Research Article

Effects of Replacement of Factor VIII Amino Acids Asp519 and Glu665 with Val on Plasma Survival and Efficacy *In Vivo*

Matthew P. Kosloski,¹ Krithika A. Shetty,¹ Hironao Wakabayashi,² Philip J. Fay,² and Sathy V. Balu-Iyer^{1,3}

Received 7 November 2013; accepted 20 May 2014; published online 17 June 2014

Abstract. Proteolytic cleavage of factor VIII (FVIII) to activated FVIIIa is required for participation in the coagulation cascade. The A2 domain is no longer covalently bound in the resulting activated heterotrimer and is highly unstable. Aspartic acid (D) 519 and glutamic acid (E) 665 at the A1–A2 and A2–A3 domain interfaces were identified as acidic residues in local hydrophobic pockets. Replacement with hydrophobic valine (V; D519V/E665V) improved the stability and activity of the mutant FVIII over the wild-type (WT) protein in several *in vitro* assays. In the current study, we examined the impact of mutations on secondary and tertiary structure as well as *in vivo* stability, pharmacokinetics (PK), efficacy, and immunogenicity in a murine model of Hemophilia A (HA). Biophysical characterization was performed with far-UV circular dichroism (CD) and fluorescence emission studies. PK and efficacy of FVIII was studied following i.v. bolus doses of 4, 10 and 40 IU/kg with chromogenic and tail clip assays. Immunogenicity was measured with the Bethesda assay and ELISA after a series of i.v. injections. Native secondary and tertiary structure was unaltered between variants. PK profiles were similar at higher doses, but at 4 IU/kg plasma survival of D519V/E665V was improved. Hemostasis at low concentrations was improved for the mutant. Immune response was similar between variants. Overall, these results demonstrate that stabilizing mutations in the A2 domain of FVIII can improve HA therapy *in vivo*.

KEY WORDS: efficacy; FVIII; Hemophilia A; immunogenicity; mutant.

INTRODUCTION

Hemophilia A (HA) is a genetic bleeding disorder caused by insufficiency in factor VIII (FVIII), an essential blood coagulation protein. The *in vivo* behavior of FVIII is complex and regulated by many processes and intermolecular interactions (1,2). Synthesized as a single-chain precursor, FVIII undergoes proteolytic processing to a heterodimer of a 90–210-kDa heavy chain (domains A1–A2-B) and a 80-kDa light chain (domains A3-C1–C2) joined by a metal cation (3). Conversion to activated FVIIIa is triggered by thrombin or factor Xa mediated cleavage forming a heterotrimer of the A1, A2, and A3-C1–C2 fragments (4). No longer covalently linked to the rest of the protein, the A2 domain is retained by a combination of weak electrostatic and hydrophobic interactions with neighboring regions in the A1 and A3 domains (5). FVIIIa associates with phosphatidylserine (PS) exposed on the surface of activated platelets adjacent to factor IXa to form the Xase complex, responsible for activation of factor X to factor Xa (6,7). The Xase complex is short lived, resulting from instabilities in

FVIIIa (8). Loss of Xase activity may result from catabolism by activated protein C (APC), factor IXa, or factor Xa, but disassociation of the loosely retained FVIII A2 domain is the primary mechanism of inactivation (2).

Analysis of the crystal structure of FVIII was used to study several polar/charged amino acids in the A1–A2 and A2–A3 interface regions (9). Spatial separation of <2.8 Å suggested involvement in hydrogen-bonding and replacement with neutral Ala (A) impaired FVIII function in >50% of the targets (10). Several residues separated by >2.8 Å were identified as potentially failing to participate in bonding interactions and targets for charge neutralization to promote hydrophobic retention of the A2 domain. Individual replacement of E665, E1984, and D519 with alanine or valine reduced the decay of FVIII and FVIIIa (8). A combinatorial study of stability-promoting mutations found that B-domain deleted human FVIII in which valine replaced D519 and E665 (D519V/E665V) demonstrated the largest improvements compared to the WT protein; decay rate of the activated FVIIIa trimer was reduced seven-fold while peak thrombin concentrations were more than doubled (11). D519 and E665 are shown with respect to the global structure of FVIII in Fig. 1 (12).

