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Preclinical efficacy and safety of rVIII-SingleChain (CSL627), a novel recombinant single-chain factor VIII☆

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ABSTRACT

Introduction: The preclinical efficacy and safety of rVIII-SingleChain (CSL627), a novel recombinant single-chain factor VIII, was assessed in a series of animal studies.
Materials and Methods: In the tail-clip bleeding model, hemophilia A mice were injected with escalating doses (1–150 IU/kg) of rVIII-SingleChain, B-domain deleted (BDD) rFVIII (ReFacto AF®), or full-length rFVIII products (Advate®, Helixate®). Total blood loss and the percentage of animals in which hemostasis occurred were assessed in this observer-blinded, randomized study. In a second non-randomized study in hemophilia A mice, thromboelastographic analysis, thrombin generation, and activated partial thromboplastin time assays were performed. General safety and toxicity were assessed in three animal species, including determination of the prothrombotic potential of rVIII-SingleChain in a rabbit venous thrombosis model.
Results: Under acute bleeding conditions, the effect of rVIII-SingleChain on total blood loss and hemostasis was indistinguishable from BDD and full-length rFVIII. rVIII-SingleChain and full-length rFVIII (both 20 IU/kg) corrected thromboelastographic parameters, activated partial thromboplastin time, and thrombin generation to a similar degree in hemophilia A mice. In a thrombosis model, the effect of rVIII-SingleChain on thrombus incidence was non-significant and comparable to BDD rFVIII at doses up to 500 IU/kg. Treatment with rVIII-SingleChain did not cause anaphylactic reaction or local intolerance in safety and toxicity studies, and demonstrated an excellent overall safety profile.
Conclusions: rVIII-SingleChain showed convincing hemostatic efficacy and excellent tolerability in animal studies, warranting continued investigation in human Phase I/III trials (AFFINITY).

Introduction

The management of bleeding disorders has improved dramatically in recent decades, resulting in great benefits for people with these conditions in terms of quality and length of life [1]. Hemophilia A is the most common form of hemophilia [2]. The mainstay of clinical management is on-demand or prophylactic replacement of deficient factor VIII (FVIII) with plasma-derived or recombinant factors [3–5]. Since recombinant FVIII (rFVIII) was first used in the 1980s, continuing changes in replacement therapy have led to recombinant products being devoid of animal and human-derived proteins in the cell culture and formulation processes [6].

Despite effective replacement therapies, which have provided a normal or near-normal life expectancy for most people with hemophilia A, key challenges remain [7]. The development of inhibitory antibodies during FVIII replacement therapy is a major problem for up to 30% of patients using these products, rendering therapy ineffective and increasing the risk of bleeding complications [8–10]. Furthermore, patients who undergo a prophylaxis regimen usually require three doses per week due to the short half-life of FVIII [11,12]. Thus, there is an unmet medical need for rFVIII therapy that offers reduced potential to elicit inhibitory antibodies combined with a more convenient dosing regimen.

rVIII-SingleChain is a novel recombinant single-chain FVIII construct comprising covalently bonded heavy and light chains. The activated form of rFVIII (rFVIIIa) produced from rVIII-SingleChain is

Abbreviations: ADA, anti-drug antibody; aPCC, activated prothrombin complex concentrate; aPTT, activated partial thromboplastin time; AUC, area under the curve; AUC_{0-24h}, AUC from 0 to 24 hours; $AUC_{0\text{-last}}$, AUC from 0 to last timepoint; BDD, B-domain deleted; CFT, clot formation time; CHO, Chinese hamster ovary; CI, confidence interval; C_{max}, maximum concentration; CT, clotting time; ELISA, enzyme-linked immunosorbent assay; ETP, endogenous thrombin potential; FVIII, factor VIII; GLP, good laboratory practice; PTF, potentiation of thrombus formation; rFVIII, recombinant factor VIII; rFVIIIa, activated recombinant factor VIII; TGA, thrombin generation assay; $t_{1/2}$, half-life; t_{max}, time taken to achieve C_{max}; VWF, von Willebrand factor.

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structurally comparable to that formed from two-chain endogenous FVIII [13]. This novel single-chain design could allow for beneficial features such as high intrinsic stability and molecular integrity, and faster and enhanced binding to von Willebrand factor (VWF), which in turn may contribute to a low potential for immunogenicity [1].

Several safety precautions are taken during the manufacture of rVIII-SingleChain, including two dedicated viral inactivation and removal steps. No human- or animal-derived proteins are added in the fermentation, purification, or formulation stages. rVIII-SingleChain is expressed in Chinese hamster ovary (CHO) cells, which are an established and standardized cell line for the production of recombinant coagulation factors. In addition, using CHO cells ensures that post-translational modifications such as glycosylation occur in a consistent manner [14].

In this series of animal studies, one of the key objectives was to determine the hemostatic efficacy and overall safety profile of rVIII-SingleChain in a range of animal species in order to guide the clinical studies (AFFINITY) of this novel rFVIII compound in humans.

Materials and Methods

Preclinical Efficacy: In vivo Characteristics of rVIII-SingleChain vs Full-Length rFVIII

Animals

Factor VIII-deficient hemophilia A mice [15], bred and supplied by Charles River Laboratories (Kisslegg, Germany), were aged more than eight weeks and weighed >19 g.

Tail-Clip Model

Hemostasis was assessed using a subaquatic tail-clip bleeding model in an observer-blinded, randomized (computer-generated pseudo-random numbers) study. Fifteen minutes before a tail clip, animals were injected intravenously with rVIII-SingleChain or marketed rFVIII products (Advate® [Baxter Bioscience, Vienna, Austria], Helixate® [CSL Behring, Marburg, Germany], or ReFacto AF® [Pfizer Inc., Sandwich, UK]) at doses of 1, 5, 15, 40, 100, or 150 IU/kg. A control group was injected with vehicle (formulation buffer of rVIII-SingleChain).

The tail was cut with a scalpel knife (start of the observation period) under deep anesthesia (sodium pentobarbital, 74.5 mg/kg), removing about 3 mm of the tail tip. Immediately upon tail clipping, the tail tip was submerged in isotonic saline solution (0.9%), which was kept at the physiologic body temperature of the mice using a water bath, until hemostasis occurred. The volume of total blood loss was calculated over an observation period of 30 minutes, or until hemostasis occurred, by measuring the hemoglobin present in the isotonic saline (0.9%) (Sysmex F-820, Sysmex Europe GmbH).

