




# Modulation of Extravascular Binding of Recombinant Factor IX Impacts the Duration of Efficacy in Mouse Models

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Thromb Haemost 2023;123:751–762.

## Abstract

**Background** There is an emerging concept that in addition to circulating coagulation factor IX (FIX), extravascular FIX contributes to hemostasis.

**Objective** Our objective was to evaluate the efficacy of extravascular FIX using animal models of tail clip bleeding and ferric chloride-induced thrombosis.

**Methods** Mutant rFIX proteins with described enhanced (rFIX<sub>K5R</sub>) or reduced (rFIX<sub>K5A</sub>) binding to extracellular matrix were generated and characterized using *in vitro* aPTT, one-stage clotting, and modified FX assays. Using hemophilia B mice, pharmacokinetic (PK) parameters and *in vivo* efficacy of these proteins were compared against rFIX wild-type protein (rFIX<sub>WT</sub>) in a tail clip bleeding and FeCl<sub>3</sub>-induced thrombosis model. Respective tissue disposition of FIX was evaluated using immunofluorescence.

**Results** *In vitro* characterization demonstrated comparable clotting activity of rFIX proteins. The PK profile showed that rFIX<sub>K5A</sub> displayed the highest plasma exposure compared to rFIX<sub>WT</sub> and rFIX<sub>K5R</sub>. Immunofluorescence evaluation of liver tissue showed that rFIX<sub>K5R</sub> was detectable up to 24 hours, whereas rFIX<sub>WT</sub> and rFIX<sub>K5A</sub> were detectable only up to 15 minutes. In the tail clip bleeding model, rFIX<sub>K5R</sub> displayed significant hemostatic protection against bleeding incidence for up to 72 hours postintravenous administration of 50 IU/kg, whereas the efficacy of rFIX<sub>K5A</sub> was already reduced at 24 hours. Similarly, in the mesenteric artery thrombus model, rFIX<sub>K5R</sub> and rFIX<sub>WT</sub> demonstrated prolonged efficacy compared to rFIX<sub>K5A</sub>.

**Conclusion** Using two different *in vivo* models of hemostasis and thrombosis, we demonstrate that mutated rFIX protein with enhanced binding (rFIX<sub>K5R</sub>) to extravascular space confers prolonged hemostatic efficacy *in vivo* despite lower plasma exposure, whereas rFIX<sub>K5A</sub> rapidly lost its efficacy despite higher plasma exposure.

## Keywords

- ▶ Hemophilia B mice
- ▶ Coagulation Factor IX
- ▶ extravascular Factor IX
- ▶ hemostatic efficacy
- ▶ tail clip bleeding model
- ▶ ferric chloride-induced thrombosis

\* Authors contributed equally.

