Thrombosis Research 134 (2014) 125–131

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Non-clinical pharmacokinetics and pharmacodynamics of rVIII-SingleChain, a novel recombinant single-chain factor VIII

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ARTICLE INFO

Article history:
Received 1 November 2013
Received in revised form 6 March 2014
Accepted 11 March 2014
Available online 19 March 2014

Keywords:
Animal models
Coagulation factors
Haemophilia A/B
Haemophilia therapy
von Willebrand factor

ABSTRACT

Introduction: rVIII-SingleChain (CSL627), a novel recombinant coagulation factor VIII (FVIII), is under investigation in a phase I/III clinical programme (AFFINITY) for the treatment of haemophilia A. Non-clinical studies were conducted to investigate the pharmacokinetic/pharmacodynamic profile of rVIII-SingleChain in comparison with full-length recombinant FVIII.

Materials and Methods: Binding affinity of rVIII-SingleChain for von Willebrand factor was investigated by surface plasmon resonance analysis. The pharmacokinetic profile of rVIII-SingleChain was compared with a marketed full-length recombinant FVIII concentrate (Advate®) in haemophilia A mice, von Willebrand factor knock-out mice, Crl:CD (SD) rats, rabbits and cynomolgus monkeys. Systemic FVIII activity or antigen levels were recorded. Procoagulant activity was measured in an $FeCl_3$-induced arterial occlusion model and by recording thrombin generation activity (ex vivo) after administration of 200–250 IU/kg rVIII-SingleChain or full-length FVIII to haemophilia A mice.

Results: rVIII-SingleChain displayed a high affinity for von Willebrand factor (K_D = 44 pM vs. 139 pM for full-length recombinant FVIII). In all animal species tested, rVIII-SingleChain had more favourable pharmacokinetic properties than full-length recombinant FVIII: clearance was decreased and area under the curve and terminal half-life were enhanced vs. full-length recombinant FVIII, while in vivo recovery and volume of distribution were equivalent. rVIII-SingleChain showed a prolonged thrombin generation potential and prolonged procoagulant activity vs. full-length recombinant FVIII in an $FeCl_3$-induced arterial occlusion model.

Conclusions: rVIII-SingleChain had a higher affinity for von Willebrand factor than full-length recombinant FVIII and displayed favourable pharmacokinetic/pharmacodynamic properties in non-clinical models.

Introduction

Treatment of patients with haemophilia A has improved significantly over recent decades. However, the development of inhibitory antibodies is still a major safety issue during factor VIII (FVIII) replacement therapy; it occurs in 15 − 30% of patients [1–3] and can lead to a higher risk of bleeding and subsequent treatment complications [4,5]. Furthermore, owing to the inherently short half-life of FVIII (approximately 10 − 14 hours), patients who undergo a prophylactic regimen usually require three doses per week [6]. Thus, there is an unmet medical need for a FVIII therapy that offers reduced potential for immunogenicity with a more convenient dosing frequency.

Under normal conditions, approximately 95% of FVIII in circulation is in complex with von Willebrand factor (VWF) [7]. VWF is the physiological partner of FVIII in plasma [7] and determines its half-life and stability, and prevents its receptor-mediated clearance in vivo [8,9]. Indeed, the half-life of FVIII is reduced to less than 3 hours in patients with type 3 von Willebrand disease [10]. The FVIII/VWF complex also plays an important role in the physiological activity of FVIII and has been shown to influence the presentation of FVIII to the immune system [8,11,12]. For instance, administration of VWF-containing concentrates

Abbreviations: AUC, area under the observed average plasma concentration vs. time curve; $AUC_{0\text{-last}}$, AUC up to the last quantifiable sampling time; CI, confidence interval; CL, clearance; C_{max}, maximum plasma concentration; ELISA, enzyme-linked immunosorbent assay; FL, full-length; FVIII, factor VIII; k_a, association rate constant; K_A, association equilibrium constant; k_d, dissociation rate constant; K_D, dissociation equilibrium constant; MRT, mean residence time; KO, knock-out; PD, pharmacodynamic; pd-VWF, plasma-derived von Willebrand factor; PK, pharmacokinetic; rFVIII, recombinant factor VIII; SD, standard deviation; $t_{1/2\beta}$, terminal half-life; V_d, apparent volume of distribution; $V_{initial}$, apparent initial volume of distribution; V_{ss}, volume of distribution at steady state; VWF, von Willebrand factor.

