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Evaluation of factor VIII polysialylation: Identification of a longer-acting experimental therapy in mice and monkeys

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Running Title: PSAylation prolongs rFVIII circulation and activity

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Nonstandard abbreviations: EHL, extended half-life; rFVIII, recombinant human Factor VIII; PSA, polysialyic acid; PSArFVIII, polysialyated recombinant human Factor VIII; PEG, polyethylene glycol; aPTT, activated partial thrombin time; VWF, von Willebrand factor; LRP1, low density lipoprotein receptor-related protein 1; CL, clearance; MRT, mean residence time.

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Abstract

Extended half-life (EHL) factor therapies are needed to reduce the burden of prophylaxis and improve treatment adherence in patients with hemophilia. BAX 826 is a novel polysialylated full-length recombinant factor VIII (PSArFVIII) with improved pharmacokinetics, prolonged pharmacology and maintained safety attributes to enable longer acting rFVIII therapy. In FVIII-deficient hemophilic mice, PSArFVIII showed a substantially higher mean residence time (>2-fold) and exposure (>3-fold), and prolonged efficacy in tail-bleeding experiments (48 vs. 30 h) compared with unmodified rFVIII, as well as a potentially favorable immunogenicity profile. Reduced binding to scavenger receptor (LRP1) and von Willebrand factor (VWF) as well as a largely VWF-independent circulation time in mice provide a rationale for prolonged BAX 826 activity. The significantly improved PK profile vs. rFVIII was confirmed in cynomolgus monkeys (mean residence time: 23.4 vs.10.1 h; exposure [$AUC_{0-\infty}$]: 206 vs. 48.2 IU/ml*h) and is in line with results from rodent studies. Finally, safety and toxicity evaluations did not indicate increased thrombogenic potential, and repeated administration of BAX 826 to monkeys and rats was well-tolerated. The favorable profile and mechanism of this novel experimental therapeutic demonstrated all the requirements for an EHL-rFVIII candidate, and thus BAX 826 was entered into clinical assessment for the treatment of hemophilia A.

Significance Statement:

Prolongation of FVIII half-life aims to reduce the burden of prophylaxis and to improve treatment outcomes in patients with hemophilia. This study shows that polysialylation of PSArFVIII resulted in prolongations of rFVIII circulation time and procoagulant activity, together with a favorable non-clinical safety profile of the experimental therapeutic.

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Introduction

A major challenge in the management of hemophilia is prolonging the half-life and reducing the immunogenicity of recombinant clotting factors and thus to reduce the frequency of complications from repeated venous access, improve patient adherence to prophylactic regimens, and enhance patient outcomes (Pipe, 2005; Peeyvandi et al., 2013). Advances in factor-based treatment of hemophilia A include conjugating human recombinant FVIII (rFVIII) to various entities to extend its half-life and circulation (Mahlangu et al., 2018). Linking B-domain deleted rFVIII to the Fc domain of human IgG_1, for example, resulted in factor half-life extension (Dumont et a., 2012; Mahlangu et al., 2014), while chemically modifying FVIII by polyethylene glycol (PEG) has been shown to decrease clearance from the circulation and extend FVIII half-life (Turecek et al., 2012; Tiede et al.; 2013; Coyle et al. 2014; Konkle et al., 2015). Most modification techniques have achieved an overall maximum EHL of FVIII of 1.5 to 1.8 times the baseline, which however remains modest, compared with that reached with other therapeutics (Laffan, 2016; Balkaransingh et al., 2018). It has been hypothesized that clearance of modified rFVIII variants is largely regulated by interaction with von Willebrand factor (VWF), and therefore this association is suggested to be a limiting factor (Pipe et al., 2016).

Modification with biodegradable polysialic acid (PSA), a polymer of N-acetylneuraminic acid, is a promising method to improve the pharmacokinetics (PK) of therapeutic proteins (Zhang et al., 2014), while maintaining their structural integrity and activity (Gregoriadis et al., 1993; Gregoriadis et al., 2005). PSAylation is thought to affect and reduce receptor-mediated clearance and albeit not yet fully understood, its mode of action probably involves cell surface receptors like low-density-lipoprotein-receptor-related-protein (LRP1), heparan sulphate proteoglycans, and asialoglycoprotein receptor (Peyvandi et al., 2013; Gregoriadis et al., 2000). However, this

technology's potential remains untapped in the development of EHL products to treat bleeding disorders. In particular, studies investigating the effects of PSAylation on PK and procoagulant activity of rFVIII have not been reported so far.

We initiated a discovery program to assess the feasibility of using PSAylation to extend the half-life of drug products to treat bleeding disorders. Evaluation of PSAylation is based on the well-known safety and efficacy of recombinant full-length FVIII (Dhillon, 2012), and achieved by covalently coupling aminooxy-PSA to carbohydrate moieties to N-linked glycans. Human rFVIII is heavily glycosylated, with most N-linked glycans (19/25) and all seven O-linked glycans located in the B-domain, a domain which is dispensable for FVIII procoagulant activity (Toole, 1986). We comprehensively assessed the degree of full-length rFVIII PSAylation that is necessary and sufficient for optimized PK, efficacy and immunogenicity attributes,

Here, we present the preclinical PK, pharmacology, and safety of this novel therapeutic approach, which led to identification of the clinical lead candidate BAX 826, the first PSAylated experimental therapeutic developed for the treatment of hemophilia.

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Materials and Methods

Materials

The full-length rFVIII and PSArFVIII formulations used in the presented studies were based on ADVATE (Baxalta US Inc., a member of the Takeda group of companies, Lexington, MA, USA). rFVIII was conjugated with a diaminooxy-linker (3-Oxa-pentane-1,5-dioxyamine) to polysyalic acid polymers (20 kDa; Xenetic Biosciences, Inc., Lexington, MA) as described (Siekmann J et al., 2014), and structure and chemistry are detailed in **Figure 1**. The PSAylation process was controlled to result in various degrees of modification (5.4, 10.4, 13.3, and 16.4 mol PSA / mol rFVIII). The defined BAX 826 PSAylation degree approximates 10 mol/mol. As a control treatment and for factor dilutions the following Tris-buffer was used (10 mM Tris; 1.7 mM $CaCl_2$; 3.2% (w/v) Mannitol; 90 mM NaCl; 0.8% (w/v) alpha-trehalose; 10 mM histidine; 0.08 mg/mL glutathione (reduced); 0.01% polysorbate-80; pH ~7.0). Human recombinant VWF (rVWF) was from Baxalta US Inc. All treatments were administered by intravenous (IV) injection. FVIII deficient mouse models are described in *Supplemental Information*. All studies complied with national laws governing animal experimentation, and were approved by the respective animal care committees.

In vitro evaluation of human PSArFVIII pharmacologic activity

Citrated rat, monkey and human plasma was diluted with citrate buffer (1+2; rat and cynomolgus) or (1+1; human), resulting in reduced levels of coagulation factors, and then spiked with increasing concentrations of PSArFVIII (0, 1, 5, and 10 U/mL). Activated partial thrombin time (aPTT) was analyzed in duplicate with Pathromtin-SL (Siemens, Erlangen, Germany) using a KC4 coagulometer (Amelung GmbH, Lemgo, Germany).

Determination of the interaction of PSArFVIII with low density lipoprotein receptor-related protein 1 (LRP1)

Interaction of PSArFVIII with LRP1 was determined using an ELISA combined with a chromogenic assay for FVIII activity. In brief, plasma-derived LRP1 (BioMac, Leipzig, Germany) is immobilized on a microtiter plate and dilutions of rFVIII or PSArFVIII added. After incubation, unbound FVIII is removed by a washing step and FVIII bound to LRP1 quantified using a chromogenic FVIII activity assay (Technochrom FVIII:C, Technoclone, Vienna, Austria).

Determination of the interaction of PSArFVIII with von Willebrand factor (VWF)

FVIII binding to plasma-derived VWF (Diagnostica Stago, Asnières sur Seine, France) immobilized on the flow cells of a CM5 biosensor chip at targeted densities of 300, 600, and 1200 response units (RU) was analyzed using Biacore T200 (GE-Healthcare, Uppsala, Sweden). Samples were diluted with running buffer (10 mM Hepes, 150 mM NaCl, 0.05% Surfactant P20, pH 7.4) in five dilutions from 0.18 to 5 nM FVIII and applied to the chip (single cycle mode, 50 µL/min constant flow rate). Association and dissociation times were 4 and 10 min, respectively. Binding was quantitatively determined by evaluating the calculated maximum binding at saturation (Rmax).