Prophylactic treatment with FVIII in HA reduces the frequency and severity of bleeding in patients, but maintenance of concentrations above the 0.01 IU/mL therapeutic threshold requires frequent infusions due to a half-life of only 10–14 h (13,14). A FVIII product that improved plasma

¹ Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York, 359 Kapoor Hall, Buffalo, New York 14214, USA.

² Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York, USA.

³ To whom correspondence should be addressed. (e-mail: svb@buffalo.edu)

Fig. 1. FVIII structure. Native structure of B-domainless FVIII generated with Polyview-3D (PDB code: 2R7E), with heavy (green) and light (blue) chains. Residues Asp519 and Glu665 (red) were replaced with Val in the stabilized mutant D519V/E665V

survival or retained efficacy at lower concentrations could substantially improve patient therapy by reducing the frequency of infusions. Multiple groups sought to engineer this next generation therapeutic by stabilizing the A2 domain, but have had limited success due to the many intermolecular actions of FVIII required *in vivo*. Removal of the FVIII A1–A2 cleavage site improved *in vitro* stability, but reduced binding to the von Willebrand Factor (vWF) carrier protein essential for survival *in vivo* (15). Another mutant with additional disulfide bridge-forming cystine residues used to covalently retain the A2 domain of FVIIIa failed to reduce overall clot times, but improved some clotting parameters at low concentrations (16).

A series of studies were designed to evaluate whether the previously characterized D519V/E665V mutant FVIII could improve therapy *in vivo*. Far-UV CD and native fluorescence were used to examine changes in the secondary and tertiary structure of D519V/E665V and WT FVIII. Comparative pharmacokinetic, hemostatic efficacy and immunogenicity studies were conducted in a mouse model of HA. Results suggest that the mutations in D519V/E665V increase hemostatic efficacy at lower concentrations without negatively impacting the *in vivo* disposition of the protein.

MATERIALS AND METHODS

Materials

WT and D519V/E665V variants of human B-domain deleted FVIII were expressed and purified in the lab of Dr. Philip Fay as previous described, or by Bayer Healthcare (San Francisco, CA) (8,11,17). Specific activities ranged from 4.8–6.5 and 6.7–8.1 IU/ μ g respectively as measured by a two-stage chromogenic assay. Complete biochemical and activity

characterization of the two protein formulations has been previously performed (8,11). Control plasmas and activated partial thromboplastin (aPTT) reagents were purchased from Precision Biologics (Dartmouth, Canada) and Tcoag (Parsippany, NJ) respectively. FVIII chromogenic assay detection kits were purchased from Chromogenix (Chapel Hill, NC). Buffer salts were purchased from Fisher Scientific (Fair Lawn, NJ).

Biophysical Characterization

The native structure of FVIII was examined as previously described (18). Briefly, secondary structure was analyzed by far-UV circular dichroism (CD) scans over 205–260 nm for solutions of WT and D519V/E665V in tris buffer (25 mM tris, 5 mM CaCl_2 , 150 mM NaCl, pH 7.0). Tertiary structure was analyzed by intrinsic fluorescence over 300–400 nm wavelengths following excitation at 265 nm. These studies were performed in triplicate and a representative profile is reported.

Animals

An inbred colony of C57BL/6J mice with a targeted deletion at exon 16 of the FVIII gene is maintained on site in accordance with the Institutional Animal Care and Use Committee of the University at Buffalo. Normal C57BL/6J mice were purchased from Charles River (Wilmington, MA). Study mice were ~12 weeks old and ~21.5 g.

Pharmacokinetic Studies

Male HA mice received a single intravenous bolus injection *via* the penile vein of 4, 10 or 40 IU/kg WT or D519V/E665V FVIII ($n \geq 3$ mice/time point) in HEPES buffer (20 mM HEPES, 5 mM CaCl_2 , 300 mM NaCl, 0.01% Tween80, pH 7.0). Blood was collected *via* cardiac puncture into a 7:1 ratio of acid citrate dextrose (ACD: 85 mM sodium citrate, 110 mM D-glucose, 71 mM citric acid) up to 8 h (10 and 4 IU/kg) or 28 h (40 IU/kg) post injection. Plasma was separated by centrifugation at $5,000 \times g$ for 5 min and stored at -80°C until analysis. FVIII activity was measured with a two-stage chromogenic assay and concentrations were determined by comparison to a standard curve of known WT or D519V/E665V FVIII activity. The lower limit of quantification of both variants in this assay was 0.006 IU/mL.

