Prolonged activity of a recombinant factor VIII-Fc fusion protein in hemophilia A mice and dogs

Running title: LONG-ACTING rFVIII-Fc FUSION PROTEIN

Jennifer A. Dumont¹, Tongyao Liu¹, Susan C. Low¹, Xin Zhang¹, George Kamphaus¹, Paul Sakorafas¹, Cara Fraley¹, Douglas Drager¹, Thomas Reidy¹, Justin McCue², Helen W. G. Franck³, Elizabeth P. Merricks³, Timothy C. Nichols³, Alan J. Bitonti¹, Glenn F. Pierce¹ and Haiyan Jiang¹*  

¹Hemophilia R&D, Biogen Idec, Waltham, MA 02451; ²Process Biochemistry, Biogen Idec, Cambridge, MA 02142; ³Francis Owen Blood Research Laboratory, Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC 27516

*Correspondence:  
Haiyan Jiang, Biogen Idec Hemophilia, 9 4th Ave, Waltham, MA 02451  
Email: haiyan.jiang@biogenidec.com, Phone: 781-522-4127, Fax: 888-619-8856

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ABSTRACT

Despite proven benefits, prophylactic treatment for Hemophilia A is hampered by the short half-life of Factor VIII. A recombinant Factor VIII-Fc fusion protein (rFVIIIFc) was constructed to determine the potential for reduced frequency of dosing. rFVIIIFc has an approximately 2-fold longer half-life than rFVIII in Hemophilia A (HemA) mice and dogs. The extension of rFVIIIFc half-life requires interaction of Fc with the neonatal Fc receptor (FcRn). In FcRn knockout mice, the extension of rFVIIIFc half-life is abrogated, and is restored in human FcRn transgenic mice. The Fc fusion has no impact on FVIII specific activity. rFVIIIFc has comparable acute efficacy as rFVIII in treating tail clip injury in HemA mice; and fully corrects whole blood clotting time (WBCT) in HemA dogs immediately post-dosing. Furthermore, consistent with prolonged half-life, rFVIIIFc shows 2-fold longer prophylactic efficacy in protecting HemA mice from tail vein transection bleeding induced 24 – 48 hrs after dosing. In HemA dogs, rFVIIIFc also sustains partial correction of WBCT 1.5-2 fold longer than rFVIII. rFVIIIFc was well tolerated in both species. Thus, the rescue of FVIII by Fc fusion to provide prolonged protection presents a novel pathway for FVIII catabolism, and warrants further investigation.
INTRODUCTION

Hemophilia A is an X-linked bleeding disorder caused by deficiency of factor VIII (FVIII) activity. The disease is characterized by spontaneous hemorrhage and excessive bleeding after trauma. Over time, repeated bleeding into muscles and joints, which begins in early childhood, results in hemophilic arthropathy and irreversible joint damage. This damage is progressive and leads to pronounced musculoskeletal morbidity. Prophylaxis significantly reduces joint damage and long-term sequelae, and improves quality of life in comparison to on-demand treatment. However, the short half-life (10-12 hrs) of FVIII necessitates dosing every other day or three times per week by intravenous injection for full prophylaxis. Therefore, a longer-acting FVIII would represent a key advancement in the management of hemophilia A.

We have developed a recombinant factor VIII-Fc (rFVIIIFc) fusion protein to extend the half-life of FVIII by leveraging a naturally-occurring biologic pathway. rFVIIIFc is a heterodimeric protein comprising a single B-domain-deleted (BDD) FVIII linked recombinantly to the Fc domain of human immunoglobulin G1 (IgG1). The Fc domain enables binding to the neonatal Fc receptor (FcRn), which is responsible for protection of IgG from degradation and facilitates its recycling, resulting in a half-life for IgG of approximately three weeks in humans. The Fc domain of IgG1 has been fused to growth factors, cytokines, enzymes and ligand-binding regions of receptors; a number of these fusion proteins have been approved as therapeutics (e.g. etanercept, abatacept, belatacept, alefacept, rilonacept, romiplostim). However, traditional dimeric Fc fusions,
created through the fusion of the monomeric effector protein to a monomer of Fc and then coupled through a disulfide bond to create a dimer, were not effective for large coagulation proteins such as FVIII. Thus, we have developed methods to create novel Fc fusion protein constructs in which a single (monomeric) effector molecule is attached to Fc\textsuperscript{11-13}. We have applied this approach to a number of proteins, including human rFIX\textsuperscript{14}, rFVIIa\textsuperscript{15}, and BDD rFVIII. This is the first report evaluating the pharmacokinetics and efficacy of rFVIIIFc compared to rFVIII in mouse and dog models of hemophilia A, in support of subsequent human studies.
METHODS AND MATERIALS