The co-primary endpoints for this study were total blood loss (hemoglobin content) after tail clip and the percentage of animals in which hemostasis occurred. The relative potency of rVIII-SingleChain versus the marketed rFVIII products with respect to total blood loss was estimated by a pairwise analysis of covariance model with dependent variables of total blood loss and covariates of sex, log dose, and treatment (rVIII-SingleChain and either Helixate®, ReFacto AF®, or Advate®). The relative potency of rVIII-SingleChain, full-length rFVIII (Helixate®, and Advate®) and two-chain BDD rFVIII (ReFacto AF®) for the percentage of animals in which hemostasis occurred was estimated in pairwise linear logistic regression models with hemostasis as the dependent variable (no/yes) and covariates of sex, log dose, and treatment (rVIII-SingleChain and either Helixate®, ReFacto AF®, or Advate®).

Thromboelastography, Thrombin Generation Assay, Activated Partial Thromboplastin Time, and FVIII Activity

In this non-randomized study in hemophilia A mice, citrated (10% v/v) FVIII-deficient mouse blood was terminally collected under deep anesthesia (vena cava puncture) 15 minutes after intravenous injection of rVIII-SingleChain or full-length rFVIII (Advate®) into the lateral tail vein (both dosed at a level of 20 IU/kg).

A ROTEG 05 analyzer (Pentapharm GmbH, Munich, Germany) was used to assess the effects of the test substances on thromboelastographic parameters in citrated whole blood. Coagulation was activated by recalcification using star-tem® 20 reagent (0.2 mol/L calcium chloride in HEPES buffer; Tem International GmbH, Munich, Germany). The thrombin generation assay (TGA) was performed by calibrated thrombinography (Calibrated Automated Thrombogram [CAT®], Thrombinoscope, Maastricht, The Netherlands). To induce intrinsic activation, samples were incubated with a mixture of phospholipid (Rossix, Mölndal, Sweden) and Pathromtin® SL (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) at 37 °C for 10 minutes; fluorescence was read for 40–60 minutes. For activated partial thromboplastin time (aPTT) analysis, samples were incubated with Pathromtin® SL reagent for 2 minutes and analyzed following addition of calcium chloride solution (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). The chromogenic FVIII activity plasma levels were determined using a Coamatic® Factor VIII test kit (Chromogenix Instrumentation Laboratory SpA, Milan, Italy). FVIII activity and aPTT measurements were performed automatically using a coagulation analyzer (BCS® XP, Siemens Healthcare Diagnostics) after an incubation time of 2 minutes.

Correction of thromboelastographic parameters, including clotting time (CT), clot formation time (CFT), maximum velocity, alfa angle, time to maximum velocity, maximum clot formation, and time to maximum clot formation, was compared between rVIII-SingleChain and full-length rFVIII. In the thrombin generation assay (TGA), lag time, endogenous thrombin potential (ETP) (0–20 minutes), thrombin peak, and time to thrombin peak were compared between products. aPTT values were compared between vehicle, rVIII-SingleChain, and full-length rFVIII using the log-transformed data.

Welch's *t*-test for unequal variances was used to compare the thromboelastography, thrombin generation variables, and aPTT values between the treatment groups. Two-sided 90% confidence intervals (CIs) for the ratio of the mean values of rVIII-SingleChain and full-length rFVIII comprised all θ values (where θ represents the hypothesized ratio), for which the null hypothesis:

$$\text{MEAN}\,[\text{rVIII} - \text{SingleChain}] - \theta\,\text{MEAN}\left[\text{Advate}^{®}\right] = 0$$

was not rejected at a 10% level by Welch's *t*-test.

All p-values refer to two-sided null hypotheses.

For statistical analysis of the FVIII chromogenic activity, a two-component mixture model was fitted to the data, due to some low outlying values. This model encompassed a normal distribution representing ordinary measurements and a Weibull distribution representing outlying data. The mean values of the normal distributions of the factor VIII groups were compared and a CI of the ratio of the mean values was derived by applying signed likelihood-ratio tests.

Safety and Tolerability: rVIII-SingleChain Characteristics in Pivotal Safety Studies – Good Laboratory Practice-Compliant Toxicology Program in Three Preclinical Animal Species

Thrombogenicity in Rabbits

The prothrombotic potential of rVIII-SingleChain was assessed in a rabbit venous thrombosis model after inducing temporary venous stasis (modified Wessler test [16]). Male and female New Zealand White rabbits (2.3–3.1 kg) were randomly allocated according to body weight to treatment groups of six animals (three males and three females), except for the placebo (negative control) group, which contained 12 animals (six males and six females). rVIII-SingleChain was evaluated at intravenous doses of 150, 300, 500, and 1000 IU/kg. FEIBA NF 500®

(activated prothrombin complex concentrate [aPCC], Baxter GmbH) was included at a dose of 200 IU/kg to act as a positive control. Two-chain BDD rFVIII (ReFacto AF®) 500 IU/kg was included as an additional comparator. The negative control (placebo) group received physiologic saline solution.

After the animals were anesthetized with an intravenous injection of sodium pentobarbital (31 mg/kg) into the marginal vein of the left ear, a segment of approximately 1 cm of both jugular veins was isolated. The study treatment was then administered via the right marginal ear vein. Stasis was produced in the previously isolated segment of both jugular veins by ligation with a cotton thread (right segment first and then the left segment) one minute after study treatment administration. The isolated segments of the right and left veins were transferred to a pre-weighed piece of paper after 10 and 20 minutes, respectively, to allow examination and evaluation. Thrombi were left to dry for 24 hours and then weighed.

Thrombosis incidence and thrombus dry weight were assessed as primary endpoints. The incidence of thrombosis was assessed using a scoring system of 0–4, where 0 = non-clotted blood, 1 = fibrin networks, 2 = ≥1 small thrombi, 3 = non-occlusive thrombus, and 4 = one occlusive thrombus.

The percentage of potentiation of thrombus formation (PTF) was calculated for each of the different doses of rVIII-SingleChain using the following formula:

$$\frac{(\text{rVIII} - \text{SingleChain mDWT}) - (\text{negative control group mDWT})}{(\text{positive control group mDWT}) - (\text{negative control group mDWT})} \times 100$$

Where mDWT is the mean dry weight of thrombus.

When a dry weight of thrombus value of ≤0.1 mg was recorded, this was considered to be 0 mg because it is at the limit of quantification for the balance used.

Scores of thrombus incidence after the different treatments were compared using the ranked Mann–Whitney test. Statistical differences between the groups in dry weight of thrombus were assessed using the Student–Newman–Keuls test.

Acute and Sub-Chronic Toxicity Studies

As part of the preclinical Good Laboratory Practice (GLP)-compliant safety pharmacology and toxicity program for rVIII-SingleChain, a series of studies were conducted in different animal species.