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http://dx.doi.org/10.1016/j.thromres.2014.03.028

hypothetically reduces the incidence of inhibitory antibodies against FVIII in patients with haemophilia A [8,11–13]. Thus, an increased interaction between FVIII and VWF may improve the stability and molecular integrity of FVIII in circulation and minimise the risk of antibody development.

rVIII-SingleChain (CSL627) is a novel single-chain recombinant coagulation FVIII (rFVIII) construct composed of covalently bonded heavy and light chains that retain all essential binding sites [14]. Following activation, the rFVIIIa produced from rVIII-SingleChain is structurally identical to that formed from two-chain rFVIII [15,16]. When produced endogenously, FVIII is processed and secreted as a heterodimer consisting of a light chain and a heavy chain joined with a metal ion bridge [17]. Under certain conditions, this structure can dissociate, resulting in the formation of inactive dissociated FVIII chains [9,18,19]. This two-chain design, although physiological for endogenous FVIII, represents a labile configuration in the manufacturing process, which can result in the formation of inactive dissociated FVIII chains.

Previous non-clinical studies of rVIII-SingleChain in several models of haemophilia A have shown excellent tolerability and convincing pharmacodynamic (PD) efficacy comparable to marketed full-length and B-domain-deleted human rFVIII [14,20,21].

The present series of studies aimed to determine the binding affinity between rVIII-SingleChain and VWF and to investigate its pharmacokinetic (PK)/PD profile in several animal species, with a view to supporting its continued clinical development in haemophilia A.

Materials and Methods

Study Materials

The compounds tested in this series of studies were rVIII-SingleChain (CSL Behring, Marburg, Germany) and full-length rFVIII (Advate®, Baxter Bioscience, Vienna, Austria). Both test materials were used directly after reconstitution or after storage below −70 °C (one freeze/thaw cycle).

Surface Plasmon Resonance Analysis

Binding affinity between rFVIII and plasma-derived (pd)-VWF was analysed by surface plasmon resonance using a Biacore 3000 instrument (GE Healthcare, Freiburg, Germany). An anti-VWF monoclonal antibody was immobilised through primary amines onto CM3 sensor chips (GE Healthcare) and used as a capture surface for reversible immobilisation of pd-VWF (prepared in house). Regeneration of the chip was performed by 250 mM $CaCl_2$ solution. Full-length rFVIII and rVIII-SingleChain were tested on the captured VWF-immobilised chip at concentrations of 250 − 340 RU. Four batches of rVIII-SingleChain and four batches of full-length rFVIII were measured at five different concentrations (0.125 − 1 nM) in triplicate. The experiments were performed at 25 °C with HBS-P buffer (GE Healthcare) as running buffer.

All binding curves measured for the VWF–rFVIII interaction were fitted into a 1:1 Langmuir binding model to determine the association (k_a) and dissociation (k_d) rate constants. The k_a and k_d rate constants were calculated for every set of curves individually, and association and dissociation equilibrium constants (K_A and K_D) were calculated for each set of curves separately. Biacore BIAevaluation Software Version 4.1 (GE Healthcare) was used for data analysis. FVIII concentrations were determined using the chromogenic substrate (CS) FVIII:C activity assay (Chromogenix, Milan, Italy), standardised using an in-house standard calibrated against the international FVIII concentrate standard (8th International Standard Factor VIII Concentrate, NIBSC code: 07/350). The specific activity for rVIII-SingleChain was determined as the quotient of the CS FVIII activity (IU mL^{-1}) and the protein concentrations, which were measured in drug substance stage samples at an optical density of 280 − 320 nm. The measured rVIII-SingleChain batches had a mean specific activity of 12,000 IU mg^{-1}. For the full-length rFVIII molecule, a specific activity of 7000 IU mg^{-1} was taken as the mean of literature-based values (4000 − 10,000 IU mg^{-1}) [22]. Molar concentrations were determined using the molar mass of 170 kDa for rVIII-SingleChain and 250 kDa for the full-length rFVIII molecule.

Pharmacokinetics

Animals

The PK profiles of rVIII-SingleChain and full-length rFVIII were determined following a single intravenous injection to FVIII knock-out (KO) mice (100 IU kg^{-1}; Charles River Laboratories, Kisslegg, Germany) [23], VWF KO mice (100 IU kg^{-1}; Charles River Laboratories) [24], Crl:CD (SD) rats (400 IU kg^{-1}; Charles River Laboratories), Chinchilla Bastard rabbits (150 IU kg^{-1}; Bauer, Neuental, Germany) and cynomolgus monkeys (*Macaca fascicularis*) (250 IU kg^{-1}; Huntingdon Life Sciences, Huntingdon, UK). Test animals were dosed according to a CS FVIII assay system for rVIII-SingleChain (FVIII:C; Chromogenix) and according to labelling for the comparator. Plasma samples were drawn predose (monkeys and rabbits) and at various timepoints after treatment until 72 hours in haemophilia A mice (n = 5 per timepoint), 48 hours in rats (n = 3 per timepoint), 72 hours in rabbits (n = 3 for full-length rFVIII, n = 4 for rVIII-SingleChain) and 24 hours in VWF KO mice (n = 6 per timepoint) and monkeys (n = 2 for full-length rFVIII, n = 10 for rVIII-SingleChain). Blood samples from all species were immediately processed in 10% citrate (9:1 mixing ratio, 3.13% w/v) for mice and monkeys and 20% citrate (8:2 mixing ratio, 3.13% w/v) for rats and rabbits.