PK studies in FVIII knockout (KO) mice

Male (m) and female (f) hemophilic B6.129S4-*F8*^{tmKaz} mice (FVIII KO mice; Bi et al., 1995) were used (body weights 17 - 39 g). All stock formulations were diluted to final concentrations of 20 U/ml and administered via single IV bolus dose (10 mL/kg) at a nominal dose level of 200 U/kg. Blood was collected from anesthetized mice (n=6/time point/group) via cardiac puncture at 5 min,

1, 3, 6, 16, 24, 32, and 40 h. Plasma FVIII activity was measured and analyzed as described in *Supplemental Information*.

Pharmacodynamic studies in mouse models of human hemophilia A

Efficacy study (tail-bleeding assay) treating acute bleeds in FVIII KO mice

FVIII KO mice were administered buffer or rFVIII or PSArFVIII of various PSAylation degrees at 10, 100, or 200 U/kg in the lateral tail vein 5 min before bleeds were induced by tail clip (N=16/group, 8m/8f).

Efficacy study (tail-bleeding assay) in FVIII KO mice with prophylactic treatment mode

The prolongation of efficacy of rFVIII PSAylation was assessed in FVIII KO mice in the same tail bleeding model. Mice were administered buffer or 200 U/kg rFVIII (18, 24, 30 or 40 h) or BAX 826 (24, 30, 40, 48 or 54 h) before bleeds were induced by clipping tail-tips. Blood loss was assessed gravimetrically (mg) over 60 min and adjusted for body weight (mg/g) as described (Schiviz et al., 2014).

Prophylactic efficacy study (arterial-thrombosis assay) in FVIII KO mice

The study methodology (Baumgartner et al., 2010) to test the efficacy of PSArFVIII or rFVIII via thrombogenic activity is detailed in *Supplemental Information*.

Comparative immunogenicity studies in murine FVIII KO transgenic mice

PSArFVIII immunogenicity was assessed in a hemophilic mouse model with a knockout of the murine FVIII that expresses a liver directed human F8 cDNA transgene (E17 FVIII-KO human F8 cDNA transgenic mice; van Helden et al., 2011), and in a hemophilic mouse model that expresses

the human MHC-class II protein HLA-DRB1*1501 on the background of a knockout of the murine MHC-class II complex (E17 FVIII-KO human MHC-class II (HLA DR15; Steinitz et al., 2012). Mice (5m/5f per group) were IV administered eight weekly doses of buffer or PSArFVIII (10.4 or 13.3 mol/mol), or rFVIII. The dose levels were selected based on the clinically relevant dose of the reference item rFVIII (50 IU/kg) approximating 200 ng/mouse, and a 5-fold higher dose level (1000 ng/mouse). An irrelevant, highly immunogenic human rFVIII protein-variant was used as a control (van Helden et al., 2011). Blood was sampled before the first dose by retro-orbital bleeds and one week after the last dose via cardiac puncture. Plasma samples were tested for anti-human rFVIII, and anti-human PSArFVIII binding antibodies and their incidence were measured as described in *Supplemental Information*.

Mechanistic PK study in FVIII/VWF double-KO (dKO) mice

FVIII/VWF dKO mice (body weights 18 - 35 g) were used and randomly allocated to treatment groups dosed with BAX 826 (200 U/kg), rFVIII (200 U/kg), without or with rVWF (3000 U/kg). Formulations were premixed with buffer or rVWF and administered via single IV bolus dose (10 mL/kg). Blood was collected at 5 min, 1, 3, 5, or 7 h (rFVIII group: 5 min, 1 and 3 h). Vials dedicated to plasma FVIII activity analysis were preloaded with rVWF (1 U/mL) to stabilize FVIII proteins *ex vivo*. Plasma VWF and FVIII activity was measured and analyzed as described in *Supplemental Information*.

Comparative PK study in cynomolgus monkey administered single doses of BAX 826 or rFVIII

Cynomolgus monkeys (3m/3f per group) were IV administered a nominal dose of 350 U/kg BAX 826 or rFVIII, or buffer, and blood samples were collected at 0 (pre-dose), 5, and 30 min, and 2, 6, 12, 24, 48, 60, 72, 96, 108, and 120 h. All plasma samples were analyzed in a FVIII chromogenic assay and PSA-FVIII modification-dependent-activity-assay (MDAA) as described in *Supplemental Information*.

Safety pharmacology and toxicity assessment

Assessment of thrombogenic potential in a rabbit venous stasis model (Wessler et al., 1959), and of repeated dose toxicity in rats and cynomolgus monkeys is described in *Supplemental Information*.

Statistical evaluation

All calculations were performed with R version 3 or higher (R Development Core Team, 2016) and SAS version 9.2 for Linux. The level of statistical significance was set to 5%. All comparisons are considered to be exploratory; therefore, no adjustment for multiplicity was applied *(Supplemental Information).*

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Results

Confirmation of PSArFVIII-retained procoagulant activity in vitro

Activated PTT in native rat, cynomolgus monkey, or human plasma was experimentally prolonged by dilution with citrate buffer. Supplementation with PSArFVIII (10.4 mol PSA/mol FVIII) led to a dose-dependent reduction and normalization of clotting time in all species (**Table 1**).

Polysialylation of rFVIII reduces interaction with LRP1 and VWF

Binding of PSArFVIII to LRP1 was tested using an ELISA-based format (**Fig. 2A**). All rFVIII and PSArFVIII samples investigated showed concentration-dependent binding to LRP1, which however, was markedly reduced vs. unmodified rFVIII, with increasing degrees of PSAylation reducing binding further.

Association between PSArFVIII and VWF is shown in **Fig. 2B**. All rFVIII and PSArFVIII samples investigated showed concentration-dependent binding to VWF. Binding of PSArFVIII to VWF was markedly reduced and correlated negatively with increasing PSA degree.

Effect of polysialylation degree on rFVIII PK in FVIII KO mice

When administered as a single IV dose to FVIII KO mice, Cmax estimates were higher in dose groups that received PSArFVIII than in the rFVIII group (**Table 2**). Clearance was profoundly reduced for PSArFVIII (9.25 to 11.01 ml/h/kg vs. 34.41 ml/h/kg for rFVIII). Statistically superior estimates for $AUC_{0-\infty}$ and MRT were detected in all PSArFVIII dose groups (**Fig. 3**). Estimates for terminal half-lives for PSArFVIII ranged from 5.14 to 7.38 h and thus were up to 2.3-fold longer than for rFVIII. Group differences for terminal-half-lives (vs. rFVIII group) were significant except for the PSArFVIII formulation with the lowest PSA degree (5.4 mol/mol).

Effect of polysialylation degrees on acute rFVIII pharmacodynamics in FVIII KO mice

We next assessed the potency of rFVIII formulations with different degrees of PSA in a tail bleeding model. Tail bleeds were initiated 5 min after IV dosing to ensure testing at peak plasma concentrations in all dose groups (**Fig. 4**). The decrease in blood loss by treatment with rFVIII and PSArFVIII was dose-related. Treatment with rFVIII was effective at all doses investigated and treatment with PSArFVIII was effective at the 100 and 200 U/kg dose (vs. buffer control group). However, no statistically significant difference (at the 5% level) in blood loss was observed between PSArFVIII and rFVIII treated groups at equivalent dose levels. Blood loss in PSArFVIII groups treated at the intermediate dose level (100 U/kg) showed larger within-group variability at higher PSAylation degrees (13.3 and 16.4 mol/mol) (**Fig. 4**). Thus, a relatively less robust pharmacologic effect might be inferred by the variable pharmacological activity at higher degrees of rFVIII-PSAylation. Guided by the PK and efficacy data in mice, formulations with PSAylation degrees of 10.4 and 13.3 were selected for further evaluation.