Local Tolerance

Local tolerance was assessed in rabbits after administration of rVIII-SingleChain by different routes. Nine New Zealand White rabbits (aged 16–18 weeks; weighing 3.15–3.84 kg) each received a single injection of both rVIII-SingleChain (at a nominal concentration of 359 IU/mL) and placebo on Day 1. Three animals received a single intravenous administration of 1.0 mL into the lateral ear vein of the right ear. Three animals received a single intra-arterial administration of 1.0 mL against the blood flow into the median auricular artery of the right ear. Three animals received a single perivenous administration of 0.2 mL alongside the lateral ear vein of the right ear. For each rVIII-SingleChain administration route tested, the same volume of control solution (isotonic saline solution [0.9%]) was injected into the corresponding site in the left ear at the same time. The animals were observed for signs of ill health or toxicity at least twice daily for four days. Examination of the injection sites was made one hour after injection and approximately 24, 48, and 72 hours later. A histologic examination was performed on both ears and surrounding tissues for each animal.

Single-Dose Toxicity Studies

The potential for systemic toxicity and the toxicokinetic profile of rVIII-SingleChain after a single administration was assessed in Crl:CD (SD) rats and cynomolgus monkeys. Exposure to and toxicokinetics of the compound were assessed using two methods, a direct analysis of rVIII-SingleChain concentrations by enzyme-linked immunosorbent assay (ELISA) (Cedarlane Laboratories, Hornby, Ontario, Canada) in rats and a chromogenic measurement of FVIII activity (Coamatic® Factor VIII, Chromogenix, Milan, Italy) in monkeys. Three groups of rats each comprising five males and five females, and three groups of monkeys each comprising three males and three females received a single dose of rVIII-SingleChain 50, 250, or 1500 IU/kg into the left or right caudal and cephalic/saphenic vein, respectively on Day 1. For both rats and monkeys, the similarly constituted control groups received isotonic saline (0.9%). The rats were observed for a total of five days and the monkeys for 10 days. In the monkey study, two males and two females from each group were killed on Day 6 (interim kill, prior to the onset on a confounding immune response against the human protein), and one male and one female from each group were killed on Day 11. In the rat study, a further three males and three females were allocated to the control group, and nine males and nine females were allocated to each treated group and were used for toxicokinetic evaluation (satellite animals). During the study, clinical condition, bodyweight, food consumption (rat only), hematology, blood chemistry, toxicokinetics, urinalysis, organ weight, macropathology, and histopathology investigations were undertaken. In rats, rVIII-SingleChain was not detected in the plasma samples from control animals by ELISA so correction for endogenous FVIII was not undertaken in toxicokinetic studies. In monkeys, as untreated animals showed detectable endogenous FVIII concentrations, the endogenous FVIII concentration measured before dosing was subtracted from the FVIII concentration measured after dosing to obtain corrected plasma FVIII levels.

Repeated-Dose Toxicity Studies

The systemic toxic potential and toxicokinetics of repeated doses of rVIII-SingleChain were also assessed in Crl:CD (SD) rats and cynomolgus monkeys. Over 28 days, three groups, each comprising 10 male and 10 female rats received rVIII-SingleChain at doses of 50, 250, or 1250 IU/kg/day, and three groups of three male and three female monkeys received doses of 50, 150, or 500 IU/kg. For both rats and monkeys, the similarly constituted control groups received isotonic saline (0.9%). A further one male and one female monkey were assigned to each group; these animals were dosed for five days and were killed on Day 6. In the rat study, a further five male and five female rats were assigned to each of the control and 1500 IU/kg groups; these animals were treated for four weeks followed by 14 days without treatment to assess recovery from any treatment-related effects. A further three male and three female rats were allocated to the control group and nine males and nine females were allocated to each treated group and were used for toxicokinetic evaluation (satellite animals). During the studies, mortality (rats only), clinical condition, body weight, food consumption (rats only), ophthalmic examinations, electrocardiogram (monkeys only), blood pressure (monkeys only), hematology, blood chemistry, neurobehavioral assessment (Irwin screen; rats only), toxicokinetics, anti-drug antibody (ADA) development, urinalysis, organ weight, macropathology, and histopathology investigations were undertaken.

Safety Pharmacology

A study was conducted to assess cardiovascular endpoints under telemetered conditions in monkeys that received rVIII-SingleChain at a cumulative dose of 1550 IU/kg. Four telemetered monkeys (two males and two females) received three separate infusions of rVIII-SingleChain (50 [first infusion], 250 [second], and 1250 IU/kg [third]) over three 30-minute periods. The animals also received vehicle (0.9% saline) as control.

Results

Preclinical Efficacy: In vivo Characteristics of rVIII-SingleChain vs Licensed Human rFVIII

Tail-Clip Model

Overall, a comparable effect on primary hemostasis endpoints was found with rVIII-SingleChain compared with licensed FVIII products in the tail-clip model in hemophilia A mice (Fig. 1).

With respect to total blood loss, the estimated potency of rVIII-SingleChain was similar to that of Helixate® (factor 1.04) and slightly higher than that of ReFacto AF® (factor 1.34) and Advate® (factor 2.15) (Table 1 and Fig. 1). These minor differences between rVIII-SingleChain and the licensed FVIII concentrates (used as active comparators) were not statistically significant ($p > 0.4$), indicating an equipotent effect.

The percentage of animals in which hemostasis occurred was similar for the compared products. All human rFVIII products showed a dose-dependent effect on this endpoint. The dose dependency of hemostasis was significant ($p < 0.0001$) in all comparisons. The estimates of the relative potency values are summarized in Table 2. With respect to the percentage of animals achieving hemostasis, the estimated potency of rVIII-SingleChain was the same as that of Helixate® (factor 1.00), and similar to ReFacto AF® (factor 1.17), and Advate® (factor 0.87). The differences between rVIII-SingleChain and ReFacto AF® or Advate® were not statistically significant ($p > 0.7$ for all comparisons) (data not shown).

Thromboelastography, Thrombin Generation Assay, Activated Partial Thromboplastin Time, and FVIII Activity

In the thromboelastography analysis, rVIII-SingleChain, and Advate® (both 20 IU/kg) corrected all ROTEM® parameters, including CT, CFT, maximum velocity, and alfa angle (Fig. 2). Estimated ratios of thromboelastographic parameters for rVIII-SingleChain:full-length rFVIII were 1.27 (90% CI: 1.11–1.48; $p < 0.01$) for CT, 1.18 (90% CI: 0.97–1.47; $p = 0.16$) for CFT, 0.86 (90% CI: 0.66–1.17; $p = 0.36$) for maximum velocity, and 0.92 (90% CI: 0.84–1.02; $p = 0.16$) for alfa angle. In addition to the thromboelastographic parameters shown in Fig. 2, both active drugs corrected mean time to maximum velocity (759 and 598 vs 3040 s for rVIII-SingleChain and Advate® vs vehicle, respectively), mean maximum clot firmness (55.6 and 56.0 vs 43.0 mm), and mean time to maximum clot firmness (3550 and 3529 vs 2649 s) compared with vehicle.