Recombinant FVIII–Fc fusion protein (rFVIIIFc). The rFVIIIFc expression plasmid pBUDCE4.1 (Invitrogen) contains two expression cassettes. One expresses, under the control of CMV promoter, native human FVIII signal sequence followed by BDD FVIII (S743 to Q1638 fusion) directly linked to the Fc region of human IgG1 (amino acids D221 to K457, EU numbering) with no intervening sequence. The other uses the EF1α promoter to express the Fc region alone with a heterologous mouse IgκB signal sequence. Human embryonic kidney 293 cells (HEK293H, Invitrogen) were transfected with this plasmid, and a stable clonal suspension cell line was generated that expressed rFVIIIFc. Protein was purified from defined cell culture harvest media using a three column purification process, including a FVIII-specific affinity purification step followed by a combination of anion exchange and hydrophobic interaction chromatographic steps.

Recombinant FVIII (rFVIII) Recombinant BDD FVIII (ReFacto® and Xyntha®), and full length FVIII (Advate®) were purchased from Novis Pharmaceuticals (Miami, FL) and reconstituted according to the manufacturer’s instructions.

Animals The hemophilia A (HemA) mice bearing a FVIII exon 16 knockout on a 129 x B6 background were obtained from Dr. H. Kazazian at the University of Pennsylvannia and bred at Biogen Idec Hemophilia. Murine FcRn knockout (FcRn KO) and human FcRn transgenic (Tg32B) mice were derived from C57BL/6J mice and were obtained from Dr. Derry Roopenian of The Jackson Laboratory in Bar Harbor, ME.
The genotypes for FcRn KO mice are mFcRn (-/-) and mβ2m (-/-), and for Tg32B are mFcRn (-/-), mβ2m (-/-), hFcRn (+/+), and hβ2m (+/+). C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). All animal activities were approved by the Institutional Animal Care Committees and performed in accordance with the “Guide to the Care and Use of Laboratory Animals.”

Hemophilia A dogs were from the in-bred colony maintained at the Francis Owen Blood Research Laboratory at the University of North Carolina, Chapel Hill. These dogs have a severe hemophilic phenotype comparable to the severe form of the human disease.

**Pharmacokinetic (PK) Studies in mice**

The PK of rFVIIIFc and rFVIII (Xyntha) was evaluated in HemA, C57BL/6, FcRn KO, and Tg32B mice after an intravenous dose of 125 IU/kg. Blood was collected from the vena cava in one-tenth volume of 4% sodium citrate at 5 minutes, and 4, 8, 16, 24, 32, 48, 54, and 72 hrs post-dosing for rFVIIIFc and at 5 minutes, and 1, 4, 8, 16, 20, 24, 32, and 48 hrs post-dosing for rFVIII (4 mice/time point/treatment). Plasma was snap frozen in an ethanol/dry ice bath and stored at -80°C until analysis for FVIII activity using a human FVIII-specific chromogenic assay (FVIII Coatest SP kit from DiaPharma [West Chester, OH]). The pharmacokinetic parameters were estimated by non-compartmental modeling using WinNonLin version 5.2 (Pharsight, Mountain View, CA).

**Efficacy studies in HemA mice**

All efficacy studies were performed blinded. Acute efficacy was studied in the tail clip bleeding model. Male HemA mice (8-12 weeks old)
were anesthetized with a cocktail of 50 mg/kg of Ketamine and 0.5 mg/kg of Dexmedetomidine. The tail was then immersed in 37°C saline for 10 minutes to dilate the lateral vein followed by tail vein injection of rFVIIIFc, rFVIII (Advate), or vehicle. Five minutes later, the distal 1 cm of the tail was clipped and the shed blood was collected into 13 mL of warm saline for 30 minutes. The blood loss was quantified gravimetrically.