Pharmacokinetic Analysis

PK analysis was performed using WinNonlin version 6.0 (Pharsight, St Louis, Missouri, USA) and FUNCALC version 2 (Prolytic GmbH, Frankfurt am Main, Germany) software. In mice and monkeys, plasma levels of FVIII were determined by measuring CS FVIII activity (FVIII:C chromogenic assay, Chromogenix). In rabbits and rats, antigen determination of FVIII levels was undertaken by enzyme-linked immunosorbent assay (ELISA) (rats: Asserachrom Stago, S.A.S., France; rabbits: polyclonal affinity purified anti-FVIII:C immunoglobulin G, Cedarlane Hornby, Ontario, Canada). PK parameters were determined for the average FVIII plasma concentration vs. time curve of each group by non-compartmental methods in FVIII KO mice, rats and rabbits. Compartmental models were used in VWF KO mice (one compartment) and monkeys (two compartments) to estimate and adjust for endogenous FVIII activity. Further details of the PK analysis can be found in the Supporting information.

Statistical Analysis

All statistical analyses were conducted with SAS® version 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Geometric and arithmetic means, and standard deviations (SDs) were calculated.

In the studies in haemophilia A and VWF KO mice, the bioavailability of rVIII-SingleChain was compared with that of full-length rFVIII by calculating the ratio of the areas under the observed average plasma concentration vs. time curves (AUCs), i.e., without employing extrapolation. Mixed analysis of variance models was used to calculate p-values and confidence intervals (CIs). Arterial occlusion rates were analysed in a linear mixed logistic regression model with treatment group and log(time) as covariates and animal as random effect.

Thrombin Generation Assay (ex vivo)

Haemophilia A mice were treated with rVIII-SingleChain or full-length rFVIII with a single intravenous administration of 250 IU kg^{-1}

on Day 0. Blood was terminally collected under deep anaesthesia at different timepoints (Days 1 — 8). Blood samples were processed in 10% citrate plasma (9:1 mixing ratio, 3.13% w/v) and stored at room temperature until centrifugation. Samples were centrifuged at 3000 *g* in a Megafuge® 1.0R centrifuge fitted with a swing-out rotor #2704 (Thermo Scientific Heraeus, Langenselbold, Germany) for 10 minutes at room temperature. Plasma was then pipetted off and stored at approximately −70 °C until analysis. The thrombin generation assay was performed by calibrated automated thrombography (CAT, Thrombinoscope, Maastricht, The Netherlands) after intrinsic activation in the presence of phospholipids (Rossix, Mölndal, Sweden)/Pathromtin® SL (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Thrombin peak levels were recorded and the average AUC of peak thrombin levels for Days 1 — 8 calculated by the linear trapezoidal rule. The AUCs of the two FVIII products were compared using an approximate F-test for the difference in AUC in a linear model with variable variances per timepoint and treatment group, resulting in an estimated time until peak levels of thrombin dropped below a defined limit ranging between 50 and 250 nM.

$FeCl_3$-induced Arterial Occlusion

Arterial occlusion was induced in haemophilia A mice by placing a patch of filter paper saturated with 10% $FeCl_3$ on the carotid artery for 3 minutes. Blood flow was assessed for 40 minutes using a perivascular flow module TS420 and perivascular flow probes (Transonic System Inc., Ithaca, New York, USA) to monitor thrombotic occlusion. Mice were dosed with 200 IU kg^{-1} of rVIII-SingleChain or full-length rFVIII according to labelled FVIII activity at 24, 32, 40, 48, 54 and 64 hours before induction of thrombosis. A vehicle (buffer)-treated group served as control.