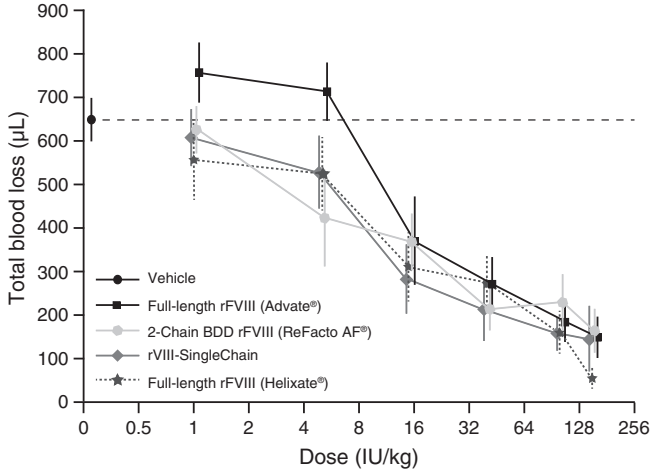

Fig. 1. Mean total blood loss in FVIII knockout mice (hemophilia A model). Mice received intravenous administrations of 1–150 IU/kg of rVIII-SingleChain, BDD (ReFacto AF®), or full-length rFVIII (Helixate®, Advate®) in a tail-clip model (n = 10–20 per group; FVIII-deficient mice). Data shown are mean ± SEM. BDD = B-domain deleted; rFVIII = recombinant factor VIII.

Table 1
Relative potency estimated from an analysis of covariance model for total blood loss in FVIII knockout mice (hemophilia A model). Mice received intravenous administrations of 1–150 IU/kg of rVIII-SingleChain, B-domain deleted rFVIII (ReFacto AF®), or full-length rFVIII (Helixate®, Advate®) in a tail-clip model (n = 10–20 per group).

Treatment	Comparator	Relative potency	90% confidence interval
rVIII-SingleChain	Helixate®	1.04	0.52–2.08
rVIII-SingleChain	ReFacto AF®	1.34	0.67–2.77
rVIII-SingleChain	Advate®	2.15	1.23–3.98

rFVIII = recombinant factor VIII.

In the TGA, rVIII-SingleChain and full-length rFVIII (both 20 IU/kg) corrected all thrombin generation parameters (Fig. 3). For lag time, ETP, and time to thrombin peak, endogenous background activity was only measurable in some individual animals in the vehicle group. The effects of rVIII-SingleChain and full-length rFVIII on thrombin peak (mean ± standard deviation (SD)) were similar (372 ± 46 and 363 ± 47 nM, respectively), with an estimated ratio of 1.02 (90% CI: 0.93–1.13) between the two products. In comparison, the mean ± SD thrombin peak for vehicle was 3.1 ± 5.7 nM. Estimated ratios of thrombin generation parameters for rVIII-SingleChain: full-length rFVIII were 1.13 (90% CI: 0.91–1.44) for lag time, 1.02 (90% CI: 0.96–1.08) for ETP, and 1.12 (90% CI: 0.93–1.35) for time to thrombin peak; all ratios were non-significant.

To further investigate the minor and apparently significant difference in CT between rVIII-SingleChain and full-length rFVIII that was evident in the thromboelastography analysis, aPTT values were measured in plasma derived from FVIII-deficient mice (ex vivo) that were treated with both rFVIII products. As shown in Fig. 4, there was no significant difference in aPTT between the rVIII-SingleChain (geometric mean 44.0 s) and full-length rFVIII (40.8 s; $p = 0.08$) groups. The 90% CI for the ratio of the geometric means extended from 1.01 to 1.16. However, aPTT was significantly reduced in both groups versus vehicle (rVIII-SingleChain: $p = 0.006$; full-length rFVIII: $p = 0.001$).

Administration of 20 IU/kg rVIII-SingleChain or full-length rFVIII led to measurable FVIII chromogenic activity in plasma. Vehicle-treated animals showed values below the limit of detection (<0.015 IU/mL). Mean values (±SD) derived from 16 animals treated with either rVIII-SingleChain or full-length rFVIII were 0.248 ± 0.052 IU/mL (range: 0.08–0.29 IU/mL) and 0.217 ± 0.06 IU/mL (range: 0.04–0.28 IU/mL), respectively with an 11% higher chromogenic factor VIII activity for rVIII-SingleChain ($p = 0.007$). However, chromogenic factor VIII activity of the two products was equivalent (within 80% to 125%; $p = 0.002$) and the 90% CIs of the mean ratio extended from 104% to 118%.

Safety and Tolerability

Thrombogenicity in Rabbits

The positive aPCC control (FEIBA NF 500®) showed the greatest procoagulant effect in both jugular veins compared with rVIII-SingleChain, two-chain BDD rFVIII (ReFacto AF®), and saline solution (negative control). The mean thrombus incidence score with rVIII-

Table 2
Relative potency of rVIII-SingleChain and comparators estimated from a logistic regression model for percentage of FVIII knockout mice in which hemostasis occurred (hemophilia A model). Mice received intravenous administrations of 1–150 IU/kg of rVIII-SingleChain, B-domain deleted (ReFacto AF®), or full-length rFVIII (Helixate®, Advate®) in a tail-clip model (n = 10–20 per group; FVIII-deficient mice).

Treatment	Comparator	Relative potency	90% confidence interval
rVIII-SingleChain	Helixate®	1.00	0.46–2.17
rVIII-SingleChain	ReFacto AF®	1.17	0.53–2.65
rVIII-SingleChain	Advate®	0.87	0.45–1.70

rFVIII = recombinant factor VIII.