Comparative immunogenicity studies in hemophilic mouse models

A FVIII KO mouse model was used to study the potential immunogenicity of the modified rFVIII where FVIII peptides are presented by HLA-DRB1*1501, as described earlier (Steinitz et al., 2012). The incidence of mice evaluated as positive for anti-FVIII binding antibodies was 5/10 and 7/9 mice treated weekly with rFVIII (200 ng or 1000 ng) after eight repeat doses (**Table 3**). At the same time point, the incidence of anti-FVIII antibodies in PSArFVIII (10.4 mol/mol) groups was only 1/10 and 2/10 respectively, and the incidence in PSArFVIII (13.3 mol/mol) groups was 4/9 and 3/10 respectively. The titer ranges were similar in all treatment groups.

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To mimic the situation in previously FVIII-treated patients without inhibitors, human rFVIII-immunotolerant hemophilic mice were used, that develop antibodies against human FVIII only when immune tolerance breaks down (van Helden et al., 2011). 4/10 and 5/10 mice treated weekly with rFVIII (200 ng or 1000 ng) were evaluated as positive after eight repeat doses. The incidence of anti-FVIII antibodies in PSArFVIII (10.4 mol/mol) groups was 0/10 (200 ng/week) and 2/10 (1000 ng/week), and all mice in PSArFVIII (13.3 mol/mol) treatment groups were evaluated as negative for binding antibodies. The titer ranges were similar in all treatment groups (**Table 3**). In summary, no substantial differences in immunogenicity were observed between the two PSArFVIII formulations, and therefore the studies below focused on that with a relatively lower PSAylation degree of 10.4 mol/mol (BAX 826).

Prolonged efficacy of BAX 826 vs. rFVIII in prophylactic treatment of hemophilic mice

We next tested the prophylactic use of BAX 826 assessing reductions in blood loss as well as for an extended period of effectiveness. Treatment was administered up to 54 h before inducing bleeds in the tail-bleeding model. The median total blood loss in buffer-treated mice was 40 mg/g and significantly reduced in rFVIII-treated groups when administered up to 30 h before tail-clip (22 mg/g; P=0.0018) (**Fig. 5A and B**). BAX 826 was efficacious up to 48 h (20 mg/g; P=0.0212) and, while the effect size was largely diminished at the last time point tested (54 h), group differences (vs. buffer) were still significant (P=0.0377).

Prolonged efficacy of BAX 826 in the carotid occlusion model in FVIII KO mice

Prolonged BAX 826 activity was further substantiated in a murine model of ferric chloride-induced arterial thrombosis (Baumgartner et al., 2010). Within the 30-min observation period, no vessel occlusions were observed in buffer-treated animals. Pharmacologic activity by rFVIII and BAX 826 was demonstrated by a distinct decrease in time to occlusion (**Supplemental Table S1**). rFVIII

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was efficacious in groups treated up to 18 h before denudation of the carotid endothelium (median time to occlusion 12.0 min; $P<0.001$ vs. buffer). Treatment with BAX 826 was efficacious in groups treated up to 30 h before denudation (median time to occlusion 6.0 min; $P<0.001$).

Prolonged circulation time of BAX 826 is largely independent of interaction with VWF

To investigate the mechanistic basis for the prolonged efficacy, BAX 826 PK was tested in FVIII/VWF dKO mice in the absence or presence of co-administered human rVWF. Due to the deficiency of VWF, administered rFVIII is rapidly cleared from the circulation, and rFVIII activity was below the lower limit of quantification (LLOQ) 3 h after administration (**Fig. 6A**), with an estimated terminal half-life of 0.33 h. Co-administration of rVWF improved the FVIII PK profile (**Fig. 6A**) and terminal half-life as expected, and significantly reduced the clearance of rFVIII (**Table 4**). Although pharmacologic intervention with rVWF did not fully normalize rFVIII clearance (CL) to the levels seen in VWF-competent mice (*conf.* **Table 2**), terminal half-life and MRT were essentially comparable (**Table 4**). In stark contrast, BAX 826 was detectable in the absence of VWF at all time points (**Fig. 6B**) and terminal half-life was profoundly prolonged (5.5 h), suggesting reduced CL of BAX 826 in VWF-deficient mice and similar to that seen in VWF-competent animals (*conf.* **Table 2**). Co-administration with rVWF resulted in modestly increased Cmax, and changes in terminal half-life and MRT were not statistically significant ($P\geq0.767$).

Comparative PK studies in cynomolgus monkeys and rats

To fully assess the potential of factor half-life extension by PSAylation, single doses of BAX 826, rFVIII or buffer were administered in parallel. Activity was determined in the presence of cynomolgus FVIII and corrected accordingly. Similar Cmax values were determined after administration of BAX 826 or rFVIII **(Table 5)**. However, reduced CL (2.25 vs. 6.72 ml/h/kg),

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prolonged FVIII activity terminal half-life (18.5 vs. 8.0 h) and MRT (23.4 vs. 10.1 h) resulting in a substantial increase in exposure to FVIII activity ($AUC_{0-\infty}$ 206 vs. 48.2 U/ml h) were recorded in the BAX 826-treated group. FVIII activity increased over baseline up to 48 to 72 h in BAX 826-treated animals and up to 12 to 24 h in the rFVIII-treated group (**Fig. 7A**). The ratios of geometric means (BAX 826/rFVIII) and corresponding two-sided 95% confidence intervals (CIs) confirm similar C_{max} and significantly improved $AUC_{0-\infty}$, terminal half-life, and MRT (**Fig. 7B**).

To further and specifically assess BAX 826 activity in FVIII-competent cynomolgus monkeys, the same plasma samples were tested in an MDAA assay format (**Table 5**). Activity was detected in plasma from all treated animals and above LLOQ (0.05 U/ml) up to 72 to 96 h after dosing. Overall, the PK results using this assay format substantiated the characteristics for BAX 826 assessed above. Additional comparative PK evaluations in rats further supported the extended circulation time observed for BAX 826 over rFVIII **(Supplemental Table S2)**.

Evaluation of BAX 826 thrombogenic potential in a rabbit venous stasis model

Assessment of the thrombogenic potential (Wessler et al., 1959) at an exceedingly high dose level of BAX 826 (900 U/kg) showed no evidence of heightened thrombogenicity in individual scores (range 0 – 2, mean 0.42) or compared with scores in the rFVIII (900U/kg)-treated group (range 0 – 1, mean 0.67). The percentage of BAX 826 and rFVIII-treated animals without evidence of thrombi formation was 44.5% and 16.7% respectively. All animals treated with the activated prothrombin complex concentrate FEIBA showed evidence of thrombi formation (range 2 – 4, mean 3.50) with the majority at the highest possible score (4), confirming the validity of the model.

Toxicology studies in rats and cynomolgus monkeys

Intravenous administration of BAX 826 once every five days for 31 days in rats (80, 350, or 800 U/kg) and cynomolgus monkeys (80, 350, or 600 U/kg) was well-tolerated. The no observed adverse effect levels (NOAEL) were set at the highest dose levels in each species as detailed in *Supplemental Information*. Repeated dosing of human BAX 826 protein to rats and cynomolgus monkeys was associated with development of anti-rFVIII/PSArFVIII antibodies in most animals.

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Discussion

Available FVIII-based treatment options for hemophilia are being further developed to enhance aspects of their biology, most notably their half-life, functional activity and immunogenicity (Laffan, 2016).

Protein modifications with PSA have been proposed to maintain the structural integrity and activity of therapeutic proteins (Gregoriadis et al., 1993; Gregoriadis et al., 2005), while the degree of PSAylation can influence their immunological and PK profiles (Fernandes and Gregoriadis, 2001; Constantinou et al., 2008). In accordance, the procoagulant potential of PSArFVIII was essentially maintained as demonstrated by normalization of an artificially prolonged aPTT *in vitro*; however, binding to LRP1 and VWF was diminished and correlated inversely with degree of PSAylation.

PSArFVIII formulations also showed striking improvements in circulation time and exposures (mean ratio estimates up to 3-fold), although there was no clear tendency that increasing degrees of PSAylation further improved PSArFVIII PK behavior. While the procoagulative potential of PSArFVIII in the acute tail-bleeding assay did not differ from that for rFVIII, analysis of all available data suggested that moderate PSAylation approximating 10 mol/mol is necessary for optimal pharmacologic activity and sufficient to prolong circulation time.