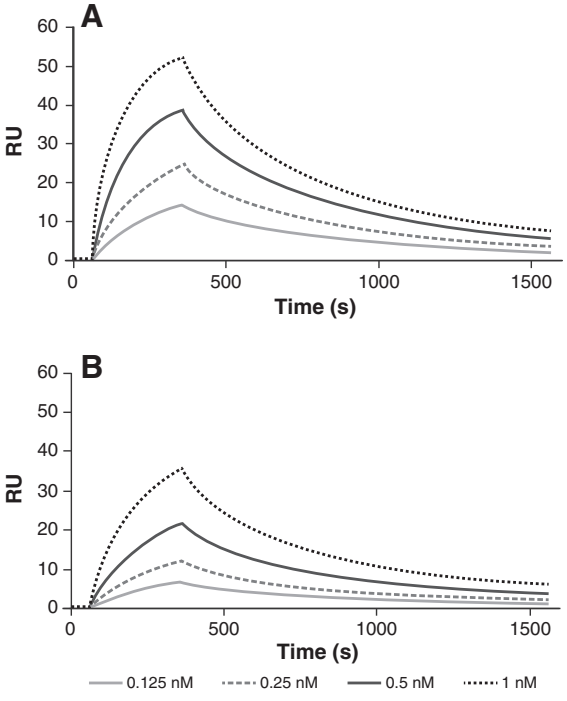

Fig. 1. Binding curves of: (**A**) rVIII-SingleChain and (**B**) full-length rFVIII (Advate®) to plasma-derived von Willebrand factor. rFVIII concentrations from 0.125 nM to 1 nM were measured. rFVIII, recombinant factor VIII; RU, relative units.

Table 1
Mean (standard deviation) kinetic parameters and equilibrium constants of the binding of rVIII-SingleChain and full-length rFVIII (Advate®) to plasma-derived von Willebrand factor. rVIII-SingleChain was from four different development lots and full-length rFVIII (Advate®) was from four commercially available lots. Each lot was measured in triplicate.

Mean (±standard deviation)	rVIII-SingleChain (n = 4)	Full-length rFVIII (n = 4)
k_a (M^{-1} s^{-1})	5.36×10^6 ($\pm 1.65 \times 10^6$)	2.12×10^6 ($\pm 1.25 \times 10^5$)
k_d (s^{-1})	2.18×10^{-4} ($\pm 4.06 \times 10^{-6}$)	2.92×10^{-4} ($\pm 6.47 \times 10^{-6}$)
K_A (M^{-1})	24.7×10^9 ($\pm 7.84 \times 10^9$)	7.27×10^9 ($\pm 5.34 \times 10^8$)
K_D (pM)	43.64 (±12.65)	139.42 (±11.59)

Abbreviation: FVIII, factor VIII; k_a, association rate constant; K_A, equilibrium association constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant; rFVIII, recombinant factor VIII.

Results

Surface Plasmon Resonance Analysis

Surface plasmon resonance analysis showed that rVIII-SingleChain had a greater affinity for VWF than full-length rFVIII (Fig. 1). The means and SDs of the different lots are shown in Table 1.

The dissociation equilibrium constant of rVIII-SingleChain and pd-VWF was determined at 44 pM; for full-length rFVIII, the dissociation equilibrium constant was 139 pM. Thus, the affinity of rVIII-SingleChain for pd-VWF was 3-fold higher than the affinity of the full-length rFVIII molecule, mainly originating from a higher k_a.

Pharmacokinetics

Haemophilia A Mice

The PK parameters of rVIII-SingleChain vs. full-length rFVIII in haemophilia A mice are given (Table 2 and Fig. 2A). There was an approximately 2-fold enhancement of AUC up to the last quantifiable sampling time ($AUC_{0\text{-last}}$) with rVIII-SingleChain vs. full-length rFVIII. Likewise, mean residence time (MRT) and terminal half-life ($t_{1/2\beta}$) were approximately 2-fold higher with rVIII-SingleChain. $AUC_{0\text{-last}}$ and $t_{1/2\beta}$ results obtained after rVIII-SingleChain treatment were significantly higher compared with full-length rFVIII, with an $AUC_{0\text{-last}}$ ratio of 1.97 (90% CI, 1.7–2.3; $p < 0.0001$) and a $t_{1/2\beta}$ ratio of 1.65 (90% CI, 1.11–2.70; $p = 0.036$). In accordance with these changes, clearance (CL) of rVIII-SingleChain was lower than with full-length rFVIII. Both volume of distribution at steady state (V_{ss}) and maximum plasma concentration (C_{max}) were similar between treatment groups.

VWF KO Mice

The $AUC_{0\text{-last}}$ and $t_{1/2\beta}$ of both rVIII-SingleChain and full-length rFVIII decreased by a factor of approximately 30 in VWF KO mice compared with haemophilia A mice (Table 2 and Fig. 2B). However, the ratios of PK parameters for rVIII-SingleChain compared with full-length rFVIII in VWF KO mice were similar to those observed in haemophilia A mice. The estimated $AUC_{0\text{-last}}$ ratio for rVIII-SingleChain:full-length rFVIII was 2.04 (90% CI, 1.47–2.99; $p = 0.001$) in VWF KO mice. This was despite the slightly higher in vivo recovery for rVIII-SingleChain vs. full-length rFVIII in the initial phase after administration in VWF KO mice (Table 2 and Fig. 2B) compared with similar in vivo recovery for both molecules in haemophilia A mice.

