIIa, but as expected there is a prolonged presence in plasma, tissues and joints (39).

rVIIa-FP: clinical studies

Results from the phase I double-blind study were reported recently, showing rVIIa-FP was well tolerated after single-dose administration to 40 healthy individuals, with no incidences of inhibitor or anti-drug antibody development (40). In humans, the half-life was increased to 8.5 h with a dose of 1,000 µg/kg, and the clearance was reduced by three- to four-fold (40). It should be noted that higher doses of rVIIa-FP (µg/kg) are required compared with rFVIIa, due to increased molecular weight following the addition of albumin. A phase II/III trial is due to commence in 2015.

rVWF-FP: preclinical studies

A recombinant fusion protein linking VWF with recombinant albumin, via a flexible glycine-serine linker sequence (rVWF-FP, CSL Behring), is at a preclinical stage of development. Data have suggested that production of the fusion protein does not adversely affect the ability of VWF to multimerise or its binding activity to collagen or ristocetin (41). In vivo results from rabbits indicated that the half-life of rVWF-FP was five-fold longer than that of plasma-derived VWF, and further investigation is ongoing to confirm these results.

Direct PEGylation and glycoPEGylation

There are several products in development that use the covalent addition of PEG to prolong their half-life, utilising site-specific PEGylation or non-site-directed PEGylation, which can influence biological activity, with non-site-specific PEGylation having the disadvantage that the PEG may happen to block the active binding sites. PEG increases the molecular weight of a product and can be used to improve solubility, increase stability and protect from degradation as well as extending circulating half-life.

N8-GP: preclinical studies

A B-domain truncated modified O-glycoPEGylated recombinant FVIII is in development (N8-GP, NN7088, Novo Nordisk) for haemophilia A, which is predicted to reach the market in 2015. N8-GP has a 40 kDa PEG attached to the B-domain truncated turoctocog alfa; upon activation by thrombin, the B-domain containing PEG is removed, releasing activated FVIII. The binding capacity of N8-GP to VWF was similar to that of rFVIII (42) and the efficacy in chromogenic assays was similar to that of non-PEGylated turoctocog alfa (43). Preclinical studies evaluating the PK and PD properties of N8-GP in dogs with congenital haemophilia demonstrated a half-life of 22 h and extended PD activity...
The half-life of N8-GP was also shown to be two-fold longer than rFVIII in mice, rabbits and cynomolgus monkeys, but was less prolonged in rats (42), and a linear dose-response relationship was confirmed in mice (45).

N8-GP: clinical studies

Similarly, a dose-escalation study in 26 previously treated patients with haemophilia A showed a linear-dose PK profile, a mean half-life of 19 h and a low frequency of adverse events (46). No inhibitors or binding antibodies were developed after a single dose (up to 75 U/kg) of N8-GP (46). The pathfinder™ N8-GP phase III program is underway (NCT01480180), as is a paediatric study (NCT01731600), and a trial evaluating efficacy during surgery is recruiting (NCT01489111).

BAY 94–9027: clinical studies

Another B-domain-deleted PEGylated FVIII in development is BAY 94–9027 (Bayer), which contains a single, large-branched PEG molecule conjugated to a specific amino acid on rFVIII (47). Introduction of a mutated cysteine through site-directed mutagenesis creates a site-specific PEGylated FVIII (47). Phase I results have been published; however, the PK data for this compound showed only a modest increase (30%) in the half-life compared with rFVIII (18.7 vs 14.6 h with a single 50 IU/kg dose) in previously treated patients with haemophilia A (48). In this study, no patients developed inhibitors or antibodies against BAY 94–9027 or PEG. It has been hypothesised that BAY 94–9027 has reduced immunogenicity vs rFVIII due to the PEG moiety shielding antigenic portions of the protein (49).