The prophylactic efficacy was studied in the tail vein transection (TVT) bleeding model as described previously 20 except that HemA mice received a single intravenous administration of 12 IU/kg of rFVIIIFc, rFVIII (Advate), or vehicle at 24 or 48 hrs prior to the transection of a lateral tail vein. The dose of 12 IU/kg was identified from a prior dose response experiment with rFVIII in which 12 IU/kg achieved 50% protection of HemA mice from a TVT injury inflicted 24 hours post dosing (data not shown).

**Hemophilia A Dog Studies**  In a single dose PK/PD study of rFVIIIFc, two naïve hemophilia A dogs (M10 and M11) received an intravenous dose of 125 IU/kg. Blood samples were collected pre-dosing and post-dosing at 5 and 30 min, and 1, 2, 4, 8, 24, 32, 48, 72, 96, 144, and 168 hrs for whole blood clotting time (WBCT). Blood collections for FVIII activity (aPTT and chromogenic assay), rFVIIIFc antigen (ELISA), hematology, and blood chemistry included the time points listed above for WBCT as well as 15 min and 3, 6, and 12 hours post-dosing.

In the following sequential design study, rFVIII (ReFacto) was administered intravenously at 114 IU/kg for dog M12 and 120 IU/kg for dog M38. WBCT was measured until clotting times were ≥ 20 min (the time consistent with FVIII:C ≤ 1%), and samples were also collected at the specified time points for FVIII activity (aPTT and
chromogenic assay), antigen (ELISA), and hematology tests. Then 125 IU/kg rFVIIIFc was administered intravenously to the same dogs and blood samples were collected for WBCT, aPTT, ELISA, hematology, and serum chemistry. Time points for WBCT included pre-dosing, and 5 and 30 min and 1, 2, 4, 8, 24, 32, 48, and 72 hrs post-dosing of rFVIII and rFVIIIFc. Blood was also collected at 96, 120, 144, and 168 hrs post-dosing with FVIIIFc. Blood collections for FVIII activity and antigens included the time points listed above for WBCT as well as 15 min and 3, 6, 12 hours after dosing. The WBCT and aPTT were performed as previously described 21.

**FVIII chromogenic assays**  FVIII activity in hemophilia A dog plasma was tested by an automated chromogenic assay on a Sysmex CA1500 instrument (Sysmex, IL) with reagents from Siemans Healthcare Diagnostics (Dallas, TX). The standard curve was generated with the 7th International Standard Factor VIII Concentrate (NIBSC code 99/678) spiked into human FVIII-depleted plasma (Stago USA) at concentrations ranging from 1.5 – 0.016 IU/mL.

FVIII activity in HemA mouse plasma was measured using the Coatest SP FVIII assay from Chromogenix (DiaPharma, Lexington, MA), following the manufacturer’s instructions. The standard curve was generated using rFVIIIFc or rFVIII serially diluted from 100 mU/mL to 0.78 mU/mL in buffer containing naive HemA mouse plasma. To measure the human FVIII activity in C57BL/6, FcRn KO, and Tg32B mouse plasma, the infused rFVIIIFc or rFVIII in mouse plasma was first captured by human FVIII-specific mAb GMA8016 (Green Mountain Antibodies, VT) followed by the standard Coatest assay.
rFVIII- and rFVIIIFc-specific ELISA  

rFVIII and rFVIIIFc antigen levels in hemophilia A dog plasma were measured by ELISA following the standard protocol. The FVIII A1 domain-specific mAb GMA-8002 (Green Mountain Antibodies, Burlington, VT) was used as the capture antibody. HRP-conjugated polyclonal anti-FVIII Ab F8C-EIA-D (Affinity Biologicals) was used to detect rFVIII. HRP-conjugated donkey anti-human (F(ab)’2) 709-036-098 (Jackson Immunologicals) was used to detect rFVIIIFc.