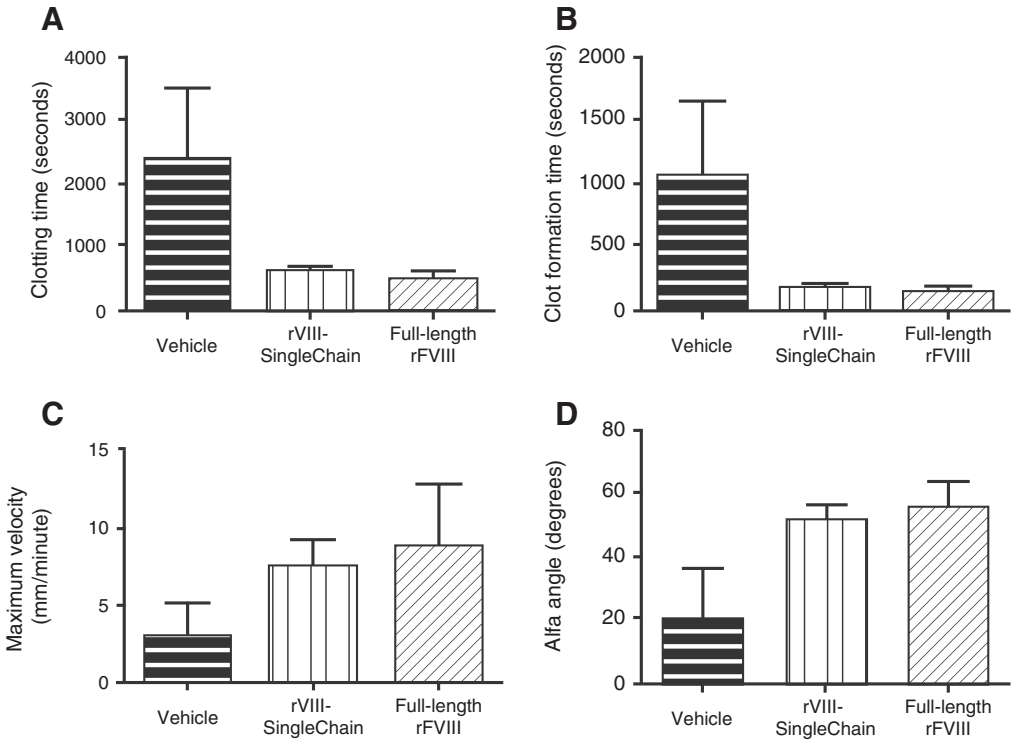

Fig. 2. Thromboelastography analysis showing ROTEM® parameters in FVIII knockout mice (hemophilia A model) following intravenous administrations of 20 IU/kg of rVIII-SingleChain or full-length rFVIII (Advate®), or vehicle. (A) Clotting time (CT; n = 10/group), (B) clot formation time (CFT; n = 10/group), (C) maximum velocity (n = 8–10/group), and (D) alfa angle (n = 10/group). Data shown are mean ± SD. The range of the mean vehicle values were: 570–3939 s (CT); 163–2012 s (CFT); 2.0–8.0 mm/min (maximum velocity); and 8.0–59.0° (alfa angle). rFVIII = recombinant factor VIII.

SingleChain increased with rising dose in the 150–500 IU/kg dose range, but was not significantly different from the negative control in either jugular vein (Table 3). Indicative of a minimal biologic effect level, rVIII-SingleChain only showed significantly ($p < 0.05$) greater thrombus formation compared with negative control at the highest dose (1000 IU/kg) and only in the left jugular vein when submitted

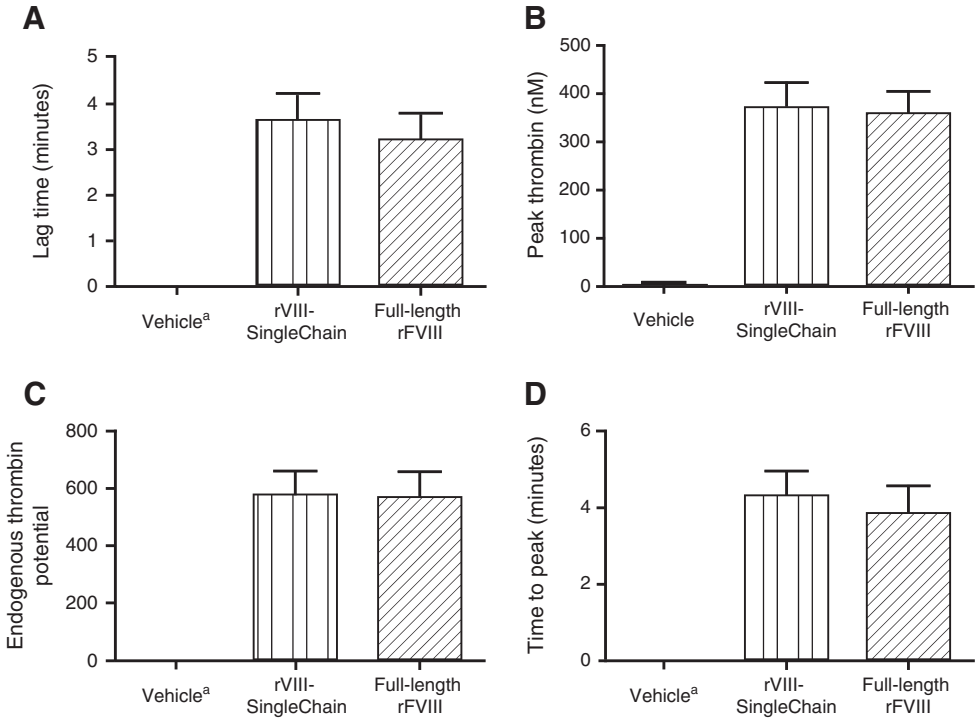

Fig. 3. Thrombin generation assay results in FVIII knockout mice (hemophilia A model). Mice received intravenous administrations of 20 IU/kg of rVIII-SingleChain or full-length rFVIII (Advate®), or vehicle (where measurable). (A) Lag time (n = 10/group), (B) thrombin peak (n = 10/group), (C) endogenous thrombin potential (ETP; n = 10/group), and (D) time to thrombin peak (n = 10/group). Data shown are mean ± SD. [a]Endogenous background activity was measurable in some individual animals in the vehicle group only. rFVIII = recombinant factor VIII.

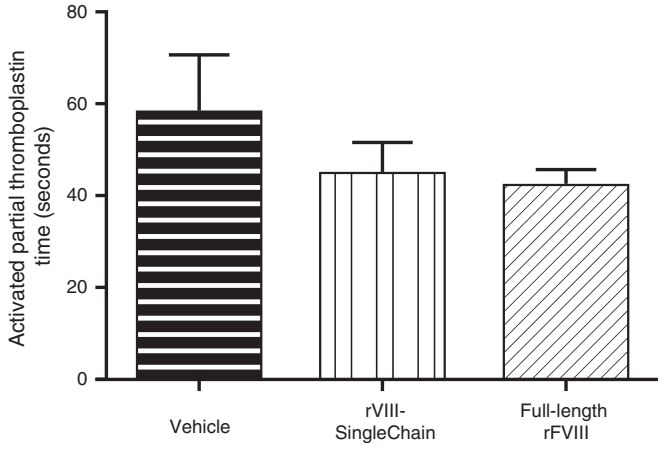

Fig. 4. aPTT (ex vivo) following administration of 20 IU/kg rVIII-SingleChain (n = 16) and Advate® (n = 15) in FVIII-deficient mice versus vehicle (n = 10). Data shown are mean ± SD. aPTT = activated partial thromboplastin time; FVIII = factor VIII.

to the longer stasis time (20 minutes) (i.e., more stringent conditions). Administration of BDD rFVIII 500 IU/kg showed an intermediate effect, similar to that observed with rVIII-SingleChain at doses of 500 and 1000 IU/kg. As with rVIII-SingleChain, this marginal effect was not significantly different from the negative control (Table 3).