BAX 855: preclinical studies

Advate® is a full-length rFVIII currently marketed by Baxter. Using Nektar Therapeutics’ proprietary technology, Baxter is developing a PEGylated version of Advate® (BAX 855) with an extended half-life. This technique incorporates two moles (~20 kDa) of PEG per FVIII in a non-site-specific manner, which improves the PK profile without compromising specific activity (50). Preclinical experiments showed no drug-related changes observed in rats and no signs of toxicity in macaques. However, neutralizing antibodies against endogenous FVIII were developed after repeated administration, although this is expected when administering a human protein to animals (51). The level of PEG incorporated into BAX 855 is below the threshold for macrophage vacuolation (52) and, in a Wessler rabbit stasis model, no thrombogenicity or adverse effects were observed (51). In a tail-tip bleed model, the efficacy of BAX 855 is below the threshold for macrophage vacuolation (52) and immunogenicity profile in murine models was similar to rFVIII (53). Assays to characterise BAX 855 have shown that there is consistency between batches and retention of functional capacity, despite the non-site-directed nature of the PEGylation (54). The reduced binding of BAX 855 to the low-density lipoprotein (LDL) receptor suggests that PEGylation may interfere with the endogenous FVIII clearance pathway, suggesting a mechanism of half-life prolongation (50, 54). The protein portion of BAX 855 is degraded by proteolysis, leaving the PEG portion, which is rapidly eliminated (52).

BAX 855: clinical studies

A phase II/III clinical trial of BAX 855 (NCT01736475) in previously treated patients with severe haemophilia A is ongoing, with a further trial currently recruiting patients undergoing surgical procedures (NCT01913405). If successful, BAX 855 is expected to reach the market towards the end of 2016.

N9-GP: preclinical studies

Novo Nordisk’s 40 kDa-glycoPEGylated FIX (N9-GP) has been modified to include a site-directed PEG molecule attached to the FIX activation peptide. When N9-GP is activated, the activation peptide and the attached PEG moiety are cleaved, thus leaving the activated FIX (55). In vivo studies have shown that N9-GP has a prolonged half-life vs rFIX (56).

N9-GP: clinical studies

The first human dose trial assessed three doses of N9-GP in 16 patients with haemophilia B and demonstrated an extended half-life (mean 93 h), five-fold higher than the patient’s previous product. In this study, three mild/moderate adverse events were assessed to be probably or possibly due to the trial product (fatigue and myalgia), and one serious hypersensitivity event occurred (55). Population modelling based on these results has suggested that weekly prophylaxis may be possible with N9-GP (57). Results of the phase III paradigm™ program were presented recently, assessing prophylaxis and on-demand treatment in 74 patients with haemophilia B, with no patients developing inhibitors. However, non-inhibitory antibodies were developed in three patients and the consequences of this are not currently clear (58). The mean half-life was 110 h and median ABR with prophylactic therapy of 40 U/kg and 10 U/kg per week were 1.0 and 2.9 episodes per year, respectively, and 15.6 for on-demand treatment, with 99% of bleeds resolved following a single injection (59). An extension trial (NCT01395810) and a paediatric trial (NCT01467427) are ongoing, and a trial in surgical patients has recently finished (NCT01386528).

N7-GP: preclinical studies

The future of PEGylated clotting factors was brought into question following the discontinuation of a glycol-PEGylated FVIIIa (N7-GP, Novo Nordisk) in 2011 (60). N7-GP consisted of rFVIIa with site-specific PEGylation of two N-glycans that maintained the protein structure in a native conformation (61). Binding studies showed that the binding of N7-GP to tissue factor and endothelial cell protein C receptor were impaired vs rFVIIa, presumably due to steric hindrances induced by the 40 kDa PEG moiety, but acti-
vation of FX was similar to rFVIIa (62). Preclinical studies in rabbits and mice indicated that the half-life of N7-GP was five- to six-fold longer than that of rFVIIa and there was prolonged efficacy (observed at 12 and 24 h post-dosing) (63–65).

N7-GP: clinical studies
The promising preclinical studies were continued in early clinical studies, where N7-GP was well tolerated in a phase I study, displaying measurable activity up to 72 h post-dosing and a half-life of 15 h (66). Results of the phase II trial in 23 patients were subsequently published, showing that the N7-GP was well tolerated, PK properties consistent with the phase I study, and the ABR using a prophylactic regimen, where patients were dosed every other day, was decreased compared with the pre-trial observation period (67). However, a dose-response relationship could not be established and for this reason the product development has been discontinued.

PEGylated liposomes
PEGylated liposomes (PEGLip) are artificial phospholipid vesicles with PEG side chains enabling the liposome to act as a carrier protein. This has the advantage of not requiring amino acid changes, covalent modifications or stabilising agents, simplifying the formulation production and retaining protein structural integrity (68). Therapies that operate via PEGLip are available, such as doxorubicin and daunorubicin for the treatment of cancer and amphotericin B for fungal infections (69, 70).