**SPR analysis of rFVIIIFc-FcRn interactions**  

Surface plasmon resonance (SPR) experiments were performed with a Biacore T100 instrument. Research-grade CM5 sensor chips, buffers, and immobilization reagents were purchased from Biacore (GE Heathcare, Piscataway, NJ). Single-chain human, canine, and murine FcRn preparations were immobilized using standard amine coupling on adjacent flow cells of a single chip at a density of approximately 370 resonance units (RU), followed by blocking with ethanolamine. The steady-state association of Fc-containing analytes (FVIIIFc and IgG) with immobilized FcRn of different species was evaluated by sequential injection of analytes at 16 concentrations (0.0625-2000 nM) in pH 6.0 running buffer (50 mM MES [4-morpholineethanesulfonic acid], 250 mM sodium chloride, 2 mM calcium chloride, 0.01% Tween 20 [polyethylene glycol sorbitan monolaurate]). Each cycle was performed in duplicate and comprised a 45 min association phase and a 15 min dissociation phase, both at a flow rate of 5 µL/min, followed by regeneration with two 60 sec injections of 1M Tris-HCl at 25 µL/min. After double reference-subtraction (blank flow cell and
running buffer alone), binding responses recorded near the end of the association phase were plotted as a function of analyte concentration, and EC$_{50}$ values (50% of R$_{max}$) were derived by non-linear regression analysis.

Statistical Analyses Unpaired t-test, one-way ANOVA, Mann-Whitney test, Kruskal-Wallis test with Dunn post-test, survival curves and associated log-rank test were performed in GraphPad Prism 5 (Graph-Pad Software Inc., La Jolla, CA). A 2-tailed $P$ value less than 0.05 was considered statistically significant.
RESULTS

Recombinant FVIII Fc fusion protein (rFVIIIIFc)

rFVIIIIFc is a recombinant fusion of human B-domain deleted FVIII with Fc from human IgG1, with no intervening linker sequence (Figure 1), that was produced in well characterized HEK 293H cells. The rFVIIIIFc is proteolytically cleaved intracellularly to yield an ~90 kDa heavy chain and ~130 kDa light chain-Fc that are bound together non-covalently through a metal bond interaction mediated by the A1 and A3 domains of FVIII.

The average specific activity of rFVIIIIFc from fourteen separate batches was 8460±699 IU/mg by the one stage clotting (aPTT) assay, and 9348±1353 IU/mg by the chromogenic assay, corresponding to 1861±154 and 2057±298 IU/nmol, respectively. The specific activity of rFVIIIIFc is comparable to that of wild type human FVIII in plasma (1429 IU/nmol) 22. Thus the FVIII activity of rFVIIIIFc is not affected by fusion of the C-terminus of human FVIII to the N-terminus of human Fc, and the results obtained with the aPTT and chromogenic assays are within approximately 10% of one another.

Binding of rFVIIIIFc to FcRn

The affinity of rFVIIIIFc for single-chain mouse, canine, and human FcRn was evaluated using surface plasmon resonance (Figure S1). The rates of association and dissociation for the complex between rFVIIIIFc and mouse FcRn were much slower than those for canine and human FcRn. Half-maximal binding (EC₅₀) of rFVIIIIFc to human FcRn was approximately 4-fold weaker than that to canine FcRn, and more than 20-fold weaker.
than that to mouse FcRn (Table 1). Similarly, human IgG1 also showed the highest
affinity to murine FcRn, while binding affinity to canine FcRn was less compared to
murine FcRn, but greater compared to human FcRn (Table 1).

**FcRn-dependent improvement in pharmacokinetics of rFVIIIFc in mice**

Interaction of Fc with FcRn is considered the underlying mechanism for extending half-
life for IgG and Fc-fusion proteins. To confirm that this mechanism of action is also
responsible for extending half-life of rFVIIIFc, we compared pharmacokinetic (PK)
profiles of rFVIIIFc with rFVIII in FVIII-deficient (HemA) mice, normal (C57BL/6)
mice, FcRn-deficient (FcRn KO) mice, and human FcRn transgenic (Tg32B) mice
following a single intravenous administration of 125 IU/kg (Figure 2).

The PK parameters (Table 2) were determined by the chromogenic measurement
of the human FVIII activity in mouse plasma. The $t_{1/2}$ of rFVIIIFc was 1.8 – 2.2 fold
longer than rFVIII in HemA mice (13.7 vs 7.6 hrs) and normal mice (9.6 vs 4.3 hrs). The
$t_{1/2}$ extension of rFVIIIFc relative to rFVIII was abolished in FcRn KO mice (6.4 vs 6.9
hrs) and restored in human FcRn transgenic Tg32B mice (9.6 vs 4.1 hrs). The results
thus confirm that the interaction of rFVIIIFc with the FcRn receptor is responsible for its
extended $t_{1/2}$. Furthermore, consistent with the improved $t_{1/2}$, rFVIIIFc also showed a 1.6
– 2.4 fold longer MRT and a 1.2 – 1.8 fold increased systemic exposure (AUC) compared
to rFVIII in FcRn-expressing (HemA, C57Bl/6 and Tg32B) mice but not in FcRn KO
mice.