Pharmacokinetic modeling was performed with Phoenix WinNonlin v6.3 (Pharsight Corporation, Sunnyvale, CA). Non-compartmental analysis was used to generate estimates for basic PK parameters such as area under the curve (AUC_t), peak plasma concentration (C_{max}), half-life ($t_{1/2}$), clearance (CL), and volume of distribution (V_{ss}). Standard error estimates of AUC_t were obtained with the sparse sampling option within WinNonlin (19,20). In addition, data were fit to a previously described (21) one-compartment model with Michaelis-Menten elimination.

Pharmacodynamic Studies

Hemostatic efficacy was assessed in normal C57BL/6J and HA mice with a tail clip assay. Mice were placed under

isoflurane anesthesia and laid on a heating pad. Naïve male HA mice received 4, 10, 20, or 40 IU/kg WT or D519V/E665V FVIII *via* a penile vein injection ($n=3/\text{dose}/\text{group}$) or were untreated ($n=5$). Normal mice were untreated ($n=4$). Tails of mice were cleaned and sanitized before transection 5 min after injection with a sterile blade 6 mm from the posterior tip. Following transection, the tail was immediately inserted into 12 mL of normal saline maintained at 37°C and bleeding observed for 45 min. Blood loss was determined gravimetrically.

Immunogenicity Studies

Immunogenicity testing of WT and D519V/E665V was conducted as previously described (18). Briefly, male mice ($n=8/\text{group}$) received four weekly intravenous injections of either FVIII variant at a dose of 1 $\mu\text{g}/\text{injection}$. Animals were sacrificed at week 6, plasma was isolated and inhibitory as well as total antibody responses were quantified with the Bethesda assay and a previously developed ELISA (22) respectively.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA). Blood loss as a function of dose and FVIII variant was compared by two-way ANOVA. Comparison of inhibitory and total titers was made using unpaired two-sample *t* tests.

RESULTS

Biophysical Characterization

Native structures of D519V/E665V and WT were investigated with biophysical analysis. Although the mutations in D519V/E665V were previously associated with increased thermal stability of the dimeric and trimeric forms measured by activity, slight changes in the organization of the protein may have implications on long-term stability (11). Far-UV CD spectra overlap for both variants and were described by a large negative ellipticity band around 215 nm characteristic of beta-sheets, the predominant secondary structure in FVIII (Fig. 2a) (23). Fluorescent emission profiles for D519V/E665V and WT following excitation at 265 nm were also superimposable indicating similar global positioning of aromatic amino acids (Fig. 2b). Together, these profiles suggest that mutation did not significantly alter the native secondary or tertiary structure of FVIII. It is appropriate to mention here that due to the large size and complex structure of the FVIII molecule, it is possible that these methods are insensitive to detect small conformational changes that these mutations could have on overall structure.

Pharmacokinetic Studies

FVIII in plasma exists in a complex equilibrium balancing vWF binding, enzymatic function, and clearance mechanisms (2). In order to determine whether the mutations could have an impact on *in vivo* disposition/catabolism, comparative

PK studies were conducted. In PK studies of WT (previously published (21)) and D519V/E665V (Fig. 3), doses of 10 and 40 IU/kg exhibited close agreement in observed profiles and resulting PK parameters (Table I). However, following 4 IU/kg injections, D519V/E665V was reproducibly detected up to 2 h, but WT could not be recovered in any samples even as early as 5 min. We have previously reported that the PK of full length FVIII as well as the B-domain deleted WT protein is non-linear in the HA mice used in these studies (21,24). This is also apparent from the fact that non-compartmental analyses show an increase in clearance at lower doses (it is appropriate to acknowledge here that the PK could have been inadequately estimated at lower doses due to limited recovery of plasma FVIII levels at these doses). We hypothesize that this non-linearity is attributable to saturable clearance of FVIII by the primary clearance receptor Low density lipoprotein receptor-related protein (LRP). A model with one distributional compartment and Michaelis-Menten clearance describes the disposition of both variants reasonably well at the higher doses of 40 and 10 IU/kg. At the 4 IU/kg dose however, the model slightly overestimates circulating D519V/E665V concentrations possibly due to complexities in macromolecular interactions—such as those with vWF—which are not accounted for within the Michaelis-Menten paradigm. The estimated parameter values of volume of distribution (51.8 vs. 66.2 mL/kg) and Michaelis-Menten parameters, IU/h.kg (2.74 vs. 2.52 mL/h.kg) and IU/mL (0.216 vs. 0.126 IU/mL kg) of the WT and D519V/E665V were comparable. Overall, the PK profile of D519V/E665V does not show any negative consequences of the mutation and may offer some distinct advantages over WT at low concentrations.