Significant ($p < 0.05$) differences were observed between the positive (FEIBA NF 500®) and negative controls for dry weight of thrombus (Table 3). In contrast, rVIII-SingleChain at all doses tested and BDD rFVIII 500 IU/kg did not show any significant differences compared with the negative control (Table 3). Dry weight of thrombus was also similar between rVIII-SingleChain (all doses) and BDD rFVIII 500 IU/kg (Table 3). The percentage PTF was greater in male than female rabbits (data not shown).

Therefore, in comparison with vehicle (control), rVIII-SingleChain showed a minimal procoagulant effect only at the highest dose of 1000 IU/kg, with no statistically significant effects at lower doses of 150, 300, or 500 IU/kg.

Acute and Sub-Chronic Toxicity Studies

In rabbits, intravenous, intra-arterial, and perivenous injections of rVIII-SingleChain were well tolerated with no local or systemic signs of reaction to treatment. No adverse effects were found with rVIII-SingleChain at single doses of 50, 250, or 1500 IU/kg in CD rats or cynomolgus monkeys (Table 4).

In rats, toxicokinetic investigations confirmed that the maximum concentration (C_{max}) of FVIII and extent (AUC and AUC_{0-24h}) of systemic exposure, increased dose proportionally with escalating doses over the range 50–1500 IU/kg: mean C_{max} values ranged from 0.88 to 8.26 IU/mL; mean AUC values ranged from 3.74 to 31.44 IU · h/mL; and mean AUC_{0-24h} values ranged from 1.27 to 30.44 IU · h/mL. The AUC parameters showed higher values for males than for females at all doses tested, but C_{max} values were in a comparable range for both sexes. The time taken to achieve C_{max} (t_{max}) values for females and males was similar across all doses tested. Over the doses tested, the half-life ($t_{1/2}$) values showed no indication of dose- or sex dependency.

In cynomolgus monkeys, the measured rVIII-SingleChain plasma concentrations, based on chromogenic activity and corrected for endogenous FVIII levels, increased with escalating doses. Dose-proportional exposure of the treated animals to rVIII-SingleChain was clearly shown in the single-dose toxicity study. The C_{max} and $AUC_{0\text{-last}}$ increased with dose for both males and females and the increase in exposure was proportional to the dose after a single administration of up to 1500 IU/kg of rVIII-SingleChain: mean C_{max} values ranged from 1.51 to 38.8 IU/mL and mean $AUC_{0\text{-last}}$ values ranged from 13.1 to 293 IU · h/mL. Higher exposure was observed at all dose levels in male versus female monkeys. Mean t_{max} was 0.25 hours for all doses and in both sexes. Similar to the study in rats, the mean $t_{1/2}$ values showed no indication of dose- or sex dependency over the dose range tested.

In the repeated-dosing studies, no systemic toxicity related to rVIII-SingleChain was observed following sub-chronic dosing of rats and cynomolgus monkeys over 28 days at dose levels up to 1250 IU/kg (Table 4). The investigation of potential neurobehavioral effects of rVIII-SingleChain in rats (Irwin test) did not reveal any treatment-related macroscopic or histopathologic changes indicative of an effect on the central nervous system. In rats and monkeys, there were no treatment-related effects for the safety endpoints assessed.

Exposure to and toxicokinetics of rVIII-SingleChain were assessed using two methods: a direct analysis of rVIII-SingleChain concentrations by ELISA in plasma samples from rats and a chromogenic measurement of FVIII activity in plasma samples from monkeys. In the rat repeated-dosing study, the rVIII-SingleChain concentrations measured in most of the plasma samples obtained on Day 28 were found to be below the lower limit of quantification, rendering it impossible to perform toxicokinetic evaluation. In the single-dose rat study (see above), dose-proportional pharmacokinetics were demonstrated. The

Table 3
Procoagulant activity of rVIII-SingleChain and BDD rFVIII (ReFacto AF®) compared with positive (aPCC; FEIBA NF 500®) and negative (saline solution) controls after venous stasis in rabbits by different endpoints (Wessler modification).

Treatment	Dose (IU/kg)	Right jugular vein			Left jugular vein		
		Global thrombus incidence score[a]	Mean dry weight of thrombus (mg) ± SD	PTF[b] (%)	Global thrombus incidence score[a]	Mean dry weight of thrombus (mg) ± SD	PTF[b] (%)
Saline (negative control)	–	1	0.02 ± 0.025	0	3	0.09 ± 0.054	0
aPCC (positive control)	200	21[d]	18.12 ± 7.111[c]	100	23[d]	22.12 ± 5.502[c]	100
BDD rFVIII	500	2	0.07 ± 0.067	0	6	0.25 ± 0.115	1
rVIII-SingleChain	150	0	0.00 ± 0.000	0	0	0.00 ± 0.000	0
	300	4	0.10 ± 0.155	0	4	0.17 ± 0.117	0
	500	0	0.00 ± 0.000	0	6	0.20 ± 0.0093	0
	1000	4	0.13 ± 0.084	1	13[d]	1.47 ± 0.398	6

aPCC = activated prothrombin complex concentrate; BDD = B-domain deleted; PTF = potentiation of thrombus formation; rFVIII = recombinant factor VIII.
[a] Scoring of thrombus incidence: blood = 0; fibrin network = 1; ≥1 small thrombus = 2; non-occlusive thrombus = 3; occlusive thrombus = 4.
[b] PTF (potentiation of thrombus formation) calculated as:

$$\frac{(\text{rVIII} - \text{SingleChain mDWT}) - (\text{negative control group mDWT})}{(\text{positive control group mDWT}) - (\text{negative control group mDWT})} \times 100$$

Where mDWT is the mean dry weight of thrombus. A DWT value of 0.1 mg or less was considered to be equal to 0.
[c] Statistically significant per Student–Newman–Keuls test ($p < 0.05$).
[d] Statistically significant per Mann–Whitney test ($p < 0.05$).

Table 4
Good Laboratory Practice-compliant safety pharmacology and toxicity program for rVIII-SingleChain in three different preclinical species.