Rats

The PK parameters of rVIII-SingleChain and full-length rFVIII in Crl:CD (SD) rats are given (Table 2 and Fig. 2C). $AUC_{0\text{-last}}$ was increased approximately 6-fold with rVIII-SingleChain compared with full-length rFVIII. Similarly, $t_{1/2\beta}$ and MRT were approximately 2- to 3-fold higher and CL was more than 5-fold lower compared with full-length rFVIII. C_{max} was marginally higher with rVIII-SingleChain than full-length rFVIII, while V_{ss} was more than 2-fold lower.

Table 2
Pharmacokinetic characteristics of rVIII-SingleChain and full-length rFVIII (Advate®) in haemophilia A and VWF KO mice, Cri:CD (SD) rats, cynomolgus monkeys and Chinchilla Bastard rabbits.

Parameter	Haemophilia A mice*		VWF KO mice*		Cri:CD (SD) rats†		Rabbits‡		Cynomolgus monkeys§	
	rVIII-SingleChain (n = 5/ timepoint)	FL rFVIII (Advate®) (n = 5/ timepoint)	rVIII-SingleChain (n = 4–6/ timepoint)	FL rFVIII (Advate®) (n = 4–6/ timepoint)	rVIII-SingleChain (n = 3/ timepoint)	FL rFVIII (Advate®) (n = 3/ timepoint)	rVIII-SingleChain (n = 4)	FL rFVIII (Advate®) (n = 3)	rVIII-SingleChain (n = 10)	FL rFVIII (Advate®) (n = 2)
$AUC_{0\text{-last}}$ (h · IU mL^{-1})	35	18	1.25	0.59	43.5	7.0	40.1	10.7	76.1	48.7
C_{max} (IU mL^{-1})	2.3	2.2	1.52	1.24	5.9	5.0	2.6	2.6	8.0	11.6
CL (mL h^{-1} kg^{-1})	2.7	5.5	80	170	8.8	51.7	3.1	8.0	2.6	4.7
MRT (h)	18.3	10.3	0.82	0.48	7.4	2.9	21.6	9.6	15.3	9.2
$t_{1/2\beta}$ (h)	15.9	9.7	0.57	0.33	5.2	2.7	14.8	6.9	10.8	6.9
$V_{initial}$ (mL kg^{-1})	43	43	66	81	67	76	55	56	31	22
V_{SS} (mL kg^{-1})	50	57	66	81	66	151	66	77	39	43
V_d (mL kg^{-1})	61	67	66	81	66	204	66	80	40	47

Abbreviation: $AUC_{0\text{-last}}$, area under the plasma concentration vs. time curve up to the last quantifiable sampling time; CL, clearance; C_{max}, maximal plasma concentration; ELISA, enzyme-linked immunosorbent assay; FL, full-length; FVIII, factor VIII; KO, knock-out; MRT, mean residence time; rFVIII, recombinant factor VIII; $t_{1/2\beta}$, terminal half-life; V_d, apparent volume of distribution; $V_{initial}$, apparent initial volume of distribution; V_{ss}, apparent volume of distribution at steady state.
*At a dose of 100 IU kg^{-1}, FVIII activity was measured by the chromogenic system and corrected for endogenous levels in VWF KO mice. †At a dose of 400 IU kg^{-1}, FVIII antigen was measured by ELISA. ‡At a dose of 150 IU kg^{-1}, FVIII antigen was measured by ELISA geometric means of individual pharmacokinetic parameters. §At a dose of 250 IU kg^{-1}, FVIII activity was measured by the chromogenic system and corrected for endogenous levels; geometric means of individual pharmacokinetic parameters.

Rabbits

The PK parameters of rVIII-SingleChain and full-length rFVIII in rabbits are given (Table 2). $AUC_{0\text{-last}}$ was increased almost 4-fold with rVIII-SingleChain compared with full-length rFVIII. Similarly, $t_{1/2\beta}$ was more than 2-fold higher, MRT was more than 2-fold higher and CL was more than 2-fold lower with rVIII-SingleChain vs. full-length rFVIII. C_{max} and V_{ss} were similar between treatment groups.

Cynomolgus Monkeys

Plasma levels of FVIII declined from a maximum at the first sampling time in an apparent bi-exponential manner, with a $t_{1/2\beta}$ of 10.8 hours (range: 6.8 − 15.6 hours) for rVIII-SingleChain and 6.9 hours (range: 6.5 − 7.3 hours) for full-length rFVIII, and an MRT of 15.3 hours and 9.2 hours, respectively (Table 2 and Fig. 2D). Mean $AUC_{0\text{-last}}$ following administration of rVIII-SingleChain was approximately 1.5-fold higher than that after administration of full-length rFVIII and CL was approximately 2-fold lower. In vivo recovery and V_{ss} appeared similar between groups. Both treatments were well tolerated and there were no adverse clinical signs, reactions to treatment or treatment-associated injection-site reactions.