PEGLip FVIII: preclinical studies
Animal studies have demonstrated that PEGLip extends the haemostatic efficacy and half-life of FVIII, and maintains the binding and biological properties of FVIII (71–75).

PEGLip FVIII: clinical studies
In a study of 18 patients with severe haemophilia A, sucrose-formulated (FS) PEGLip-FVIII-FS (Omri Laboratories Ltd) was well tolerated with similar infusion rates to existing clotting factor concentrates (76). Clinical studies have demonstrated the prolonged properties of an FS-PEGLip-FVIII (Bayer, BAY 79–4980) compared with rFVIII, and studies investigating the optimal dose, safety and pharmacokinetics of PEGLip indicated that it was well tolerated and no inhibitor formation was detected (77–79). These studies also noted that the PK properties cannot fully explain the prolonged activity of PEGLip-FVIII (78). A 52-week phase II study comparing BAY 79–4980 administered once-weekly vs thrice-weekly rFVIII-FS was halted in 2010 after it did not meet its efficacy endpoint (80).

PEGLip-FVIIa
Bayer and Omri Laboratories Ltd were pursuing a PEGLip-FVIIa formulation, which has prolonged efficacy without affecting activity or stability in animal and in vitro studies (81). A phase I/II study assessing efficacy revealed shortened clotting times and increased clot firmness following PEGLip-FVIIa-FS administration compared with rFVIIa (82) but no further clinical trials are currently registered.

Single-chain design
The in vivo half-life of FVIII is largely determined by the presence of and binding to VWF, which protects FVIII from degradation (83). Based on this knowledge, a different approach has been adopted to prolong the half-life of FVIII. rVIII-SingleChain (CSL Behring) is a recombinant single-chain FVIII protein comprising covalently bound heavy and light chains, which was designed to have improved stability and integrity versus traditional FVIII two-chain proteins (84).

rVIII-SingleChain: preclinical studies
Biochemical characterisation has shown that the activated FVIIIa produced from rVIII-SingleChain is structurally comparable to endogenous FVIII (84). Preclinical experiments showed that the half-life of rVIII-SingleChain was improved (1.6- to 2-fold longer) compared with rFVIII (85). rVIII-SingleChain was found to have a higher affinity for VWF than rFVIII, which is likely to be the mechanism of half-life extension, as the half-life of VWF is 1.5-fold longer than that of rFVIII (41). rVIII-SingleChain at doses of 1–150 IU/kg demonstrated equivalent efficacy compared with rFVIII (86). Acute and sub-chronic toxicity studies in rats, rabbits and cynomolgus monkeys revealed that rVIII-SingleChain was well tolerated (86).

rVIII-SingleChain: clinical studies
Preliminary results from the AFFINITY phase I/III clinical trial in 27 previously treated patients showed it has a longer half-life and lower clearance than rFVIII, supporting a twice-weekly dosing regimen for prophylaxis therapy (87). At the time of writing, the phase I/III study (NCT01486927) was recruiting patients.

CTP
Carboxyl-terminal peptide (CTP) technology is being investigated by PROLOR Biotech as a means of stabilising proteins in the bloodstream, extending half-life without loss of the biological activity or toxicity. It involves the fusion of the C-terminus peptide of the hormone human chorionic gonadotrophin (hCG) to one or both ends of the target protein. As hCG is found naturally, there is a low likelihood that fusion proteins will be immunogenic or toxic.

CTP: preclinical studies
Two products are in preclinical development: a FIX-CTP being developed for once-weekly injections for patients with haemophilia B, and an FVIIa-CTP designed for twice-weekly injections.
in patients with haemophilia with inhibitors. FIX-CTP demonstrated a four-fold longer half-life than rFIX and a reduction in bleeding intensity and duration was observed in FIX knockout mice, but there was a reduction in FIX-specific activity (88). Preclinical data suggest that FVIIa-CTP may have the potential for subcutaneous as well as i.v. delivery (unlike currently available FVIIa products), making prophylactic administration more convenient for patients. In haemophilia mice, FVIIa-CTP had a five-fold longer half-life than rFVIIa and a prolonged haemostatic effect; however, the FVIIa-specific activity was slightly reduced vs rFVIIa (88). Bioavailability was superior to rFVIIa and toxicological studies showed that FVIIa-CTP was safe and well tolerated in animal models (89).