**rFVIIIFc is fully active in treating acute bleeds in HemA mice**
To evaluate the acute efficacy of rFVIIIFc in comparison to rFVIII, HemA mice (16 – 20 mice/group) were treated with escalating doses (24, 72, and 216 IU/kg) of rFVIIIFc or rFVIII and injured by tail clip 5 min post-dosing. In comparison to vehicle-treated mice (n=18) that had a median blood loss of 1 mL, both rFVIIIFc and rFVIII treatments resulted in significantly improved protection (P<0.05, Kruskal-Wallis test with Dunn post-test) (Figure 3). The median blood loss progressively decreased with increasing doses, reaching maximum reduction to 0.23 mL at 72 IU/kg of rFVIIIFc, and 0.20 mL at 216 IU/kg of rFVIII. Overall, the blood loss was comparable in animals treated with equal doses of rFVIIIFc or rFVIII, indicating that both therapeutics are comparably active in resolving acute arterial bleeds.

**Prolonged prophylactic efficacy of rFVIIIFc in HemA mice**

To determine if prolonged PK leads to prolonged protection from injury, we compared the prophylactic efficacy of rFVIIIFc and rFVIII in HemA mice. Twenty-four hours after an intravenous dose of 12 IU/kg, one lateral tail vein in HemA mice was transected (Figure 4). Following injury, 49% of rFVIII-treated mice (n=39) survived, compared with 100% survival of rFVIIIFc-treated mice (n=19) (P<0.001, Log-Rank test). To further demonstrate that rFVIIIFc sustains a longer duration of efficacy, HemA mice were injured 48 hrs post-dosing with 12 IU/kg of rFVIIIFc. Nevertheless, 58% of rFVIIIFc-treated mice (n=40) survived, which is similar to that achieved in rFVIII-treated mice (49%) injured at 24 hrs post dosing. Both rFVIIIFc and rFVIII treatments are significantly better than the HemA vehicle-control group (n=30) in which only 3% of mice survived the injury (P<0.0001). The improved and prolonged prophylactic efficacy
of rFVIIIFc is also evident by the measurement of rebleeding post injury (Figure 4B). Whereas 100% of vehicle-treated HemA mice rebled within 10 hrs following tail vein transection, 87% of rFVIII-treated and 47% of rFVIIIFc-treated mice rebled after the injury inflicted at 24 hrs post dosing, respectively (P=0.002, rFVIIIFc vs rFVIII). The rebleed profile for rFVIIIFc-treated mice injured at 48 hrs is largely comparable to that for rFVIII-treated mice injured at 24 hrs post dosing. In contrast, both the survival and rebleed profile for rFVIII-treated mice injured at 48 hrs are indistinguishable from the profile for the vehicle-control group (data not shown). Therefore, the results indicate that rFVIIIFc protects HemA mice from tail vein injury twice as long as that achieved by the same dose of rFVIII.

**Improved PK/PD of rFVIIIFc in Hemophilia A dogs**

The PK and pharmacodynamics (PD) of rFVIIIFc were also studied in hemophilia A dogs. Following an intravenous dose of 125 IU/kg of rFVIIIFc, the WBCT was immediately corrected to normal, which is in the range of 8 – 12 min in normal dogs (Figures 5A and B). The WBCT remained below 20 min, indicating FVIII activity >1%, through approximately 4 days in 3 out of 4 rFVIIIFc-treated dogs and 3 days in the remaining dog (Figure 5A). In dog M12 treated with 114 IU/kg of rFVIII and dog M38 with 120 IU/kg of rFVIII, the WBCT was also corrected to normal immediately after dosing. However, the WBCT remained below 20 min for 2 days in M12 and 3 days in M38, approximately 1.5 - 2-fold shorter than that achieved by rFVIIIFc (Figure 5B). Furthermore, both rFVIIIFc and rFVIII treatment also improved aPTT clotting time similarly at 5 min post dosing (Table S1).
The PK of rFVIIIFc antigen (Figure 6A) was determined by measuring the concentration of rFVIIIFc in plasma with a rFVIIIFc-specific ELISA that detects both the FVIII and Fc portions of the molecule. The t1/2 of rFVIIIFc antigen is 15.7 ± 1.7 hr (Figure 6A), similar to the t1/2 of rFVIIIFc activity (Figure 6B), as measured by the chromogenic assay: 15.4 ± 0.3 hr (Table 3). There is a good correlation between the FVIII activity and the rFVIIIFc antigen data, thereby demonstrating that rFVIIIFc protein was fully active in vivo.