Pharmacodynamic Studies

Hemostatic efficacy of WT and D519V/E665V *in vivo* was measured with a tail clip blood loss assay (Fig. 4). This model has been shown to mimic the spontaneous bleeding characteristic of severely hemophilic patients, is sensitive to the plasma levels of FVIII and its utility in comparative hemostatic efficacy studies has been extensively reported in the literature (25,26).

At 40 IU/kg both FVIII variants restore the normal clotting phenotype, but below doses of 20 IU/kg, response diverged. WT FVIII displayed a sharp loss in clotting activity and at concentrations ≤ 10 IU/kg, bleeding was uninhibited by treatment. In contrast, D519V/E665V maintains partial hemostatic efficacy at doses as low as 4 IU/kg. Treatment with WT or D519V/E665V had a significant effect on blood loss at the lower doses ($p<0.005$, two-way ANOVA at the 10 IU/kg dose). FVIII concentrations at the time of tail vein transection, 5 min after injection, are dose dependent (Fig. 3), thus these results suggest that similar clotting function can be maintained with a lower concentration of D519V/E665V than WT FVIII.

Immunogenicity Studies

To evaluate the immunogenicity of the FVIII variants, a series of *i.v.* injections were administered to HA mice with WT or D519V/E665V. HA mice are routinely used to study relative immunogenicity owing to a FVIII immune response qualitatively similar to humans (27–29). Inhibitory and total

Fig. 2. **a** Circular dichroism of WT and D519V/E665V. Secondary structural analysis by far-UV CD of WT (dashed black line) and D519V/E665V (solid gray line). No changes were observed in the profiles characterized by the large negative 215 nm band corresponding to beta-sheet structures. **b** Intrinsic fluorescence of WT and D519V/E665V. Tertiary structural analysis following fluorescent excitation at 265 nm of WT (dashed black line) and D519V/E665V (solid gray line). Overlapping peak position and shape indicates similar microenvironments of aromatic amino acids

anti-FVIII titers were measured in plasma collected from mice with the clinical Bethesda assay and an ELISA respectively (Fig. 5a, b). No significant differences were observed between mean inhibitory titers (273 ± 209 vs. 260 ± 183 BU) or mean total titers (823 ± 680 vs. 722 ± 361 Arbitrary titer units) of the D519V/E665V and WT treatment groups ($p > 0.5$). The lack of statistical significance and the qualitatively similar pattern of distribution indicate that the mutations in D519V/E665V did not significantly alter immunogenicity of the protein.

DISCUSSION

Factor VIII is a large, complex protein and its *in vivo* disposition is governed by numerous macromolecular interactions including those with antigen presenting cells, carrier proteins, clearance receptors, platelet surfaces, and other coagulation factors (for review, see (2,30–32)). Unlike previous mutant forms of FVIII that employed covalent linkage of the A2 domain thus inherently altering the protein structure and intermolecular

Fig. 3. Pharmacokinetics of D519V/E665V FVIII. PK profiles of D519V/E665V FVIII in HA mice after i.v. doses of 4, 10, and 40 IU/kg. Profiles for the two variants at 10 and 40 IU/kg were similar, but D519V/E665V substantially extended plasma survival at 4 IU/kg (no samples containing measurable WT were recovered following the 4 IU/kg dose)

interactions, D519V/E665V does not cause any significant changes in the native secondary or tertiary structure of FVIII (15,33).