received

July 8, 2022

accepted after revision

January 11, 2023

accepted manuscript online

May 10, 2023

article published online

June 8, 2023

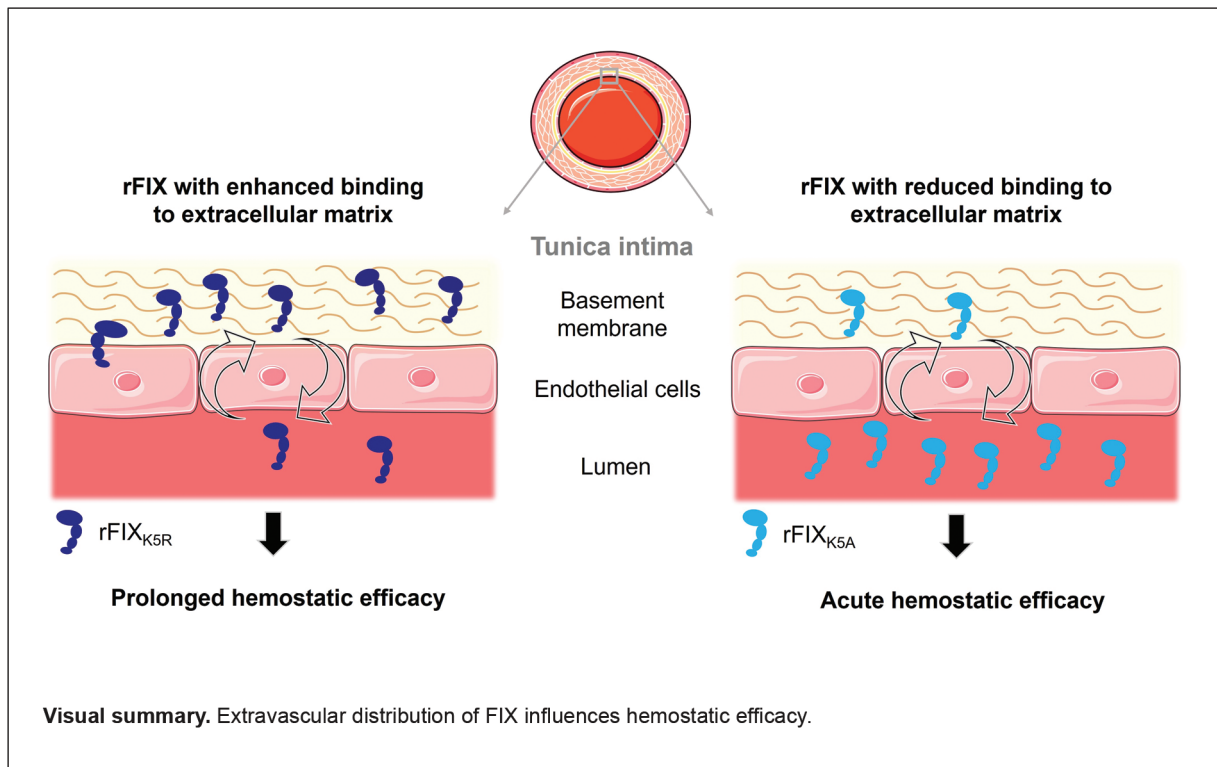
DOI <https://doi.org/10.1055/a-2090-9739>.

ISSN 0340-6245.

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Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany



## Introduction

Hemophilia B (HB) is an X-linked recessive hemorrhagic disorder occurring in about 1 in 25,000 male and is caused by a deficiency or lack of clotting factor IX (FIX). The severity of bleeding in HB is generally correlated with plasma FIX activity.<sup>1</sup> This led to the development of half-life-extended FIX products with reduced frequency of dosing which in turn made prophylactic treatment more feasible and the preferred therapy for individuals with severe HB.<sup>1</sup> Recent studies had indicated that circulating plasma levels of FIX may not be the only contributors to hemostatic efficacy in individuals with HB.<sup>2–4</sup> This is a challenging debate since plasma activity levels of FIX are routinely used in the clinical setting to determine the frequency of dosing in HB individuals,<sup>5</sup> and there are no suitable biomarkers beyond plasma FIX activity levels to determine therapeutic efficacy.

Studies in HB mice showed that hemostatic efficacy could be achieved even in the absence of circulating FIX.<sup>6</sup> These studies implied that the deposition of recombinant human FIX (rFIX) in the extravascular compartment may play a decisive role in hemostasis.<sup>7</sup> The notion of extravascular FIX was first reported in 1983, when direct binding of FIX to endothelial cells was demonstrated<sup>8,9</sup>. Few years later Stern et al showed a rapid, reversible equilibrium between blood and extravascular FIX by injecting bovine FIX into baboons which resulted in increased baboon FIX in the circulation.<sup>10</sup> Later studies demonstrated altered binding of FIX variants to cultured endothelial cells. A single point mutation of lysine to alanine (FIX<sub>K5A</sub>) or arginine (FIX<sub>K5R</sub>) at residue 5 in the Gla domain of the FIX molecule resulted in altered endothelial cell binding affinity.<sup>11</sup> The FIX<sub>K5R</sub> variant was shown to have a