In two of the dogs (M12 and M38) that also received a single dose of rFVIII 72 hrs prior to dosing with rFVIIIFc, the t1/2 of FVIII antigen was determined to be 6.9 hrs and rFVIII activity 7.4 hrs. Therefore, the plasma half-life of rFVIIIFc was approximately twice as long compared to that for rFVIII by both antigen and activity measurements.

In addition, platelet count and fibrinogen were assessed to serve as preliminary tests for thrombogenicity. After dosing with either rFVIIIFc or rFVIII, platelet numbers and plasma fibrinogen concentration did not change from pre-dose values (data not shown).
DISCUSSION

In this report, rFVIIIIfc has been shown to be fully active in treating acute bleeds in HemA mice, in addition to retaining normal specific activity. Other studies, not reported here, have shown that rFVIIIIfc is also fully functional in interacting with FIXa, FX, and phospholipids in forming the Xase complex (Peters et al, manuscript in preparation). Furthermore, the binding affinity to von Willebrand Factor (VWF) is comparable between rFVIIIIfc and rFVIII, with a Kd of approximately 1.4 and 0.8 nM for rFVIIIIfc and rFVIII, respectively (Peters et al, manuscript in preparation). It is somewhat surprising that the activity of rFVIIIIfc is not affected by fusion of the C-terminus of FVIII with the N-terminus of Fc since the C1 and C2 domains of FVIII are involved in phospholipid binding which is essential for the formation of prothrombinase complex on activated platelet surfaces. However, the finding is consistent with the observation that residues thought to bind phospholipids, ie, K2092/F2093 in C1, M2199/F2200 and L2251/L2252 in C2, all appear to form a surface that is distant from the C-terminal residues of FVIII.

In our studies, the half-life of rFVIIIIfc was doubled only in mice expressing either endogenous murine or transgenic human FcRn, but not in FcRn KO mice (Figure 2 and Table 2), demonstrating that the mechanism of prolonging half-life of rFVIIIIfc is clearly mediated by FcRn. While it is known that both endothelial and hematopoietic cells contribute equally in recycling internalized IgG to the cell surface to facilitate the longevity of IgG and protection from degradation, it is not known specifically which
FcRn-expressing cell type(s) are responsible for the uptake and recycling of rFVIIIIFc. FcRn is broadly expressed in the vascular endothelium, epithelium of kidney, liver, spleen, as well as in bone marrow-derived APCs including macrophages. Since FVIII circulates largely (~98%) in complex with VWF, and both proteins were colocalized to the macrophages in liver and spleen when recombinant FVIII and VWF were co-injected into VWF-deficient mice, it is possible that macrophages may play a role in rescue of rFVIIIIFc from degradation and prolongation of half-life. However, the results may also suggest a previously unrecognized pathway for FVIII catabolism, and rescue of the protein permitted by Fc fusion.

Approaches in development to extend the half-life of clotting factors include pegylation, glycopegylation, and conjugation with albumin. Interestingly, regardless of the protein engineering utilized, the half-life of modified rFVIII variants appears to be maximally twice as long as wild-type FVIII in a variety of preclinical animal models. Consistent results have been demonstrated in humans, eg, rFVIIIIFc was reported to improve half-life approximately 1.7-fold compared to Advate in hemophilia A patients. This limitation of extending FVIII half-life appears to be related to VWF. In FVIII and VWF knockout mice, preliminary experiments observed a 5-fold increase in the half-life of rFVIIIIFc compared to rFVIII (Liu, T et al, unpublished results). Similar findings were reported previously in VWF knockout mice utilizing Pegylated-FVIII. Taken together, these results suggest that VWF may be a limiting factor for further extending FVIII half-life.
Beyond extending half-life, rFVIIIFc may provide additional benefits. One major challenge with FVIII replacement therapy is the development of neutralizing anti-FVIII antibodies (inhibitors). This occurs in 15-30% of previously untreated patients. rFVIIIFc has the potential to induce immune tolerance and thus prevent the development of neutralizing antibodies. It has been reported that retroviral vector-transduced B-cells, presenting FVIII domains as Ig fusion proteins, specifically prevent or decrease existing FVIII antibodies in HemA mice \(^{40}\). It was also found that Fc contains regulatory T-cell epitopes capable of inducing Treg expansion and suppression of antigen-specific immune responses in vitro \(^{41}\). In addition, the FcRn-mediated transfer of maternal IgG and Fc-fusion proteins across placenta to fetal circulation \(^{42,43}\) could induce neonatal tolerance to rFVIIIFc while also providing needed protection in the newborn from bleeding during delivery.