Pharmacokinetic profiles for both WT and D519V/E665V after administration of 40 and 10 IU/kg doses were comparable. Plasma survival was similar between the two variants as reflected by the PK parameter estimates (Table I). The stabilizing effect of the mutant was apparent only after the low dose of 4 IU/kg. We hypothesize that the observed improvement in plasma survival at this dose might be due to enhanced stability of the mutant in the absence of vWF binding. Prior to activation, elimination of FVIII is believed to be primarily controlled by the vWF carrier and the LRP clearance receptor (30,34,35). Neither D519 nor E665 are within recognized sites of interaction with vWF (1,680, 2,303–2,332) or LRP (484, 1,804–1,834, 2,065, 2,092, 2,173–2,332) (36). When these are the predominant macromolecular interactions controlling FVIII disposition, the mutations are expected to have little impact on the overall kinetics of the FVIII dimer. At the low dose of 4 IU/kg dose, theoretical initial concentrations ($\text{Dose}/V_{ss} \approx 0.1 \text{ nM}$) are less than the K_d reported for interaction between human FVIII and human vWF of 0.32 nM (37). At these concentrations, a larger portion of FVIII may be unprotected by vWF and rapid catabolism may explain the inability to detect WT in any recovered plasma samples (38). Prolonged survival of D519V/E665V at this dose is likely a function of the stabilized FVIIIa trimer unassociated with vWF. In order to test this hypothesis, we conducted plasma

survival assessments of the WT and D519V/E665V variants in vWF knock-out mice. vWF^{-/-} mice were administered a 200 IU/kg dose of WT or D519V/E665V. Plasma samples were collected 2 h post dose and analyzed for FVIII activity using a two-stage chromogenic assay. The mean FVIII activity levels of WT ($0.06 \pm 0.001 \text{ IU/mL}$) and D519V/E665V ($0.07 \pm 0.01 \text{ IU/mL}$) in these samples were comparable. The baseline FVIII activity in untreated vWF^{-/-} animals has previously been measured and found to be 0.01 IU/mL. Given these results, the stabilizing effect of the mutant cannot be attributed to improved survival in the absence of vWF binding. Further work is required to ascertain the molecular mechanisms driving the improvement in PK at low doses of the D519V/E665V variant. Because, there is a constant turn-over of FVIII to FVIIIa under steady state physiological conditions in order to maintain hemostasis, we speculate that after the low dose, FVIIIa is likely a significant fraction of the circulating species and therefore under these experimental conditions enhanced stability of mutant becomes apparent.

The increased stability of the mutant was also readily evident in the pharmacodynamic studies where the hemostatic challenge presented by laceration of the tail vein results in increased generation of FVIIIa. D519V/E665V demonstrated substantial improvement in efficacy over WT FVIII in the dose-response study. Both variants resulted in similar blood loss above 20 IU/kg, but at 10 IU/kg, mice treated with WT displayed the same blood loss as untreated HA mice. Mice receiving 10 IU/kg

Table I. Non-compartmental Pharmacokinetic Parameters

Formulation	Dose (IU/kg)	AUC _t ^a (IU·h/mL)	Cmax (IU/mL)	t _{1/2} (h)	CL (mL/h/kg)	Vss (mL/kg)
WT	40	8.69 ± 1.34	1.29	4.3	4.5	35.1
WT	10	0.841 ± 0.088	0.20	2.0	11.3	32.6
WT	4	— ^b	— ^b	— ^b	— ^b	— ^b
D519V/E665V	40	6.97 ± 0.54	1.09	3.9	5.7	39.4
D519V/E665V	10	0.531 ± 0.159	0.16	2.4	17.1	52.8
D519V/E665V	4	0.049 ± 0.006	0.04	— ^c	55.6	97.4

^a AUC_t ± standard error

^b WT could not be recovered after 4 IU/kg injection

^c Duration of plasma survival too short to reliably estimate t_{1/2}

Fig. 4. Efficacy of WT and D519V/E665V FVIII. Blood loss in a tail clip assay was measured in HA mice that received no treatment (open squares), WT FVIII (closed circles), or D519V/E665V FVIII (open circles). Normal mice (open triangles) are shown for reference. FVIII variant type was statistically significant ($p < 0.005$) by two-way ANOVA at the 10 IU/kg dose

Fig. 5. Immunogenicity of WT and D519V/E665V FVIII. Inhibitory titers (a) and total titers (b) were determined by a Bethesda assay and ELISA, respectively in HA mice treated intravenously for 4 weeks with either WT or D519V/E665V FVIII ($n=8/\text{group}$). Mean and standard deviation are shown. No significant differences were observed

of D519V/E665V lost 72% less blood than untreated mice. These results are supportive of observations from the PK studies that below some threshold concentration, likely where stability of the FVIIIa heterotrimer becomes a rate limiting step, the handling of WT drastically changes causing rapid elimination and loss of efficacy. Although D519V/E665V also displays a loss of efficacy and increase in catabolism at lower concentrations, the impact is more gradual than for WT. This is evidenced by the retention of partial efficacy at doses as low as 4 IU/kg.