The PK profile was dose proportional over the range of 50 − 1500 IU/kg and PK parameters derived from all other dose groups (i.e. besides the 250 IU/kg group) support the favourable PK properties. Median $t_{1/2\beta}$ of rVIII-SingleChain calculated from 48 monkeys in two PK and two Good Laboratory Practice-compliant toxicology studies [21] was 10.4 hours and median clearance was 2.64 mL h^{-1} kg^{-1} (data not shown).

Thrombin Generation Assay

In haemophilia A mice, rVIII-SingleChain showed favourable haemostatic activity compared with full-length rFVIII, as indicated by the longer duration (average of 20 hours) for the thrombin peak levels to drop below each limit between 50 and 250 nM (Fig. 3). When assessing the area under the peak curve between Days 1 and 8, the thrombin generation activity of rVIII-SingleChain was also significantly higher than full-length rFVIII (estimated ratio 1.26, 90% CI, 1.14–1.39; $p[AUC_{TGA\ Peak}\ ratio = 1] = 0.0002$).

$FeCl_3$-induced Arterial Occlusion

Similarly, higher and longer PD effects of rVIII-SingleChain were evident compared with full-length rFVIII in an $FeCl_3$-induced arterial occlusion model in haemophilia A mice over 64 hours (Fig. 4). In both treatment groups the occlusion rates decreased with time, indicating the loss of FVIII activity over time. On average, the time to reach a particular occlusion rate was approximately 29 hours later in the rVIII-SingleChain-treated mice than in the mice treated with full-length rFVIII, however, the difference was not significant ($p = 0.13$).

Discussion

The present series of non-clinical studies assessed the binding affinity of rVIII-SingleChain to VWF and investigated its PK and PD profiles in animals. rVIII-SingleChain showed improved binding affinity for VWF compared with full-length rFVIII in haemophilia A mice. Furthermore, rVIII-SingleChain had a favourable PK profile in four non-clinical animal species vs. marketed full-length rFVIII and prolonged procoagulant effects in haemophilia A mice.

The binding of VWF to FVIII in plasma plays a vital role in its stability, half-life and presentation to the immune system [8,11,12]. In the present study, a 3-fold increase in the binding affinity of rVIII-SingleChain for VWF, resulting in more rapid and stronger binding to VWF vs. full-length rFVIII, represents a favourable finding in comparison with other rFVIII products currently under development. Similar studies with