In conclusion, we have demonstrated that rFVIIIFc, provides approximately 2-fold longer efficacy duration relative to rFVIII in protecting HemA mice from tail vein transection injury and improving WBCT in HemA dogs. The prolonged efficacy correlates well with a 2-fold extended \(t_{1/2}\) of rFVIIIFc, a result of recycling of the Fc fusion protein via a specific and well established intracellular pathway. Future studies in humans will evaluate the potential benefit of rFVIIIFc in patients with hemophilia A for decreasing dosing frequency for prophylaxis, treatment of episodic bleeds, and possible protection from the generation of neutralizing antibodies.
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AUTHORSHIP

J.A.D., S.C.L., G.F.P., and H.J. designed research, analyzed data, and wrote the manuscript; T.L. designed research, conducted research, and analyzed data; X.Z., G.K., P.S., C.F., and T.R. conducted research and analyzed data; J.Mc. provided vital reagents; D.D., H.G.F., E.P.M., and T.N. conducted research; A.J.B. designed research and analyzed data.

REFERENCES


### Table 1. Surface Plasmon resonance analysis of murine, canine, and human FcRn with rFVIIIFc and human IgG1

<table>
<thead>
<tr>
<th>Fc Sample</th>
<th>FcRn</th>
<th>FcRn Density (RU)</th>
<th>EC$_{50}$ (nM)</th>
<th>Rmax (RU)</th>
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<tr>
<td>rFVIIIFc</td>
<td>murine</td>
<td>370</td>
<td>&lt; 1.5  ‡</td>
<td>581.4</td>
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<tr>
<td>rFVIIIFc</td>
<td>canine</td>
<td>367</td>
<td>8.6</td>
<td>499.3</td>
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<tr>
<td>rFVIIIFc</td>
<td>human</td>
<td>369</td>
<td>33.4</td>
<td>365.4</td>
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<tr>
<td>Human IgG1</td>
<td>murine</td>
<td>378</td>
<td>&lt; 22.4 ‡</td>
<td>320.0</td>
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<tr>
<td>Human IgG1</td>
<td>canine</td>
<td>367</td>
<td>196.3</td>
<td>282.2</td>
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<tr>
<td>Human IgG1</td>
<td>human</td>
<td>378</td>
<td>558.4</td>
<td>211.0</td>
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</tbody>
</table>

* rFVIIIFc or IgG1 were injected over a flow cell to which various FcRn molecules were chemically conjugated at approximately equal densities (~370 RU).

† EC$_{50}$ values (50% of R$_{max}$) were the average derived from non-linear regression analysis of the binding response curves fitted to 16 analyte concentrations (0.0625-2000 nM) repeated in duplicate (Figure S1).

‡ Due to the high affinities, the low binding curves at low analyte concentrations did not reach equilibrium under the normal operating conditions of the instrument.
Table 2. Summary of PK parameters for rFVIIIFc and rFVIII in different mouse strains

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>HemA †</th>
<th>C57BL/6 †</th>
<th>FcRn KO †</th>
<th>hFcRn Transgenic (Tg32B) †</th>
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<tbody>
<tr>
<td></td>
<td>rFVIIIFc*</td>
<td>rFVIII*</td>
<td>rFVIIIFc*</td>
<td>rFVIII*</td>
</tr>
<tr>
<td>Cmax (mIU/mL)</td>
<td>2613.6</td>
<td>2710.4</td>
<td>2356.2</td>
<td>2000.1</td>
</tr>
<tr>
<td>Half-life (hr)</td>
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<td>7.6</td>
<td>9.6</td>
<td>4.3</td>
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<tr>
<td>MRT (hr)</td>
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<td>11.0</td>
<td>9.8</td>
<td>5.4</td>
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<tr>
<td>Vss (mL/kg)</td>
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<td>49.2</td>
<td>67.5</td>
<td>50.8</td>
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<tr>
<td>CL (mL/hr/kg)</td>
<td>3.9</td>
<td>4.5</td>
<td>6.9</td>
<td>9.3</td>
</tr>
<tr>
<td>AUC (hr*mIU/mL)</td>
<td>32332.4</td>
<td>28026.8</td>
<td>18089.1</td>
<td>13404.0</td>
</tr>
</tbody>
</table>

Cmax: maximum plasma FVIII activity post infusion; MRT: mean residence time; Vss: volume of distribution at steady-state; CL: clearance; AUC: area under the curve.