Development of an immune response to FVIII in the HA patient population is a major concern with up to 30% of patients developing some level of inhibitory response towards FVIII, drastically complicating the management of bleeding episodes (39). Immunodominant epitopes have been identified in the A2, A3, and C2 domains of FVIII (40–42). Within the A2 domain alone, seven unique immunoreactive epitope sequences have been identified including the 513–523 region in which the D519V substitution is located (43). Although no significant difference was observed in the immunogenicity for a limited sample of mice, the higher specific activity and improved hemostasis at low concentrations for D519V/E665V compared to WT could decrease the amount of administered protein required to maintain equivalent efficacy; that smaller antigen load may contribute to reducing antibody formation under chronic dosing. D519V/E665V is compatible with stabilizing mutations elsewhere in the structure of FVIII (44). Combination with other mutations designed to disrupt immunogenic epitopes or formulation strategies capable of reducing immunogenicity and/or inducing tolerance such as liposomal delivery may be able to further improve FVIII replacement therapy (45,46).

CONCLUSION

Clinically, where the goal of prophylactic FVIII therapy is to maintain a minimum activity level, the stabilizing mutations associated with D519V/E665V may have a direct therapeutic impact. Increased stability and the decreased blood loss at low doses for D519V/E665V compared to equivalent doses of WT suggest that similar therapeutic efficacy may be maintained with a lower therapeutic concentration threshold, thus reducing the required frequency of injections. At a minimum, higher specific activities associated with the D519V/E665V could provide a dose-sparing effect, an important consideration for biotherapeutics where production costs constitute a non-trivial portion of a drugs expense.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (R01 HL-70227 to SVB and R01-HL38199 to PJF). MPK received pre-doctoral fellowships from Pfizer Inc. and the American Foundation for Pharmaceutical Education. Portions of the D519V/E665V protein used were supplied by Dr. John E. Murphy of Bayer Healthcare.

REFERENCES

1. Lenting PJ, Schooten CJMV, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. *J Thromb Haemost*. 2007;5(7):1353–60.