To address putative effects on immunogenicity, we used hemophilic mouse models representing either 1) relative immunotolerance to human rFVIII therapy or 2) an increased genetic risk of an immune response to rFVIII in humans (van Helden et al., 2011; Steinitz et al., 2012). The incidence of animals that tested positive for anti-rFVIII/PSArFVIII antibodies was numerically higher in rFVIII than in PSArFVIII-treated groups in both models. However, the titers appeared similar and there were no further meaningful reductions in incidence with a higher degree of PSAylation (i.e.

13.3 mol/mol). These *in vivo* data are congruent with a previous report where one or two injections of unmodified insulin generated an immune response in mice, whereas four injections of PSA-insulin were needed to elicit a modest response (Gregoriadis et al., 2005; Jain et al. 2003). Adding PSA to human FVIII does not appear to negatively affect the immunogenicity profile of FVIII and could potentially affect its immunogenicity profile favorably; however, no final proof of decreased immunogenic potential can be deduced from animal studies. BAX 826 did not increase activation of the human innate immune system or of complement pathways (C5a) compared with rFVIII *in vitro,* though potential development of anti-PSA-antibodies was noted after repeated administration of BAX 826 to rats and monkeys (manuscript in preparation).

In summary, in-vitro data suggested that increasing degrees of FVIII PSAylation correlated with reduced interactions with LRP1 and VWF and a holistic assessment was used to narrow a preferable PSAylation degree *in vivo*. PSAylation degrees ≥10.4 mol/mol provided significant half-life extension ratios *vs.* rFVIII, whereas higher PSAylation degrees ≥13.3 mol/mol resulted in more variable efficacies at intermediate dose levels and were without additional benefit when assessing immunogenicity risks in mice. These combined attributes suggested a PSAylation degree of about 10 mol/mol as a preferable target.

Restrained immunogenicity potential of novel FVIII products might be beneficial in treating hemophilia; however, systemic clearance of FVIII is largely determined by the plasma level of VWF (Pipe et al., 2016; Björkman et al., 2001). While binding of PSArFVIII to VWF is diminished *in vitro,* the circulation time of BAX 826 in FVIII KO mice (VWF-competent mice) was clearly prolonged. We further interrogated this mechanistic relationship *in vivo* using VWF-deficient mice in a reconstitution experiment with human rVWF. Compared to BAX 826 MRT in wild-type mice (9.06 h), the circulation time remained relatively prolonged (7.7 h) in VWF-

deficient animals. Co-administration of human rVWF had a non-significant effect on BAX 826 MRT (8.3 h), whereas the circulation time of unmodified rFVIII (0.33 h) was substantially restored by reconstitution with VWF. This observation is consistent with *in vitro* binding data, indicating markedly reduced PSArFVIII/VWF interaction. Previous studies with PEGylated full-length or B-domain deleted rFVIII have shown relatively unchanged interactions with VWF (Turecek et al., 2012; Tang et al. 2013). The data suggest a differentiated mechanism for the EHL of PSArFVIII that is largely, though not entirely, independent of interaction with VWF *in vivo*, whereas they do not exclude PSArFVIII/VWF interactions *in vivo* in the pharmacologic control of hemostasis. By extension, however, these results might infer a relatively maintained EHL attribute for PSArFVIII in human pathologies where circulating VWF is rate-limiting (Favaloro, 2013). In aggregate, the current data suggest that PSArFVIII EHL comprises two possibly intertwined mechanisms: reduced binding to scavenger receptors (i.e. LRP1) and a largely VWF interaction-independent circulation time. The current data do not exclude that additional physiological mechanisms and FVIII interactions (Pipe et al., 2016) might potentially be affected by PSAylation of rFVIII.

It has been recognized, that the time-consuming nature of prophylaxis regimen that typically involves two to four infusions per week is the greatest treatment-related barrier to adherence in children and adults receiving prophylaxis for hemophilia (Thornburg and Duncan, 2017). To support the effective translation of improved PK behavior into a period of extended pharmacodynamic activity, we have applied an orthogonal approach demonstrating prophylactic reductions in blood loss over an extended period of effectiveness as well as prolonged procoagulant (thrombotic) activity in hemophilic FVIII-deficient mice. The clinical relevance of these findings can be inferred from a previous study in the same mouse model that demonstrated prolonged

prophylactic efficacy for a clinical EHL FVIII therapeutic (PEG-rFVIII) over a similar period (Turecek et al., 2012).

A recent report suggests that EHL rFVIII products should have a minimum half-life extension ratio of 1.3 to allow a reduction in dosing frequency from 3× to 2×/week compared with standard rFVIII products while maintaining the same minimum FVIII trough level (Hermans et al., 2018). Comparative analyses of key PK parameters in non-human primates clearly demonstrated that treatment with BAX 826 or rFVIII shows equivalent Cmax yet produces significantly increased AUC and circulation time as indicated by a 3-fold reduction in clearance, and greater than 2-fold prolongations of MRT and half-life.

Considering the availability of safe and effective FVIII replacement therapies (Cafuir and Kempton, 2017), the safety profile of novel modifications of rFVIII, like BAX 826, needs to be scrutinized. As a first step, the evaluation of BAX 826 thrombogenicity potential during venous stasis did not show differences between BAX 826 and rFVIII. BAX 826 was also well-tolerated in rats and monkeys in repeat-dose toxicity studies up to 5 weeks, with no mortalities, clinical signs, or pathology directly attributable to BAX 826 at exposure levels up to 14.9-fold above those tested in mice. Both rat and monkey species however, showed development of binding and neutralizing antibodies against BAX 826 and rFVIII, which is an expected immune response after repeated application of a heterologous protein and not predictive of the human situation. The presence of neutralizing antibodies correlated with a decrease in exposure and a prolongation of aPTT after repeated application of BAX 826, and all sequelae were attributed to antibodies cross-reacting with monkey FVIII protein.

The rationale of the presented approach is based on unmodified rFVIII with PSA conjugation predominantly within the FVIII B-domain, a domain not essential and not present in activated

FVIII protein. Site-directed FVIII PSAylation of particular glycans or substitution of FVIII residues to allow for site-directed protein chemistries would present alternative conjugation strategies. While there are advantages to these approaches there are also caveats that would need to be considered, like a potential increase in immunogenic risks. Nevertheless, site-specific polysialylation of modified proteins is feasible (Constantinou et al., 2009) and site-specific PEGylated FVIII variants are in clinical development (Tiede, 2015).

In summary, comprehensive preclinical evaluation of PSArFVIII defined a preferable degree of rFVIII polysialylation resulting in identification of BAX 826. BAX 826 demonstrated a favorable nonclinical safety profile and all characteristics of an EHL rFVIII product compared with unmodified rFVIII. These features recommended BAX 826 for further clinical evaluation as a potential EHL treatment option for hemophilia.

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Authorship Contributions

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Footnotes

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Legends for Figures

Fig. 1: Schematic presentation of PSA and rFVIII structures in PSArFVIII (BAX 826). The 20 kDa PSA polymer is a linear homo-polymer composed of N-acetylneuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galacto-nonulo-pyranos-1-onic acid) monomers. Monomers in PSA used to manufacture BAX 826 are linked in an α-2,8 manner. The chemistry for the PSAylation of rFVIII in BAX 826 makes use of free aldehyde groups generated by mild oxidation of sialic acids of N-glycans of the protein with $NaIO_4$. PSA containing an active aminooxy group was used to react with the free aldehyde groups of the protein. PSA is preferentially coupled to N-linked glycans (complex type) within the B-domain of rFVIII. Indicated are also N- or O-linked glycans where PSAylation was not detected (Schrenk et al., 2016).

Fig. 2. In vitro evaluation of PSArFVIII interaction with LRP1 and VWF. Binding to LRP1 **(A)** was determined by an ELISA-combined chromogenic assay (means ± S.D., N=3; where not shown error bars are too small to depict). Binding to VWF **(B)** was determined by Biacore as the maximum binding at saturation (Rmax; single measurements). rFVIII (triangles), PSArFVIII with a PSA degree (mol PSA/mol FVIII) of 5.4 (diamonds), 10.4 (circles), 13.3 (squares, dotted line) and 16.4 (open diamonds). Note that affinity of rFVIII to VWF (K_D:0.19 – 0.25 nM) was only slightly affected by polysialylation but similar for all PSA degrees (K_D: 0.39 – 0.41 nM) as determined by Biacore (data not shown).