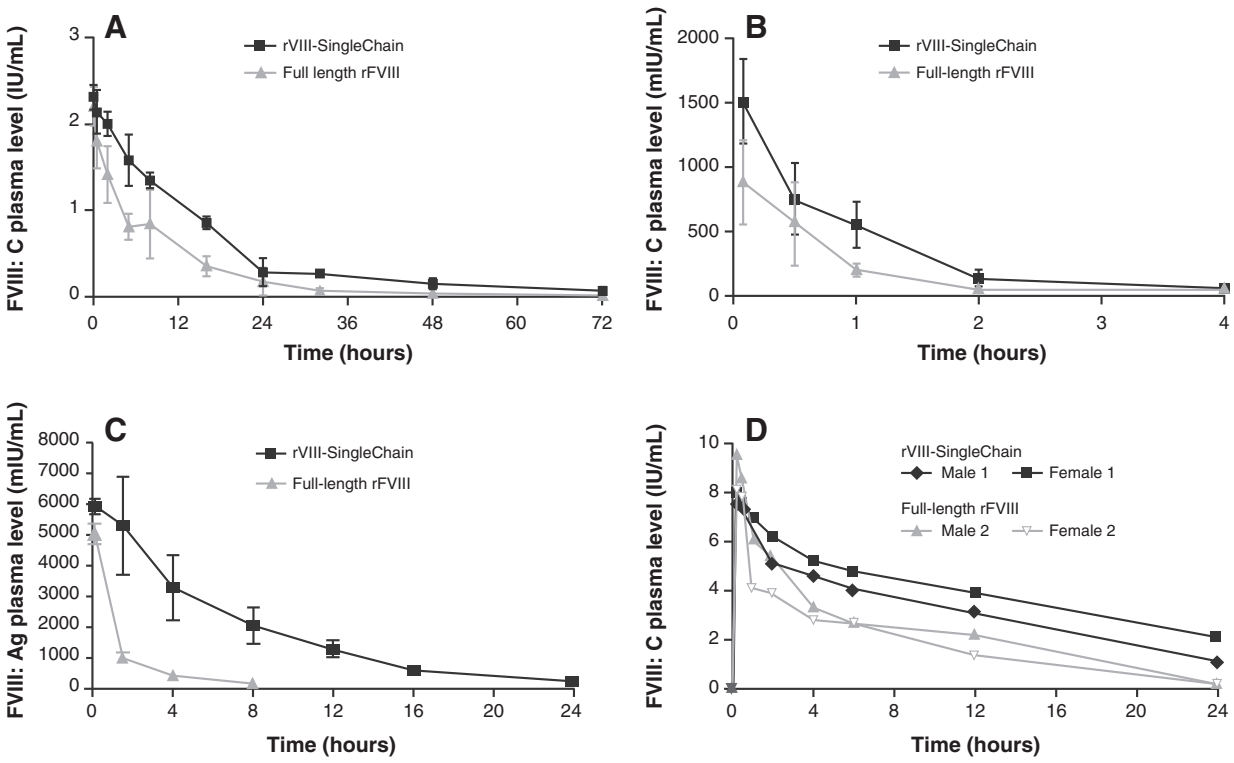

Fig. 2. Concentration–time curves following dosing with rVIII-SingleChain or full-length rFVIII (Advate®) in: (**A**) haemophilia A mice (100 IU kg^{-1}), (**B**) von Willebrand factor KO mice (100 IU kg^{-1}), (**C**) Crl:CD (SD) rats (400 IU kg^{-1}) and (**D**) cynomolgus monkeys (250 IU kg^{-1}). Data are arithmetic mean ± standard deviation. FVIII, factor VIII; KO, knock-out; rFVIII, recombinant factor VIII.

human-cl rhFVIII and N8 have shown approximately 1.5- and 2-fold increases, respectively, in binding affinity vs. full-length rFVIII [25,26].

It appears that the enhanced binding of rVIII-SingleChain to VWF may contribute to its beneficial PK characteristics. The half-life of rVIII-SingleChain was, depending on the animal model, approximately 2-fold greater than full-length rFVIII and CL was approximately 2-fold lower. Moreover, the overall PK properties of rVIII-SingleChain and full-length rFVIII were markedly different in haemophilia A mice compared with VWF KO mice. In VWF KO mice, the AUC and half-life were approximately 30 times lower, and accordingly clearance was higher compared with haemophilia A mice. This emphasises the importance of strong binding of FVIII to VWF for improving the pharmacokinetic properties of FVIII. Additionally, as rVIII-SingleChain showed similarly beneficial pharmacokinetic properties in VWF KO mice compared with full-length rFVIII, it may be assumed that other features, such as the intrinsic molecular stability and thus structural integrity of rVIII-SingleChain, also contribute to its favourable properties.

Owing to the short half-life of FVIII, current prophylaxis regimens for haemophilia A typically require injections three times per week to maintain a sufficient level of circulating clotting factor. The inconvenience of three times weekly dosing is a significant burden to people with haemophilia A. The favourable PK of rVIII-SingleChain may therefore offer the potential for more convenient dosing schedules, a hypothesis that needs to be confirmed in future human studies.

The PK properties of rVIII-SingleChain in several animal species compare favourably with several extended half-life products currently under development, which employ strategies such as PEGylation and fusion protein technology to reduce the required dosing frequency. For instance, in studies in haemophilia A mice, the $t_{1/2\beta}$ of rFVIIIFc was 13.7 hours [27], PEGylated B-domain deleted-rFVIII was 13.6 hours [28], glycoPEGylated rFVIII (N8-GP) was 14 hours [29] and PEGylated rFVIII (BAX 855) was 5.9 hours [30] compared with 15.9 hours for rVIII-SingleChain. Corresponding studies in rabbits, rats and monkeys also show similar half-lives as rVIII-SingleChain for PEGylated B-domain deleted-rFVIII [28], glycoPEGylated rFVIII (N8-GP) [29] and PEGylated rFVIII (BAX 855) [30]. Interestingly, comparison of half-life values obtained using different methods to determine plasma FVIII levels suggests that ELISA-based analysis tends to result in a longer half-life than activity-based assays. This observation has been reported for PEGylated FIX (N9-GP) [31] and PEGylated rFVIII (BAX 855) [30] in non-clinical species. Furthermore, ELISA analysis of N8-GP plasma levels in monkeys revealed a notably longer half-life (18 hours) [29] than for BAX 855 (9.4 hours) [30] and rVIII-SingleChain (10.8 hours), which were derived by FVIII chromogenic activity. In accordance with its favourable $t_{1/2\beta}$, CL of rVIII-SingleChain in non-clinical