2. Fay PJ. Activation of factor VIII and mechanisms of cofactor action. *Blood Rev.* 2004;18(1):1–15.
3. Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem.* 1988;263(13):6352–62.
4. Eaton D, Rodriguez H, Vehar GA. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. *Biochemistry.* 1986;25(2):505–12.
5. Fay PJ, Haidaris PJ, Smudzin TM. Human factor VIIIa subunit structure. Reconstruction of factor VIIIa from the isolated A1/A3-C1-C2 dimer and A2 subunit. *J Biol Chem.* 1991;266(14):8957–62.
6. Bevers EM, Tilly RHJ, Senden JMG, Comfurius P, Zwaal RFA. Exposure of endogenous phosphatidylserine at the outer surface of stimulated platelets is reversed by restoration of aminophospholipid translocase activity. *Biochemistry.* 1989;28(6):2382–7.
7. Ahmad SS, Scandura JM, Walsh PN. Structural and functional characterization of platelet receptor-mediated factor VIII binding. *J Biol Chem.* 2000;275(17):13071–81.
8. Wakabayashi H, Varfaj F, Deangelis J, Fay PJ. Generation of enhanced stability factor VIII variants by replacement of charged residues at the A2 domain interface. *Blood.* 2008;112(7):2761–9.
9. Pemberton S, Lindley P, Zaitsev V, Card G, Tuddenham EG, Kemball-Cook G. A molecular model for the triplicated A domains of human factor VIII based on the crystal structure of human ceruloplasmin. *Blood.* 1997;89(7):2413–21.
10. Wakabayashi H, Fay PJ. Identification of residues contributing to A2 domain-dependent structural stability in factor VIII and factor VIIIa. *J Biol Chem.* 2008;283(17):11645–51.
11. Wakabayashi H, Griffiths AE, Fay PJ. Combining mutations of charged residues at the A2 domain interface enhances factor VIII stability over single point mutations. *J Thromb Haemost: JTH.* 2009;7(3):438–44.
12. Shen BW, Spiegel PC, Chang CH, Huh JW, Lee JS, Kim J, *et al.* The tertiary structure and domain organization of coagulation factor VIII. *Blood.* 2008;111(3):1240–7.
13. Morfini M. Pharmacokinetics of factor VIII and factor IX. *Haemophilia: Off J World Fed Hemophilia.* 2003;9 Suppl 1:94–9. discussion 100.
14. Manco-Johnson MJ, Abshire TC, Shapiro AD, Riske B, Hacker MR, Kilcoyne R, *et al.* Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med.* 2007;357(6):535–44.
15. Pipe SW, Kaufman RJ. Characterization of a genetically engineered inactivation-resistant coagulation factor VIIIa. *Proc Natl Acad Sci U S A.* 1997;94(22):11851–6.
16. Radtke KP, Griffin JH, Riceberg J, Gale AJ. Disulfide bond-stabilized factor VIII has prolonged factor VIIIa activity and improved potency in whole blood clotting assays. *J Thromb Haemost.* 2007;5(1):102–8.
17. Jenkins PV, Dill JL, Zhou Q, Fay PJ. Clustered basic residues within segment 484–510 of the factor VIIIa A2 subunit contribute to the catalytic efficiency for factor Xa generation. *J Thromb Haemost: JTH.* 2004;2(3):452–8.
18. Peng A, Straubinger RM, Balu-Iyer SV. Phosphatidylinositol containing lipidic particles reduces immunogenicity and catabolism of factor VIII in hemophilia A mice. *AAPS J.* 2010;12(3):473–81.
19. Nedelman JR, Jia X. An extension of satterthwaite's approximation applied to pharmacokinetics. *J Biopharm Stat.* 1998;8(2):317–28.
20. Holder DJ. Comments on Nedelman and Jia's extension of Satterthwaite's approximation applied to pharmacokinetics. *J Biopharm Stat.* 2001;11(1-2):75–9.
21. Shetty KA, Kosloski MP, Mager DE, Balu-Iyer SV. Soy phosphatidylinositol containing nanoparticle prolongs hemostatic activity of B-domain deleted factor VIII in hemophilia A Mice. *J Pharma Sci.* 2014.
22. Purohit VS, Ramani K, Sarkar R, Kazazian Jr HH, Balasubramanian SV. Lower inhibitor development in hemophilia A mice following administration of recombinant factor VIII-O-phospho-L-serine complex. *J Biol Chem.* 2005;280(18):17593–600.
23. Grillo AO, Edwards KL, Kashi RS, Shipley KM, Hu L, Besman MJ, *et al.* Conformational origin of the aggregation of recombinant human factor VIII. *Biochemistry.* 2001;40(2):586–95.
24. Kosloski MP, Pisal DS, Mager DE, Balu-Iyer SV. Nonlinear pharmacokinetics of factor VIII and its phosphatidylinositol lipidic complex in hemophilia A mice. *Biopharm Drug Dispos.* 2014;35(3):154–63.