Fig. 3. Evaluation of PK parameters demonstrate improved circulation times and exposures by PSArFVIII preparations vs rFVIII in FVIII KO mice. PK data were assessed using a serial sampling design where only one sample is taken per animal at one of the time points investigated. Unmodified rFVIII or PSArFVIII were administered intravenously (n=6/time point/group) at a

dose of 200 IU/kg. Shown are the group ratios between PSArFVIII (PSAylation degrees 5.4 **(A)**, 10.4 **(B)**, 13.3 **(C)**, and 16.4 **(D)** mol/mol) and rFVIII and the corresponding two-sided 95% confidence intervals (CIs) of the PK parameters maximum concentration (C_{max}), area under curve ($AUC_{0-\infty}$), terminal half-life, and mean residence time (MRT). A two-sided 95% CI for the ratio not containing the value 1, indicated by the vertical dashed line, is equivalent to rejecting the null hypothesis of no difference against the two-sided alternative at the 5% level of statistical significance.

Fig. 4. Single-dose treatment with PSArFVIII (or rFVIII) shows dose-related procoagulant activity in hemophilic FVIII KO mice and robustness of pharmacological PSArFVIII activity affected by the degree of PSAylation. Buffer, unmodified rFVIII, or PSArFVIII (PSAylation degrees 5.4, 10.4, 13.3, and 16.4 mol/mol) were administered intravenously 5 min before bleeds were started. Treatment with rFVIII reduced median blood loss from 42.2 mg/g (control group) to 27.05 mg/g (10 U/kg), 1.47 mg/g (100U/kg) and 0.93 mg/g (200 U/kg). Administration of increasing doses of PSArFVIII (with varying PSA degree) reduced median blood loss ranging from 25.02 - 38.34 (10 U/kg), 1.31 to 7.05 (100 U/kg), and 1.27 to 1.53 (200 IU/kg). Body weight normalized blood loss over 60 minutes (n = 16/group) is displayed using boxplots (see *Supplemental Information*).

Fig. 5. Single-dose treatment with BAX 826 prolongs procoagulant activity vs. treatment with rFVIII in hemophilic FVIII KO mice**.** Buffer, unmodified rFVIII, or BAX 826 were administered intravenously at a dose of 200 IU/kg at the indicated time points before bleeds were started. (**A**) Body weight normalized blood loss over 60 minutes (n = 15 - 16/group) is displayed using boxplots. See *Supplemental Information* for a description of boxplots. (**B**) Relative effects and corresponding one sided 95% confidence intervals at the respective time points after treatment with rFVIII (grey circles) or BAX 826 (open triangles) are displayed. The relative effect provides a

probability that a randomly selected animal treated with rFVIII or BAX 826 has a lower normalized blood loss than a randomly selected animal treated with buffer. The relative effect is 0.5 if both groups are stochastically equal. A one-sided 95% CI for the relative effect not containing the value 0.5, indicated by the vertical dashed line, is equivalent to rejecting the null hypothesis against the two-sided alternative of a lower normalized blood loss than with Buffer at the 5% level of statistical significance.

Fig. 6. Prolongation of BAX 826 circulation time is largely independent of circulating VWF in FVIII/VWF dKO mice. **(A)** Unmodified rFVIII or **(B)** BAX 826 was administered at a dose of 200 IU/kg without and with 3000 U/kg of rVWF, and blood was sampled at the time points indicated. Individual FVIII activity is shown as grey circles and triangles (without VWF) or white circles and triangles (with VWF). Medians are shown as dashed lines (without VWF) or solid lines (with VWF). Medians are shown as dashed lines (without VWF) or solid lines (with VWF). PK data were assessed using a serial sampling design where only one sample is taken per animal at one of the time points investigated (n=6/time point/group).

The PK profiles of rVWF (not shown) were similar when administered in combination with either rFVIII or BAX 826 as indicated by the respective median for Cmax (35.83 and 38.62 U/mL), terminal half-life (6.3 and 6.1 hours), as well as $AUC_{0\text{-tlast}}$ (148.1 and 149.8 U/mL*hours).

Fig. 7. Comparative PK analyses in plasma of cynomolgus monkey reveals prolonged circulation of BAX 826 vs. rFVIII. Unmodified rFVIII or BAX 826 was administered intravenously (n=6/group) at a dose of 350 IU/kg and blood was sampled at the time points indicated. FVIII activity was baseline corrected to account for endogenous monkey FVIII activity. (**A**) Individual FVIII activity is shown as grey circles (rFVIII) or white triangles (BAX 826) with thin lines connecting measurements over time per animal. Medians are shown as dashed lines (rFVIII) or

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solid lines (BAX 826). (**B**) Shown are ratios of geometric means (BAX 826/rFVIII) and corresponding two-sided 95% confidence intervals (CIs) of the PK parameters maximum concentration (C_{max}), area under curve ($AUC_{0-\infty}$), terminal half-life, and mean residence time (MRT). A two-sided 95% CI for the ratio not containing the value 1, indicated by the vertical dashed line, is equivalent to rejecting the null hypothesis of no difference against the two-sided alternative at the 5% level of statistical significance.

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Tables

TABLE 1

PSArFVIII (10 mol/mol) is pharmacologically active and fully reverses aPTT prolongation in diluted rat, cynomolgus monkey, and human plasma (n=2, means).

PSArFVIII [IU/mL]	**aPTT (seconds)**		
	rat	**cynomolgus**	**human**
0	42.5	42.5	57.3
1	34.2	34.2	48.9
5	29.3	29.3	40.0
10	26.2	26.2	34.1
Undiluted native plasma	29.2	29.2	33.9

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TABLE 2

Summary of PK parameters after single-dose intravenous administration of PSArFVIII presentations or rFVIII to FVIII KO mice (n=6/group/time point). Tmax (time at Cmax), Cmax (peak plasma concentration), IR (incremental recovery), MRT (mean residence time), AUC 0 – 40h (area under the curve from 0 to 40 hours), $AUC_{0-\infty}$ (area under the curve from 0 to infinity), CL (clearance), Vdss (volume of distribution, steady state). Values in parentheses refer to two-sided 95% confidence intervals (CIs).

	rFVIII (200 IU/kg)				
PSAylation degree (mol/mol)	**5.4**	**10.4**	**13.3**	**16.4**	**0**
Tmax (min)	5	5	5	5	5
Cmax (IU/mL)	4.33 (3.89 – 4.76)	2.92 (1.83 – 4.01)	3.70 (2.90 – 4.51)	4.22 (3.82 – 4.63)	2.40 (1.14 – 3.66)
IR (IU/ml)/(IU/kg)	0.0143 (0.0129 – 0.0158)	0.0146 (0.0092 – 0.0201)	0.0176 (0.0138 – 0.0214)	0.0154 (0.0139 – 0.0169)	0.0101 (0.0048 – 0.0154)
Terminal half-life (h)	7.37 (1.54 – 13.20)	6.51 (5.19 – 7.82)	5.14 (4.65 – 5.63)	7.38 (6.55 – 8.20)	3.20 (2.71 – 3.70)
AUC 0-40h (IU/ml*h)	27.19 (22.53 – 31.85)	21.06 (18.24 – 23.89)	18.86 (17.22 – 20.50)	26.64 (24.54 – 28.74)	6.872 (5.711 – 8.034)
AUC0-∞ [IU/mL*h]	27.69 (23.34 – 32.04)	21.35 (18.48 – 24.23)	18.94 (17.28 – 20.6)	27.25 (25.0 – 29.5)	6.87 (5.71 – 8.04)
MRT (h)	8.97 (7.56 – 10.37)	9.06 (7.85 – 10.27)	7.21 (6.59 – 7.83)	10.22 (9.17 – 11.27)	4.18 (3.57 – 4.78)
CL (ml/h/kg)	10.93 (9.21 – 12.64)	9.34 (8.1 – 10.6)	11.11 (10.14 – 12.09)	10.05 (9.23 – 10.88)	34.7 (26.83 – 40.57)
Vdss (mL/kg)	97.96 (78.22 – 117.7)	84.65 (71.94 – 97.36)	80.12 73.08 – 87.15)	102.7 (93.20 – 112.3)	144.9 (121.1 – 168.8)