† PK parameters of rFVIII and rFVIIIFc were compared only within the same mouse strain not across strains, because the same molecule can display different t½ in different mouse strains.

*The PK evaluation of each molecule used a cohort of 36 mice, sampled by terminal vena cava bleeding from 4 mice at each of the 9 time points. The group means at each time point were used for non-compartment modeling in WinNonLin to derive PK parameter estimates.
**Table 3. Summary of PK parameters for rFVIIIFc and rFVIII in hemophilia A dogs**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PK by FVIII activity measurement</th>
<th>PK by rFVIII and rFVIIIFc antigen measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (IU/mL)</td>
<td>AUC (hr·IU/mL)</td>
</tr>
<tr>
<td>rFVIIIFc&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.0 ± 0.54</td>
<td>25.9 ± 6.47</td>
</tr>
<tr>
<td>rFVIII&lt;sup&gt;†&lt;/sup&gt;</td>
<td>2.0</td>
<td>18.2</td>
</tr>
</tbody>
</table>

<sup>*</sup>Results presented are Mean ± SD from 4 dogs.

<sup>†</sup>Results presented are Mean. SD not reported since two dogs were utilized.
FIGURES

Figure 1. Schematic representation of rFVIII-Fc monomer
Figure 2. Half-life extension of rFVIIIFc is dependent on FcRn. PK profiles of rFVIIIFc and rFVIII in (A) HemA mice, (B) C57BL/6 mice, (C) FcRn KO mice, and (D) human FcRn transgenic Tg32B mice following a tail vein injection of 125 IU/kg. Results shown are Mean ± SD from 4 mice per treatment at each time point. The PK parameter estimates are summarized in Table 2.
Figure 3. Comparable acute activity of rFVIII Fc and rFVIII in tail clip bleeding model. Male HemA mice received a tail vein injection of rFVIII Fc or rFVIII as specified followed by a 10 mm tail clip 5 min post dosing. Results presented are individual and median blood loss over 30 min following the tail clip from 20 mice in each treatment group. P<0.05 for Vehicle vs all other treatments, and P>0.05 for C57Bl/6 vs HemA mice treated with 72 or 216 IU/kg of rFVIII Fc, or 216 IU/kg of rFVIII.
Figure 4. Improved prophylactic efficacy of rFVIIIFc relative to rFVIII in the tail vein transection (TVT) bleeding model. Male HemA mice were injured by TVT either 24 hrs following vehicle, or rFVIII, or rFVIIIFc treatment, or 48 hrs following rFVIIIFc treatment. The survival and rebleed within 24 hrs following TVT are shown in (A) and (B), respectively. *P<0.001 by Log-Rank test of the survival curves from animals that received 12 IU/kg rFVIIIFc vs rFVIII 24 hrs prior to TVT (A). #P=0.002 by Log-Rank test of the non-rebleed curves from animals that received 12 IU/kg rFVIIIFc vs rFVIII 24 hrs prior to TVT (B).
Figure 5. WBCT of rFVIIIFc and rFVIII in hemophilia A dogs. Normal WBCT range in dogs is shown by the large dashed lines. The area above the small dashed lines (20 minutes) indicates the point at which the plasma FVIII activity is expected to be below 1% of normal.

A. rFVIIIFc

B. rFVIII followed by rFVIIIFc in a Crossover Study
Figure 6. Improved pharmacokinetics of rFVIIIfc compared to rFVIII in Hemophilia A dogs after an i.v. dose. Plasma antigen concentration was measured by ELISA (A) and plasma FVIII activity was measured by chromogenic assay (B). N = 4 for rFVIIIfc and N = 2 for rFVIII.
Prolonged activity of a recombinant factor VIII-Fc fusion protein in hemophilia A mice and dogs