CTP: clinical studies
Data from human studies for FVIIa-CTP and FIX-CTP are not yet available and the products are not expected to launch until 2018 onwards.

HES
Hydroxyethyl starch (HES) is a high-molecular-weight molecule that can be covalently conjugated to the glycans of a protein. In the medical field, HES products have been approved for use as plasma volume expanders for several decades (90), but this is different to the HESylation that would be used to create fusion proteins for haemophilia.

HES: preclinical studies
There are little published data concerning HES and clotting factors, but a long-acting B-domain-deleted FVIII is in development by Bayer/Fresenius Kabi (HES-rFVIII), which displayed efficacy following the addition of HES and the half-life was extended in animal models (91). Activity and VWF binding were maintained despite addition of up to 700 kDa HES. Similarly, results for a HES-rFVIIa (Bayer/Fresenius Kabi) were presented in 2010, reporting activity in clotting assays (92).

HES: clinical studies
No clinical trials have been registered for HES-rFVIII or HES-rFVIIa.

XTEN
Another mechanism to extend the half-life of a protein is fusion with XTEN, an unstructured, hydrophilic polypeptide that increases the hydrodynamic radius of proteins, thus reducing renal clearance. The location, length and number of XTEN insertions on a protein can be varied to modify PK and activity. Investigations of rFVIII-XTEN conjugates are underway, demonstrating a half-life of 16 h in a FVIII/VWF double-knockout mouse model (93).

XTEN: preclinical studies
Several rFVIIa-XTEN fusion proteins that target platelets using a single-chain fragment variable region (ScFv), which binds with high affinity to the αIIbβ3 receptor (Biogen Idec), have been assessed in vitro and in vivo, with the aim of developing an rFVIIa that has improved activity as well as half-life extension (94). In mice, the half-life was 9 h, representing an eight-fold extension vs rFVIIa, and preliminary data indicated that addition of the platelet-targeting ScFv enhanced clotting activity in ROTEM and thrombin generation assays (94, 95).

XTEN: clinical studies
At the time of writing, there were no registered clinical trials with XTEN.

Hyperglycosylation
Hyperglycosylation is another method of increasing the circulating half-life of a protein and has been used successfully to prolong the activity proteins such as follicle-stimulating hormone (96). N-glycosylation is an intracellular process, which glycosylates asparagine residues in specific motifs, and N-glycans can be added to a recombinant protein via the introduction of specific mutations.

Hyperglycosylation: preclinical studies
Wild-type FIX has two N-glycans at positions 157 and 167 but a recombinant version with an additional four N-glycans at sites not thought to confer biological function has been generated (97). When haemophilia mice were administered hyperglycosylated FIX (FIX-T172N-K228N-I251T-A262T, Novo Nordisk), the terminal half-life was increased 2.4-fold and the clearance reduced 5.4-fold compared with rFIX (97). However, the exact mechanism responsible for the reduced clearance is still to be elucidated and it is not known how hyperglycosylation impacts upon efficacy.

Hyperglycosylation: clinical studies
There are no clinical studies underway for hyperglycosylated clotting factors at this stage.

Discussion
Several half-life extended products are in development, which have the potential to improve the treatment of haemophilia by increasing the interval between replacement therapy doses. The techniques employed vary, but in general the approach is to create proteins that are protected from plasma elimination.
Pharmacokinetics

The addition of Fc, albumin, PEG, CTP and XTEN all successfully prolong the half-life of clotting factors. However, lengthening the half-life of FVIII has been challenging, with only modest (1.5- to two-fold) extensions observed; both FVIII-Fc (15) and N8-GP reported a half-life of 19 h (46). Therefore, frequent dosing would still be necessary under prophylactic regimens. Weekly prophylaxis with rFVIII-Fc resulted in an ABR of 3.6, suggesting that dosing every 3–5 days, where the rate was 1.6, may be better (15). The half-life extension of FIX products is much more impressive (three- to over five-fold vs rFIX) (21, 31, 59), which suggests that prophylactic therapy every 10–14 days may be possible; this would translate to a significant advantage for patients.

As the two most abundant proteins in the body, albumin and IgG make up 70% of plasma proteins. The long half-life of albumin and Fc is due to binding to FcRn, which salvages them from the lysosomal degradation pathway, resulting in recycling to the cell surface. Albumin and IgG have been shown to interact at distinct and separate sites on the FcRn (98).