Study type	Species	rVIII-SingleChain dose range (IU/kg)	Outcome
Acute, single dose[a]	Rat, monkey	50–1500	No adverse findings
Local tolerance	Rabbit	21–105	No adverse findings
Sub-chronic, repeated dose[a] (incl. Irwin test)	Rat	50–1250	No adverse findings
Sub-chronic, repeated dose[a] (incl. CV parameters)	Monkey	50–500	No adverse findings
CV endpoints by telemetry[a]	Monkey	50–1250	No adverse findings

CV = cardiovascular; FVIII = factor VIII.
[a] Studies encompassing plasma analysis by FVIII:Ag (ELISA) or FVIII activity (chromogenic assay) for toxicokinetic assessment.

toxicokinetic analysis of monkeys that received rVIII-SingleChain 50, 150, or 500 IU/kg indicated that on Day 1 the measured rVIII-SingleChain plasma concentrations increased with increasing dose. C_{max} and AUC_{0-24} increased with dose for both males and females and the increase in exposure was proportional to the dose after a single administration of up to 1500 IU/kg of rVIII-SingleChain. There was no effect of sex on plasma concentrations and toxicokinetic parameters. At Day 6 of the repeated-dosing study in rats and monkeys, there was no evidence of a measured immune response to rVIII-SingleChain. However, at Day 28 (the end of both repeated dosing studies), the majority of animals in all dose groups had developed neutralizing and non-neutralizing ADAs against the heterologous human rVIII-SingleChain. Consequently, treated animals had FVIII levels that had significantly decreased from their pre-treatment values, as well as during all sampling time points after the last application of rVIII-SingleChain. In the cardiovascular safety study in telemetered monkeys, parameters such as arterial blood pressure, left ventricular systolic pressure, left ventricular end diastolic pressure, cardiac output, and stroke volume, were considered unaffected by rVIII-SingleChain treatment (Table 4) after in-depth analysis. No effects were observed throughout the study period on ECG (lead II) waveform morphology after animals received control or rVIII-SingleChain. Furthermore, dose formulation and toxicokinetic analysis revealed proper reconstitution and adequate exposure of all animals to rVIII-SingleChain (data not shown).

Discussion

In this series of preclinical studies, rVIII-SingleChain (1–150 IU/kg) demonstrated equivalent hemostatic activity compared with full-length (Helixate® and Advate®) and BDD rFVIII (ReFacto AF®), with minor differences in total blood loss in a hemophilia A mouse tail-clip bleeding model [17]. In addition, the percentage of animals with hemostasis was comparable between products in this well-established and relevant animal model, reflecting the clinical situation in patients with hemophilia A with regard to major injury or life-threatening trauma. In an assessment of hemostasis kinetics in hemophilia A mice, rVIII-SingleChain (20 IU/kg) and full-length rVIII (Advate® 20 IU/kg) exhibited comparable FVIII hemostatic activity, correcting individual hemostatic efficacy parameters (thromboelastographic, aPTT, and thrombin generation variables) to a similar degree.

In the thromboelastography analysis, the minor and apparently significant difference in CT observed for rVIII-SingleChain compared with Advate® using this highly sensitive technique was not relevant to the hemostatic efficacy seen in vivo (i.e., in the tail-clip model). This observation is corroborated by results from TGA analysis, a similarly sensitive method, which did not show a significant difference in all parameters tested: lag time, thrombin peak, endogenous thrombin potential, and time to thrombin peak. Specifically, in the parameters that measure the early phase of hemostasis there was no significant difference. Furthermore, there was no significant difference in the measured aPTT with rVIII-SingleChain versus full-length rFVIII and both showed bioequivalence when monitoring chromogenic FVIII activity.

An excellent safety profile was observed across all studies in three different, pharmacologically relevant animal species (monkey, rabbit, and rat), with no toxicologically relevant adverse effects after administration of rVIII-SingleChain. This observation is consistent with preclinical safety and toxicity data described for marketed BDD rFVIII products such as ReFacto AF®/Xyntha® [18,19] as well as full-length rFVIII products such as Advate® [20] and Kogenate® [21]. The excellent tolerability and safety characteristics of rVIII-SingleChain were observed at doses up to 1500 IU/kg in the GLP-compliant safety and toxicology program. This represents a safety margin of ≥10-fold above the anticipated standard human dose of approximately 50 IU/kg/day, and even covers higher daily human doses of 75–100 IU/kg in clinical use for the treatment of life-threatening hemorrhages and after major surgery [18].

FVIII activity levels (as geometric means) in plasma samples from the rabbit study ranged from 2 IU/mL to 19 IU/mL across dose levels of 150 IU/kg to 1000 IU/kg. Most importantly, even at high doses exceeding the proposed human standard dose by a factor of 10 and giving rise to FVIII levels of >1.5 IU/mL (odds ratio 4–6), these supra-therapeutic investigations found no increase in prothrombotic risk with rVIII-SingleChain, while exhibiting procoagulant activity comparable to commercial BDD and full-length rFVIII. A thrombogenic risk associated with high plasma FVIII levels (250% FVIII activity) has previously been demonstrated in rodents [22]. In this thrombophilia mouse model, a mild injury was inflicted at the carotid artery by irradiation in combination with the intravenously injected dye rose bengal. However, in the current safety and toxicity program, thrombus formation was induced by stasis of jugular veins in rabbits. Furthermore, the current preclinical investigation used a venous stasis model to investigate the thrombogenic potential of rVIII-SingleChain in rabbits according to Giles et al. [16] based on an initial study by Wessler et al. [23]. The Wessler test, in which induced hypercoagulability is combined with local venous stasis, has been used extensively for over 40 years and has significantly contributed to our understanding of the pathogenesis of venous thromboembolism and its prevention. In addition, it has proven invaluable for assessing the thrombogenicity of various blood products, as well as for assaying the effectiveness of heparin and heparin functions [23,24]. A large number of different animal species have been used to study venous thrombosis and the method of thrombus formation also varies, including differences in the means of inducing vascular wall damage, stasis of blood, and local activation of coagulation. Although there is ample variation in these methods, most techniques may be considered as a variation of the Wessler model, which produces temporary venous stasis by ligating an appropriate vein and taking thrombosis incidence and thrombus dry weight as parameters for evaluation and comparison. Thus, in the context of investigating exaggerated procoagulant activity as a risk factor for thrombosis, both animal models exploring venous and arterial thrombosis appear relevant. Elevated plasma FVIII levels are recognized as an independent risk factor for both arterial and venous thrombosis in humans exhibiting a thrombophilic phenotype [25].

As human rFVIII proteins represent a heterologous protein species to animals, obstacles will be encountered during preclinical pharmacology and safety investigations. As expected, neutralizing and non-neutralizing ADAs against rVIII-SingleChain were observed at the end of the repeated-dosing studies in the majority of the rats and monkeys that received rVIII-SingleChain. This development of ADAs indicates that rVIII-SingleChain functions as an antigen in

rodents and non-rodent animals as a consequence of its heterologous nature as a human protein. In the rat repeated-dosing study, the formation of ADAs after four weeks of daily treatment was thought to be a reason for the concentrations of drug remaining below the lower limit of quantification, meaning that toxicokinetics could not be assessed. Furthermore, this lack of exposure makes toxicity data derived from longer-term chronic toxicity studies tenuous. For this reason, chronic toxicity studies of 3–6 months in duration, even though recommended for biotechnology-derived pharmaceuticals by regulatory guidelines i.e., ICH guideline S6 (R1), were not undertaken in this series of preclinical studies. Formation of alloantibodies is consistent with observations reported from the preclinical development of marketed FVIII concentrates, including BDD FVIII [18,19] and full-length rFVIII products [20]. As reported for other marketed human rFVIII concentrates (ReFacto AF®, Advate®), the ADA response to rVIII-SingleChain correlated with increased aPTT in monkeys, which indicates cross-reactivity of inhibitory ADAs to monkey FVIII clotting factor activity. In accordance with marketed FVIII concentrates, the potential for immunogenicity in humans will be assessed during the rVIII-SingleChain clinical program.