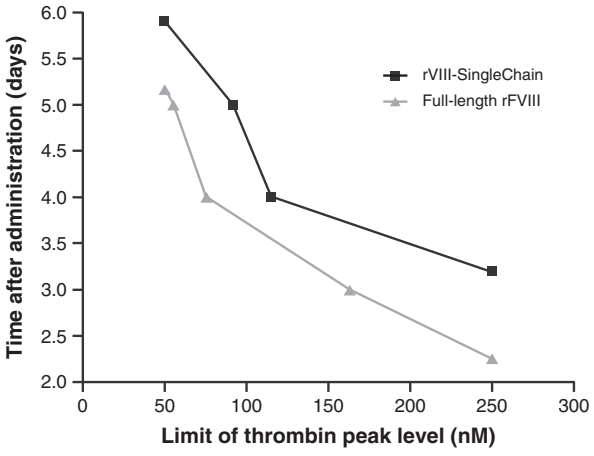

Fig. 3. Time-dependent drop in the thrombin generation assay parameter thrombin peak level ex vivo in haemophilia A mice following dosing with rVIII-SingleChain or full-length rFVIII (Advate®) (250 IU kg^{-1}). FVIII, factor VIII; rFVIII, recombinant factor VIII.

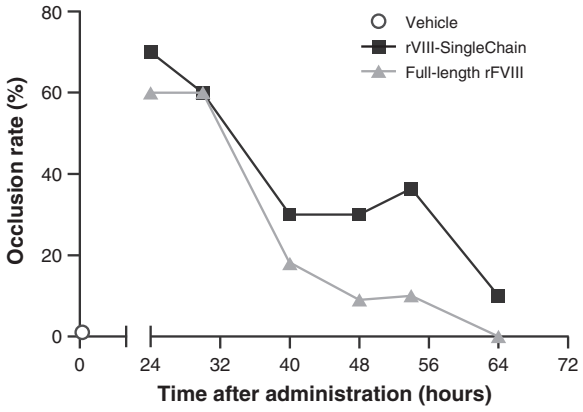

Fig. 4. Occlusion rate after $FeCl_3$-induced thrombosis in haemophilia A mice receiving rVIII-SingleChain or full-length rFVIII (Advate®) (200 IU kg^{-1}; n = 10–11/timepoint). FVIII, factor VIII; rFVIII, recombinant factor VIII.

species was similar to values reported for half-life-extended FVIII molecules [27,29,30].

Interestingly, regardless of the method of extension, the half-life of modified rFVIII variants appears to be maximally twice as long as wild-type FVIII in a variety of non-clinical animal models. This suggests that similar to the human situation, the half-life of FVIII in animals is predominantly determined by the elimination half-life of its physiological partner VWF. Intriguingly, among animal species, monkeys mimic human conditions in terms of the dependency of FVIII/VWF half-life on intrinsic variability factors for VWF half-life (e.g., ABO blood group [32] and glycosylation). To this end, the PK of rVIII-SingleChain in monkeys may be most relevant to its clinical PK, which is currently under investigation in the AFFINITY clinical trial programme.

The improved PK of rVIII-SingleChain were reflected in the improved PD vs. full-length rFVIII. In haemophilia A mice rVIII-SingleChain showed favourable haemostatic activity in a thrombin generation assay and prolonged procoagulant activity in $FeCl_3$-induced arterial occlusion. The arterial occlusion model exhibits lower variability and better sensitivity at lower FVIII activity levels than the tail-cut bleeding model [33,34], which allows measurement of smaller, but still distinguishable, differences in procoagulant activity. The arterial occlusion model was therefore considered the more suitable model for evaluating potential differences in procoagulant activity between rVIII-SingleChain and full-length rFVIII, particularly at the advanced time points investigated (up to 64 hours).

This non-clinical programme indicates favourable PK and PD for rVIII-SingleChain compared with full-length rFVIII. The ongoing AFFINITY clinical trial programme (phase I/III) will assess the PK, safety, immunogenicity and efficacy of rVIII-SingleChain in patients with haemophilia A.

Conflicts of Interest

S. Zollner is an employee of CSL Behring AG (Bern, Switzerland). E. Raquet, P. Claar, J. Müller-Cohrs, H. Metzner, T. Weimer, I. Pragst, G. Dickneite and S. Schulte are employees of CSL Behring GmbH (Marburg, Germany). The CSL Behring product rVIII-SingleChain (CSL627) was studied in this work.

Acknowledgements

This work was sponsored and funded by CSL Behring GmbH (Marburg, Germany) and the authors were fully responsible for the content of this manuscript. The authors gratefully acknowledge editorial assistance by Adelphi Communications Ltd (Bollington, UK), which was funded by CSL Behring GmbH.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2014.03.028.

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