According to the developers of XTEN fusion (Biogen Idec), this method should be able to increase the circulating half-life of coagulation factors beyond that possible with Fc fusion (95). However, as it is a novel strategy, there are no existing XTEN fusion proteins on the market and no human studies to assess possible side effects.

An improved PK profile is not only determined by half-life extension; parameters such as area under the curve (AUC), clearance and time to trough level may also be important and contribute to differences between products and their eventual use.

Modelling efforts are underway to assess the impact that the extended pharmacokinetics will have on predicted injection frequency and dose requirements to maintain factor levels above the targeted threshold (24, 99).

In the future, further health economic assessments of the benefits of the prophylactic treatment with extended half-life factors will be needed to convince payers of their benefits over currently available recombinant and plasma-derived clotting factors.

Efficacy

To confer an advantage to patients over currently available clotting factors, it is essential that half-life extended products demonstrate an activity profile that is non-inferior to current products. It is vital that the structural changes involved in producing a fusion protein do not result in loss of efficacy or unwanted side effects. Several of the preclinical studies have analysed the biophysical properties to show that the higher-order structure is not compromised (100). Clinical studies have shown that Fc fusion, albumin fusion and PEGylated clotting factors are efficacious, with prophylactic regimens resulting in lower ABR versus on-demand therapy (15, 21, 34, 59). Three FIX products have been studied under a weekly prophylaxis regimen, with ABR of 2.95 for rFIXFc, 1.26 for rIX-FP and 1.0–2.9 for N9-GP. 97.3% of bleeds were resolved with ≤2 injections of rFIXFc, 100% with ≤2 injections of rIX-FP, and 99% were resolved with a single injection of N9-GP (21, 34, 59), suggesting that rIX-FP and N9-GP may convey superior efficacy vs rFIXFc, although a head-to-head comparison would be required to confirm this. For FVIII, only rFVIII-Fc has been studied in a prophylactic regimen, although a direct comparison of efficacy to rFVIII was not provided in this study (15). No phase III results for half-life extended FVIIa products are currently available.

In spite of the fact that the overall aim of prophylaxis is to reduce bleeding frequency whilst reducing the burden of treatment, the relationship between the ideal dosing frequency and optimal trough levels of the coagulation factor is not clearly defined. Traditionally, a 1% trough level has been considered sufficient to prevent haemophilic arthropathy; however, some patients require higher factor levels to prevent bleeds and it is likely that maintaining higher levels would benefit long-term outcomes. It is probable that dosing regimens will need to be individualised due to substantial inter-patient variability and also depending on patient age and level of activity (101, 102).

The implementation of biological standards and international units have enabled the comparison of products and reduced the variation between laboratory results (103). The accurate measurement of activity is important to ensure that physicians are able to select the correct dose. However, results may differ depending on whether a chromogenic or one-stage clotting assay is adopted. It is possible that the potency of modified molecules by the one-stage assay may be dependent on the choice of reagent (104). If only one type of assay is suitable or there is considerable variation between methods, it may be that this information needs to be included in the product labelling. At present, there is debate between the regulatory authorities who have different opinions on how best to guide pharmaceutical companies as to what will be used as biological standards for the products in development, given that the current standards may not be suitable. Field studies are underway to compare results between laboratories and the solutions may vary depending on product type. It remains to be seen how pharmaceutical companies will react to this issue once their half-life extended products have launched.