Unlike the currently marketed rFVIII products, which have a two-chain structure, rVIII-SingleChain is a unique single-chain recombinant FVIII. Following activation, the rFVIIIa produced from rVIII-SingleChain is structurally comparable to that formed from two-chain endogenous FVIII [13]. When produced endogenously, FVIII is secreted from hepatic and endothelial cells and processed to a heterodimer consisting of a light chain and a heavy chain joined by a metal ion bridge [26]. Under certain conditions, this structure can dissociate, resulting in the formation of inactive dissociated FVIII chains [27]. The two-chain design, although physiologic for endogenous FVIII, represents a labile configuration in the manufacturing environment, which can result in the formation of inactive dissociated FVIII chains. Therefore, the novel single-chain design could provide a basis for beneficial product characteristics such as high intrinsic stability and molecular integrity. Whether this high molecular stability and integrity when associated with VWF (which protects and shields FVIII in systemic circulation) results in a reduced immunogenic potential will need to be investigated in clinical use.

In conclusion, this preclinical program demonstrates convincing preclinical efficacy for rVIII-SingleChain and provides a clear indication of its excellent safety profile and tolerability. The investigations did not reveal any safety concerns and support the evidence necessary for proceeding into human trials. The rVIII-SingleChain AFFINITY clinical trial program has now commenced with recruitment into and dosing of rVIII-SingleChain in a Phase I/III study.

Conflict of Interest Statement

S. B. Zollner, E. Raquet, J. Müller-Cohrs, H. J. Metzner, T. Weimer, I. Pragst, G. Dickneite, and S. Schulte are employees of CSL Behring GmbH (Marburg, Germany), whose product rVIII-SingleChain was studied in this work.

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References

[1] Schulte S. Pioneering designs for recombinant coagulation factors. Thromb Res 2011;128(Suppl. 1):S9–S12.
[2] Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. Lancet 2003;361:1801–9.
[3] Keeling D, Tait C, Makris M. Guideline on the selection and use of therapeutic products to treat haemophilia and other hereditary bleeding disorders. A United Kingdom Haemophilia Center Doctors' Organisation (UKHCDO) guideline approved by the British Committee for Standards in Haematology. Haemophilia 2008;14:671–84.
[4] Srivastava A, Brewer AK, Mauser-Bunschoten EP, Key NS, Kitchen S, Llinas A, et al. Guidelines for the management of hemophilia. Haemophilia 2013;19:e1-47.
[5] Wong T, Recht M. Current options and new developments in the treatment of haemophilia. Drugs 2011;71:305–20.
[6] Franchini M, Lippi G. Recombinant factor VIII concentrates. Semin Thromb Hemost 2010;36:493–7.
[7] Sheridan C. Hemophilia market awaits next-generation therapies. Nat Biotechnol 2011;29:960.
[8] Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, et al. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. Lancet 1992;339:594–8.
[9] Hay CR, Palmer B, Chalmers E, Liesner R, Maclean R, Rangarajan S, et al. Incidence of factor VIII inhibitors throughout life in severe hemophilia A in the United Kingdom. Blood 2011;117:6367–70.
[10] Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. Haemophilia 2003;9:418–35.
[11] Bjorkman S, Berntorp E. Pharmacokinetics of coagulation factors: clinical relevance for patients with haemophilia. Clin Pharmacokinet 2001;40:815–32.
[12] Bjorkman S, Folkesson A, Jonsson S. Pharmacokinetics and dose requirements of factor VIII over the age range 3-74 years: a population analysis based on 50 patients with long-term prophylactic treatment for haemophilia A. Eur J Clin Pharmacol 2009;65:989–98.
[13] Schmidbauer S, Witzel R, Kreuter J, Schwingal S, Robbel L, Sebastian P, et al. Characterization of recombinant single chain FVIII, rVIII-SingleChain (CSL627). Haemophilia 2012;18:37.
[14] Hossler P, Khattak SF, Li ZJ. Optimal and consistent protein glycosylation in mammalian cell culture. Glycobiology 2009;19:936–49.
[15] Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian Jr HH. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 1995;10:119–21.
[16] Giles AR, Johnston M, Hoogendoorn H, Blajchman M, Hirsh J. The thrombogenicity of prothrombin complex concentrates: I. The relationship between in vitro characteristics and in vivo thrombogenicity in rabbits. Thromb Res 1980;17:353–66.
[17] Sabatino DE, Nichols TC, Merricks E, Bellinger DA, Herzog RW, Monahan PE. Animal models of hemophilia. Prog Mol Biol Transl Sci 2012;105:151–209.
[18] EPAR ReFacto AF® Pfizer. Scientific Discussion. [online] http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000232/WC500049003.pdf/; 2012 . [Accessed 27/03/13].
[19] FDA. Pharmacology/Toxicology Review Memorandum (9/26/2007) - XYNTHA®. http://www.fda.gov/biologicsbloodvaccines/bloodbloodproducts/approvedproducts/licensedproductsblas/fractionatedplasmaproducts/ucm056051/2012 [Accessed: 27/03/2013].
[20] EPAR Advate® Baxter Healthcare. Scientific Discussion. [online] http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000520/WC500022464.pdf/; 2012 [Accessed 27/03/2013].
[21] EPAR Kogenate® Bayer. Scientific Discussion. [online] http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000232/WC500049003.pdf/; 2012 [Accessed 27/03/2013].
[22] Kawasaki T, Kaida T, Arnout J, Vermylen J, Hoylaerts MF. A new animal model of thrombophilia confirms that high plasma factor VIII levels are thrombogenic. Thromb Haemost 1999;81:306–11.
[23] Wessler S. Studies in intravascular coagulation. III. The pathogenesis of serum-induced venous thrombosis. J Clin Invest 1955;34:647–51.
[24] Thomas DP. Venous thrombosis and the "Wessler test". Thromb Haemost 1996;76:1–4.
[25] Kamphuisen PW, Eikenboom JC, Bertina RM. Elevated factor VIII levels and the risk of thrombosis. Arterioscler Thromb Vasc Biol 2001;21:731–8.
[26] Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, et al. Structure of human factor VIII. Nature 1984;312:337–42.
[27] Sawamoto Y, Prescott R, Zhong D, Saenko EL, Mauser-Bunschoten E, Peerlinck K, et al. Dominant C2 domain epitope specificity of inhibitor antibodies elicited by a heat pasteurized product, factor VIII CPS-P, in previously treated hemophilia A patients without inhibitors. Thromb Haemost 1998;79:62–8.