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TABLE 3

Anti-FVIII antibody response in hemophilic mouse models. Incidence of plasma samples evaluated as positive for anti-FVIII antibodies after 8 weekly administrations of buffer, PSArFVIII formulations, rFVIII, or treatment with a positive control rFVIII protein in respective hemophilic mouse models. Titer ranges within each study group are indicated. As expected, no anti-FVIII antibodies were detected in buffer control-treated animals of either mouse model, whereas all (9/9) positive control-treated human FVIII-transgenic mice showed breaking of immune tolerance. ND, not determined.

		buffer	**PSArVIII 10.4 mol/mol**		**PSArVIII 13.3 mol/mol**		**rFVIII**		**control**
Dose (ng/week)		0	200	1000	200	1000	200	1000	1000
FVIII KO, HLA-DRB1*1501 Tg	incidence	0/10	1/10	2/10	4/9	3/10	5/10	7/9	ND
	titer range	0-0	1-5	3-8	1-7	2-8	1-9	1-9	ND
E17 murine FVIII KO, human F8 Tg	incidence	0/10	0/10	2/10	0/10	0/10	4/10	5/10	9/9
	titer range	0-0	0-0	3-11	0-0	1-1	4-9	1-9	5-13

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TABLE 4

Summary of PK parameters after single-dose intravenous administration of 200 U/kg BAX 826 or rFVIII to FVIII/VWF dKO mice, without or with rVWF co-administration (n=6/group/time point). Tmax (time at Cmax), Cmax (peak plasma concentration), IR (incremental recovery), MRT (mean residence time), AUC 0 – tlast (area under the curve from 0 to last time point), CL (clearance), Vdss (volume of distribution, steady state). Values in parentheses refer to two-sided 95% confidence intervals (CIs). * linear regression on $\log_e$-transformed individual concentrations resulted in a terminal half-life of 0.3307 h (95% CI: 0.2955 to 0.3755). NA, not available.

	BAX 826		**rFVIII**	
rVWF (3000 U/kg)	-	+	-	+
Tmax (min)	5	5	5	5
Cmax (IU/mL)	3.36 (3.12 to 3.60)	3.87 (3.22 to 4.52)	1.61 (1.38 to 1.84)	3.23 (1.90 to 4.57)
IR (IU/ml)/(IU/kg)	0.0146 (0.0136 to 0.0157)	0.0158 (0.0132 to 0.0185)	0.0081 (0.0070 to 0.0093)	0.0134 (0.0078 to 0.0189)
Terminal half-life (h)	5.52 (3.42 to 14.42)	5.73 (4.84 to 7.04)	0.33*	2.76 (1.87 to 5.22)
AUC 0-tlast (IU/ml*h)	11.83 (10.52 to 13.14)	17.41 (16.42 to 18.39)	NA	8.80 (7.95 to 9.66)
MRT (h)	7.70 (4.10 to 11.31)	8.30 (6.73 to 9.87)	NA	3.78 (2.44 to 5.13)
CL (ml/h/kg)	11.59 (9.04 to 14.15)	7.98 (6.92 to 9.04)	NA	22.84 (19.17 to 26.51)
Vdss (mL/kg)	89.31 (64.16 to 114.5)	66.22 (60.44 to 72.00)	NA	86.43 (65.10 to 107.8)

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TABLE 5

Summary of PK parameters after single-dose intravenous administration of BAX 826 or rFVIII (350 U/kg) to cynomolgus monkeys evaluated with PSA-FVIII modification-dependent activity assay (MDDA) or chromogenic assay as described in Materials and Methods. (n=6/group). Tmax (time at Cmax), Cmax (peak plasma concentration), IR (incremental recovery), MRT (mean residence time), AUC 0 – tlast (area under the curve from 0 to last time point), AUC0 – ∞ (area under the curve from 0 to infinity), CL (clearance), Vdss (volume of distribution, steady state). Except for Tmax, all values in parentheses refer to two-sided 95% CIs.

	BAX 826		**rFVIII**
assay format	MDAA	Chromogenic	
Tmax median minutes, (range)	5 (5 – 30)	5 (5 – 30)	5 (5 – 5)
Cmax (IU/mL)	7.9 (5.6 – 11.0)	10.7 (7,9 – 14.4)	7.9 (6.8 – 9.2)
IR (IU/ml)/(IU/kg)	0.019 (0.014 – 0.026)	0.023 (0.017 – 0.031)	0.024 (0.021 – 0.028)
Terminal half-life (h)	13.5 (11.7 – 15.5)	18.5 (13.2 – 26.0)	8.0 (4.7 – 13.5)
AUC 0-tlast (IU/ml*h)	127 (91 – 178)	189 (146 – 245)	39.6 (28.0 – 55.9)
AUC 0-∞ (IU/ml*h)	129 (93 – 179)	206 (160 – 266)	48.2 (33.3 – 69.7)
MRT (h)	17.6 (14.2 – 21.9)	23.4 (18.4 – 29.9)	10.1 (6.25 -16.3)
CL (ml/h/kg)	3.24 (2.32 – 4.51)	2.25 (1.75 – 2.90)	6.72 (4.65 – 9.73)
Vdss (mL/kg)	57.1 (39.3 – 82.9)	52.8 (39.9 – 69.8)	67.9 (49.2 – 93.6)