25. Dumont JA, Liu T, Low SC, Zhang X, Kamphaus G, Sakorafas P, *et al.* Prolonged activity of a recombinant factor VIII-Fc fusion protein in hemophilia A mice and dogs. *Blood.* 2012;119(13):3024–30.
26. Pan J, Liu T, Kim JY, Zhu D, Patel C, Cui ZH, *et al.* Enhanced efficacy of recombinant FVIII in noncovalent complex with PEGylated liposome in hemophilia A mice. *Blood.* 2009;114(13):2802–11.
27. Kosloski MP, Miclea RD, Balu-Iyer SV. Role of glycosylation in conformational stability, activity, macromolecular interaction and immunogenicity of recombinant human factor VIII. *AAPS J.* 2009;11(3):424–31.
28. Pisal DS, Kosloski MP, Middaugh CR, Bankert RB, Balu-Iyer SV. Native-like aggregates of factor VIII are immunogenic in von Willebrand factor deficient and hemophilia A mice. *J Pharm Sci.* 2012;101(6):2055–65.
29. Reipert BM, Ahmad RU, Turecek PL, Schwarz HP. Characterization of antibodies induced by human factor VIII in a murine knockout model of hemophilia A. *Thromb Haemost.* 2000;84(5):826–32.
30. Lenting PJ, Vans CJ, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. *J Thromb Haemost: JTH.* 2007;5(7):1353–60.
31. Wang W, Wang YJ, Kelner DN. Coagulation factor VIII: structure and stability. *Int J Pharm.* 2003;259(1–2):1–15.
32. Pratt KP. Inhibitory antibodies in hemophilia A. *Curr Opin Hematol.* 2012;19(5):399–405.
33. Gale AJ, Pellequer JL. An engineered interdomain disulfide bond stabilizes human blood coagulation factor VIIIa. *J Thromb Haemost.* 2003;1(9):1966–71.
34. Bovenschen N, Boertjes RC, van Stempvoort G, Voorberg J, Lenting PJ, Meijer AB, *et al.* Low density lipoprotein receptor-related protein and factor IXa share structural requirements for binding to the A3 domain of coagulation factor VIII. *J Biol Chem.* 2003;278(11):9370–7.
35. Bovenschen N, van Stempvoort G, Voorberg J, Mertens K, Meijer AB. Proteolytic cleavage of factor VIII heavy chain is required to expose the binding-site for low-density lipoprotein receptor-related protein within the A2 domain. *J Thromb Haemost.* 2006;4(7):1487–93.
36. Lenting PJ, Christophe OD, Gueguen P. The disappearing act of factor VIII. *Haemophilia.* 2010;16(102):6–15.
37. Leyte A, Verbeet MP, Brodniewicz-Proba T, Van Mourik JA, Mertens K. The interaction between human blood-coagulation factor VIII and von Willebrand factor. Characterization of a high-affinity binding site on factor VIII. *Biochem J.* 1989;257(3):679–83.
38. Pisal DS, Balu-Iyer SV. Phospholipid binding improves plasma survival of factor VIII. *Thromb Haemost.* 2010;104(5):1073–5.
39. Saenko EL, Ananyeva NM, Kouliavskaya DV, Khrenov AV, Anderson JA, Shima M, *et al.* Hemophilia A: effects of inhibitory antibodies on factor VIII functional interactions and approaches to prevent their action. *Haemophilia.* 2002;8(1):1–11.
40. Fijnvandraat K, Celie PHN, Turenhout EAM, ten Cate JW, van Mourik JA, Mertens K, *et al.* A human alloantibody interferes with binding of factor IXa to the factor VIII light chain. *Blood.* 1998;91(7):2347–52.
41. Pratt KP, Qian J, Ellaban E, Okita DK, Diethelm-Okita BM, Conti-Fine B, *et al.* Immunodominant T-cell epitopes in the factor VIII C2 domain are located within an inhibitory antibody binding site. *Thromb Haemost.* 2004;92(3):522–8.
42. Zhong D, Saenko EL, Shima M, Felch M, Scandella D. Some human inhibitor antibodies interfere with factor VIII binding to factor IX. *Blood.* 1998;92(1):136–42.
43. Griffiths AE, Wang W, Hagen FK, Fay PJ. Use of affinity-directed liquid chromatography-mass spectrometry to map the epitopes of a factor VIII inhibitor antibody fraction. *J Thromb Haemost.* 2011;9(8):1534–40.

44. Wakabayashi H, Griffiths AE, Fay PJ. Enhancing factor VIII and VIIIa stability by combining mutations at the A2 domain interface and A1-C2 domain interface. *J Thromb Haemost.* 2012;10(3):492–5.
45. Gaitonde P, Peng A, Straubinger RM, Bankert RB, Balu-Iyer SV. Phosphatidylserine reduces immune response against human recombinant Factor VIII in hemophilia A mice by regulation of dendritic cell function. *Clin Immunol.* 2011;138(2):135–45.
46. Gaitonde P, Peng A, Straubinger RM, Bankert RB, Balu-Iyer SV. Downregulation of CD40 signal and induction of TGF-beta by phosphatidylinositol mediates reduction in immunogenicity against recombinant human Factor VIII. *J Pharm Sci.* 2012;101(1):48–55.