Safety and immunogenicity

Due to the lifelong duration of treatment for haemophilia, the long-term safety of replacement therapy is essential, and of paramount interest for patients and healthcare providers. Fc, albumin, PEG and hyperglycosylation have the advantage of being established technologies, used successfully for other therapeutic proteins (27, 96, 105–107), whereas CTP and XTEN are novel and therefore have limited safety data. It should be noted that some of these technologies are currently used in oncology where short-term side effects may be more acceptable compared with long-term therapy in haemophilia. Furthermore, whereas albumin and Fc are endogenous proteins, PEG is a foreign substance not normally found in the body, and has not previously been utilised for lifelong therapy. Moreover, there have been safety concerns over the use of HES as a fluid expander after patients with severe sepsis required kidney dialysis, prompting the European Medicines...
Recombinant albumin fusion has the advantage that it uses a natural pathway that protects proteins from degradation, thus keeping it in the circulation. Furthermore, albumin is a natural carrier molecule that does not exhibit enzymatic activity and plays no role in the immune system, making albumin fusion proteins less likely to elicit adverse immune responses or to be recognised as foreign proteins (109). Fc fusion proteins also utilise the FcRn pathway to protect them from degradation. However, Fc is an antibody fragment, which plays a role in the immunity as the Fc receptor is expressed on a variety of cells. Thus far, no patients have developed inhibitors following treatment with rIX-FP or rFIXFc, with no serious adverse events related to the product reported with rIX-FP and just one reported with rFIXFc (obstructive uropathy) (21, 33). Non-neutralising antibodies were developed by six patients treated with rFVIIIIFc, suggesting that there is an immune response to rFVIIIIFc, but these did not impact upon clinical outcome, and there were no reports of serious vascular thrombotic events, hypersensitivity or anaphylaxis (15). In vitro studies have indicated that the recycling pathways for Fc vary according to cell type (110); therefore, the exact pathways involved and the impact they have on half-life prolongation are not fully elucidated.

PEGylation has the advantage that it is a long-established method of decreasing renal clearance and prolonging half-life. The safety of PEGylated products may be dependent on the size of the molecule, with studies suggesting that the level of tissue penetration and rate of clearance are determined by the molecular weight (111). Site-specific PEGylation can enable extension of half-life without compromising efficacy (47) and clinical studies have reported a low incidence of adverse effects, and suggested that PEG is rapidly eliminated (52, 112). However, PEGylation can reduce efficacy, with pegamotecan (PEGylated camtothecin) being abandoned after phase II results showed that PEG release shortened the half-life (113). Additionally, PEGylated molecules can induce an immunogenic response due to the generation of antibodies against PEG, as shown in several animal studies and in patients treated with PEG-asparaginase for acute lymphoblastic leukaemia. Furthermore, anti-PEG has been observed in healthy individuals without prior exposure to PEGylated therapeutics (114, 115). It has been suggested that immunogenicity is dependent on the presentation of epitope to CD4+ cells, indicating that the site of PEGylation is crucial (116). Clearance by macrophages has also been reported in vivo, with some studies suggesting this leads to organ-specific vacuolisation or cellular damage, for example in the renal epithelium and lymph nodes (117-119), but others indicating that they consistently resolve over time (52).

Lastly, the discontinuation of PEGylated N7-GP due to the absence of a dose-response relationship, and BAY 79-4980 (PEG-Lip-FVIII) due to a lack of efficacy, suggest that PEG may hamper PK and/or efficacy. The report of non-inhibitory antibodies following treatment with N9-GP (58, 67) has also raised concerns regarding the feasibility of PEG therapies in haemophilia.

What is known about this topic?
- Replacement therapy with products containing the deficient coagulation factor is the mainstay of haemophilia care.
- Prophylaxis is the recommended regimen of replacement therapy, entailing intravenous infusions repeated at intervals of 2–3 days each week.
- Frequent intravenous infusions and the associated venous access affect the quality of life and compliance of persons with haemophilia.

What does this paper add?
- Various methods based upon genetic engineering of coagulation factors have been developed in order to increase the plasma half-life of factors VIII, IX and VIIa.
- Long-acting coagulation factors have reduced the need for frequent intravenous infusions, from twice weekly to every 10–15 days for factor IX and from thrice weekly to once or twice weekly for factor VIII.
- While the available studies of these new factors demonstrate efficacy under ideal clinical conditions, effectiveness under ordinary clinical conditions requires evidence stemming from their wider use after licensing.

Conclusion
Successful treatment of haemophilia is possible using clotting factor replacement therapy; however, the short half-life of clotting factors means that frequent infusions are necessary. Several unmet needs remain in the management of haemophilia, in particular the ability to manage the disease prophylactically with a less frequent dosing regimen that will prevent joint haemarthrosis and reduce the burden on patients and healthcare providers. Bioengineering using recombinant DNA technology to increase the circulating half-life of clotting factors for patients with haemophilia A and B, and those with inhibitors, has the potential to overcome some of these challenges. Several novel products in clinical development have demonstrated promising clinical results, with the first half-life extended products reaching the market in 2014, providing an opportunity to make a difference to the lives of patients.

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