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Figures

Figure 1

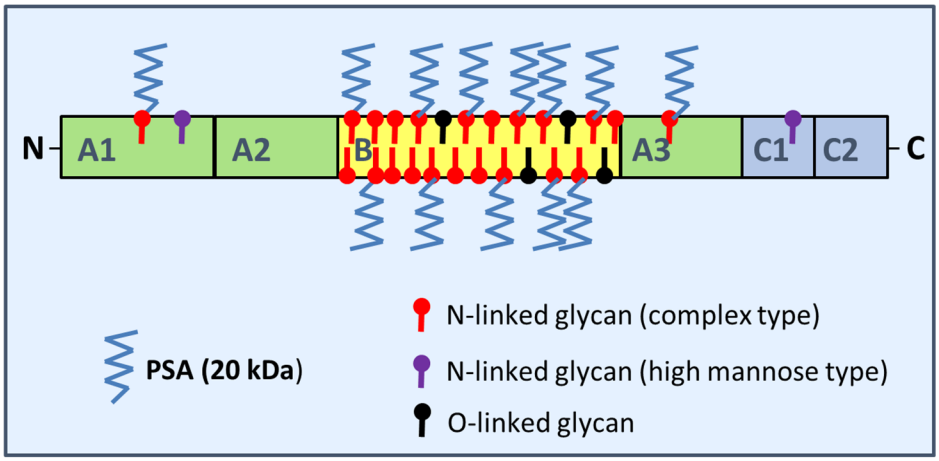

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Figure 2

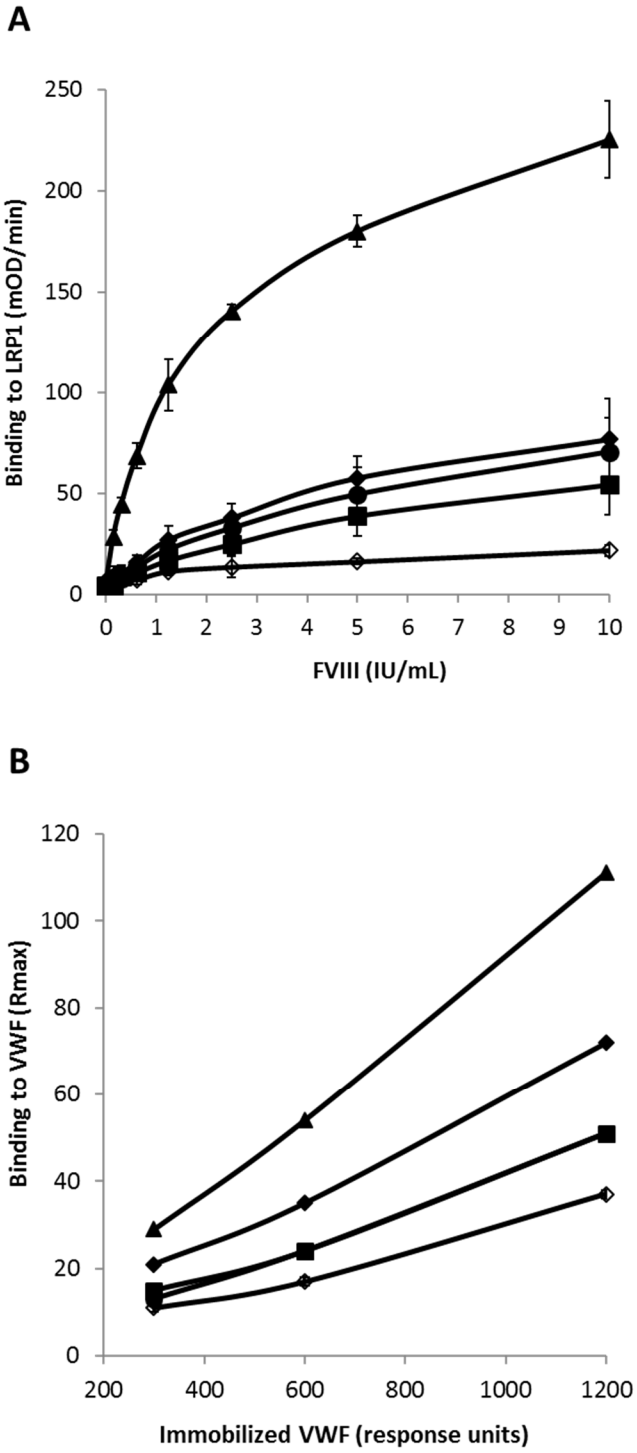

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Figure 3

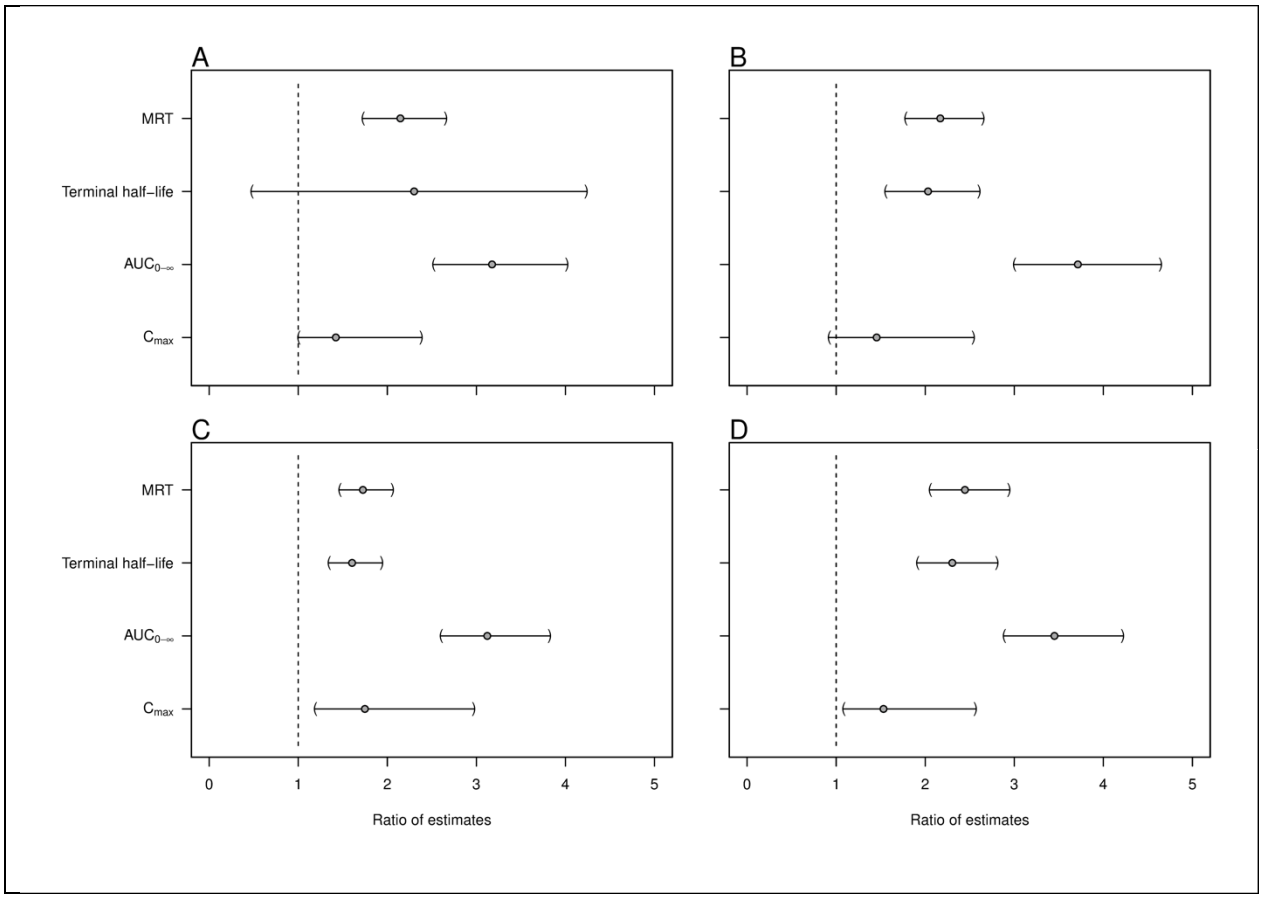

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Figure 4

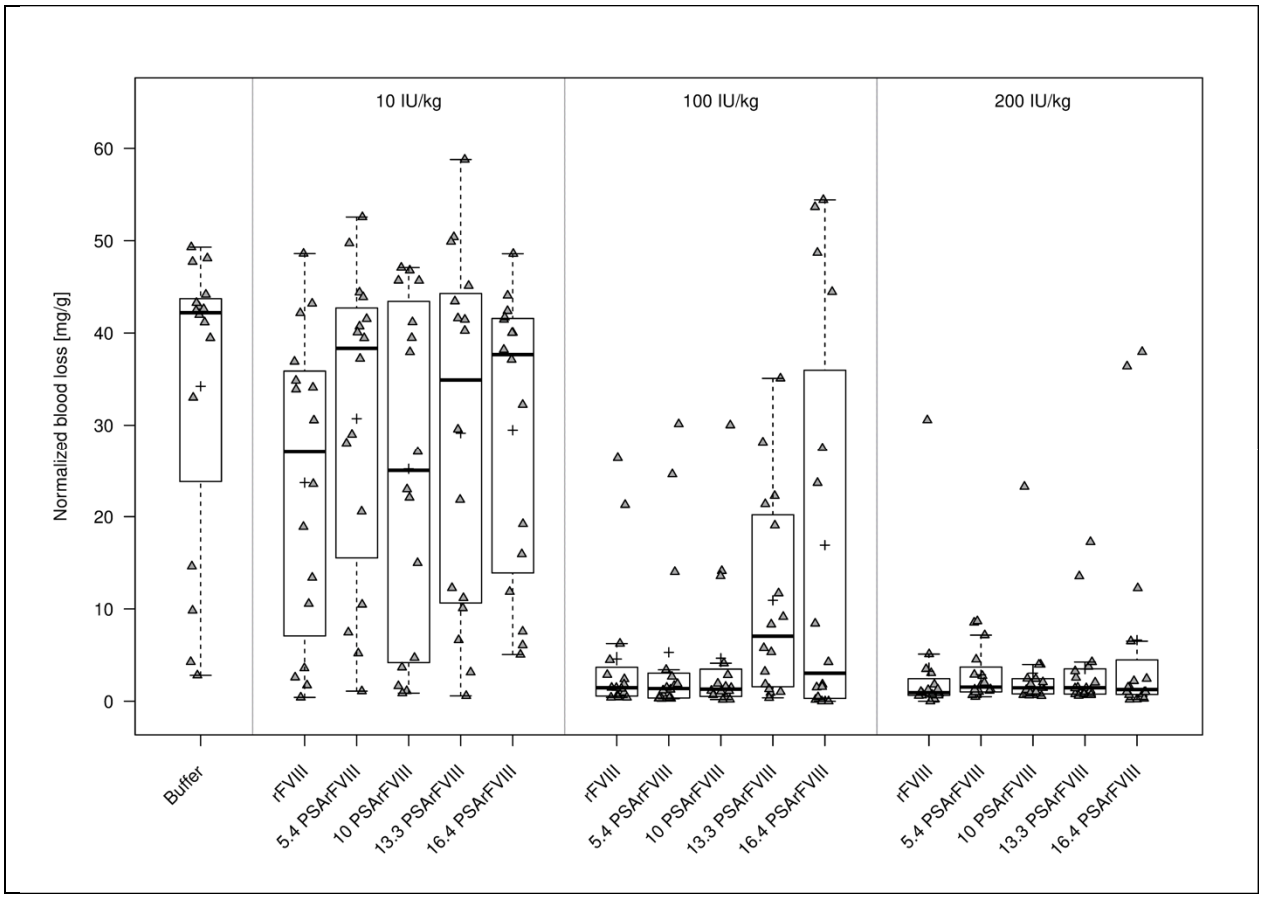

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Figure 5

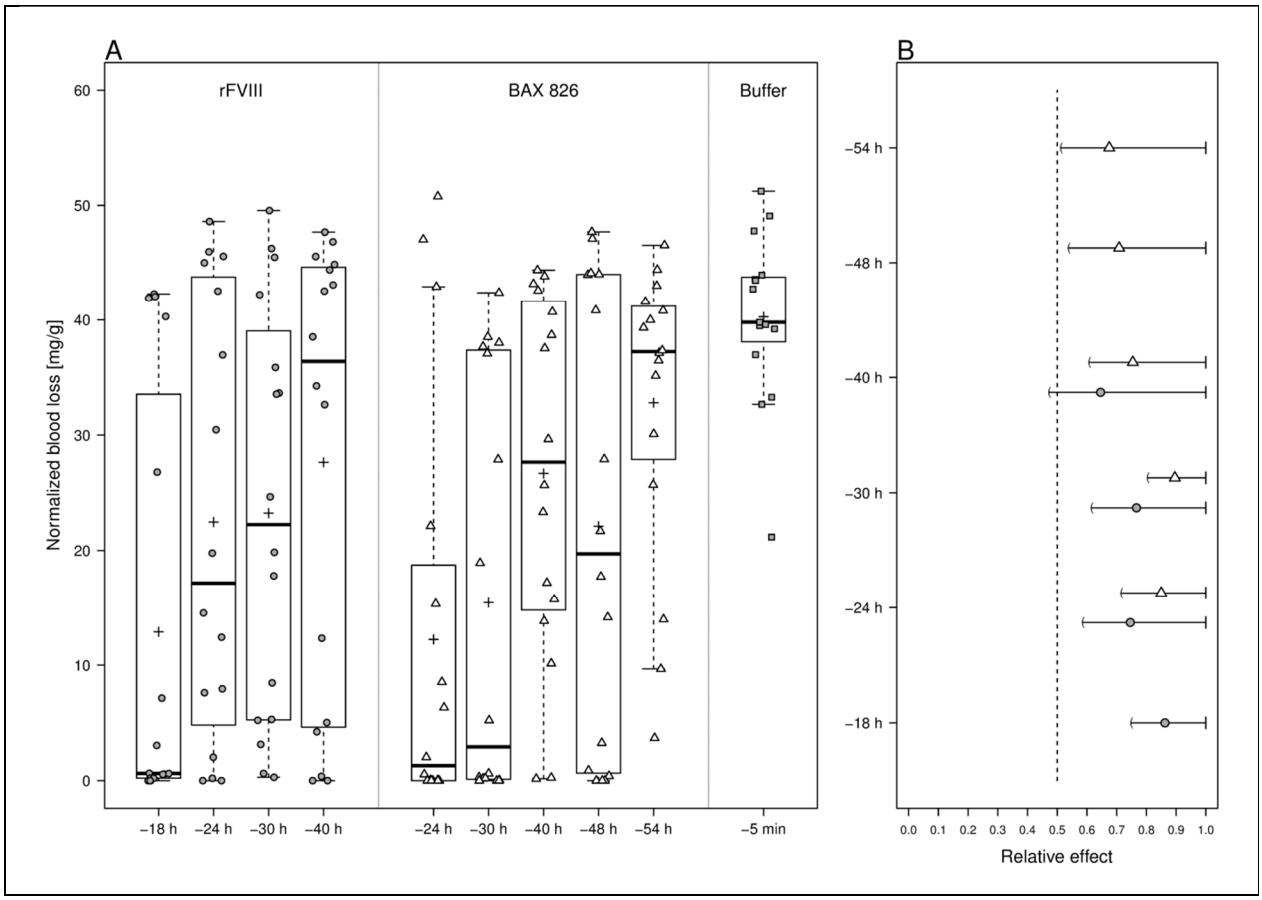

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Figure 6

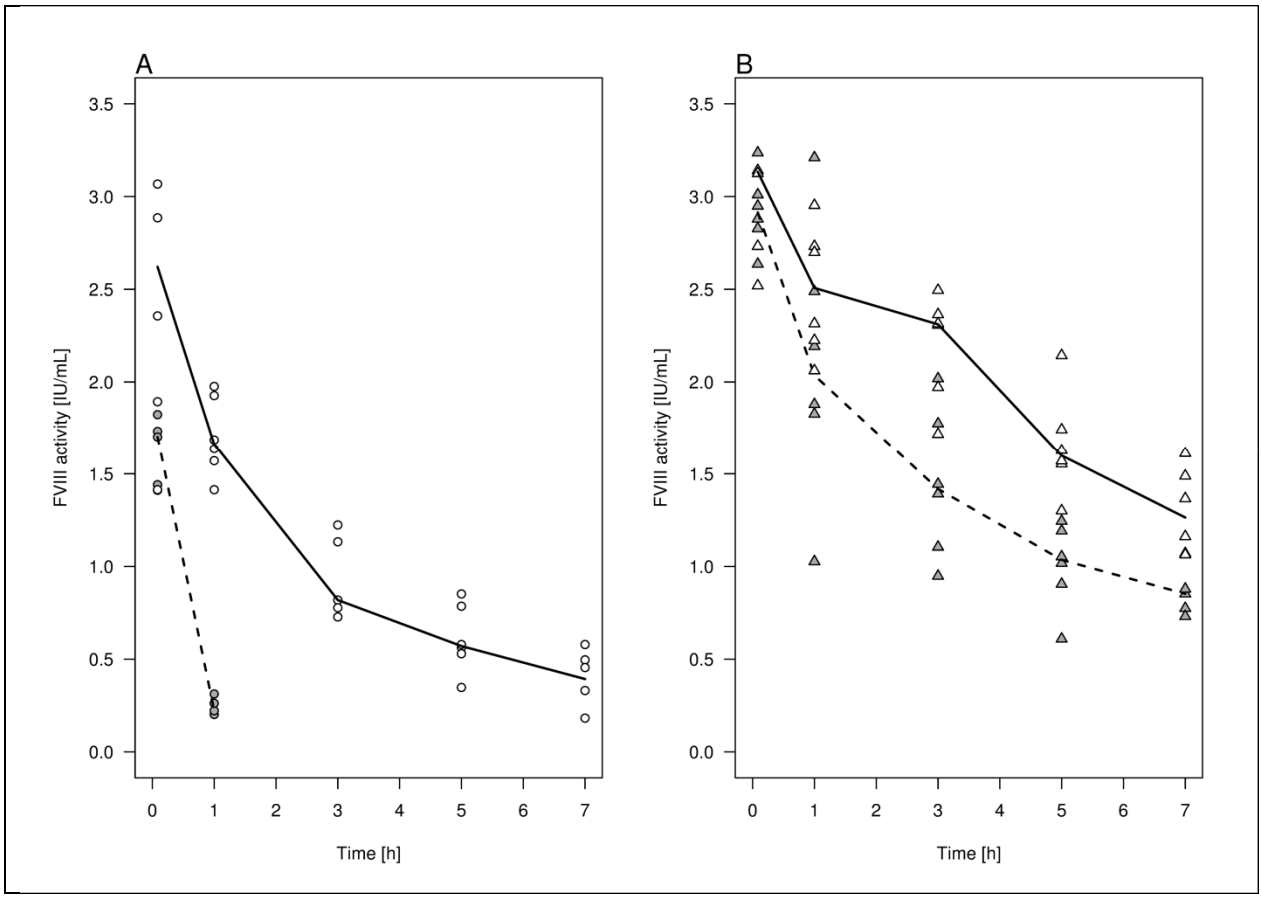

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Figure 7