higher binding affinity to endothelial cells than wild-type FIX (FIX<sub>WT</sub>)<sup>12</sup> and the FIX<sub>K5A</sub> variant failed to bind bovine aortic endothelial cells but retained normal clotting activity.<sup>11</sup> Collagen IV was postulated as the binding site for FIX binding on the extracellular matrix of endothelial cells.<sup>12</sup> In line with these findings, studies in HB mice demonstrated prolonged efficacy of FIX in a saphenous vein bleeding model despite <1% of normal plasma FIX concentrations.<sup>7</sup> This finding argues that long-term clotting could be mediated not only by circulating FIX but also by FIX bound to endothelial tissue. In addition, HB mice not expressing any endogenous FIX (CRM mice) yielded a better hemostatic efficacy compared to transgenic mice expressing a dysfunctional human FIX (CRM+ mice) when treated with exogenous rFIX, implying that saturation of extravascular FIX binding sites by dysfunctional FIX reduced hemostatic efficacy.<sup>6</sup> Whether FIX sequestered into other extravascular spaces remains biologically available at the site of clotting remains unknown.

Since the hemostatic efficacy of extravascular FIX was primarily demonstrated in a saphenous vein bleeding model, we aimed to determine if the concept of extravascular FIX can also be demonstrated in alternative models of hemostasis and thrombosis. The Gla domain mutant FIX variants (rFIX<sub>K5A</sub> and rFIX<sub>K5R</sub>) and rFIX<sub>WT</sub> were, therefore, used to demonstrate the prolonged efficacy of extravascular FIX in tail clip bleeding and arterial thrombus models. These proteins were thoroughly characterized in different *in vitro* assays to determine the impact of these mutations on the functionality of FIX protein. In this report, extravascular space refers to noncirculating FIX that can bind outside the plasma, that is, either to the vascular endothelium or sub-endothelial extracellular matrix.

## Materials and Methods

### Expression and Purification of Recombinant Human Factor IX K5 Variants

Human FIX variants were expressed in stable transfected Chinese Hamster Ovary (CHO) cells. The cell culture supernatants were purified by a sequence of chromatographic separation steps including anion exchange chromatography on a Poros50HQ resin (ThermoFisher Scientific, Germany) and size exclusion chromatography on an S200 Resin (Cytiva, Sweden). The rFIX concentration was determined by absorbance at 280 nm based on the theoretical extinction coefficient of 1.33 mg/mL/cm. Recombinant FIX (BenefIX) was used as the wild-type reference.

### Factor IX one Stage aPTT Clotting Assay

The FIX activity level, based on one stage clotting factor assay (FIX:C), of rFIX concentrates or mouse plasma samples from *in vivo* studies was determined with the silica surface activator Pathromtin SL as described before.<sup>13</sup> The test was performed on a Coagulation analyzer (BCS<sup>T</sup> XP System, Siemens Healthcare GmbH, Germany). FIX:C was estimated using a reference curve prepared from standard human plasma (Siemens Healthcare GmbH, Germany), which was calibrated by the manufacturer against the World Health Organization international standard for FIX.

### Kinetics of Activation of Factor IX by FXIa

The enzyme kinetics of FIX activation by FXIa was monitored over time using 8 to 16% Tris-glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Thermo Fisher Scientific, Invitrogen, Germany). FIX was activated by FXIa in a reaction mixture containing 2  $\mu$ M rFIX and 0.002  $\mu$ M FXIa for up to 360 minutes. Samples collected at different intervals were loaded on a nonreducing SDS PAGE gel. The time point 0 indicates the inactivated rFIX. The intensity of the bands was detected by ChemiDoc MP Image System (Bio-Rad Laboratories Inc., United States) and quantified using Image Lab (Version 6.0 Software, Bio-Rad).

### Activation of Factor X by rFIXa

The rate of activation of FX by FIXa was determined using a commercially available Biophen Kit (CoaChrom Diagnostica GmbH, Germany). We used a modified format of this intrinsic Xase assay to compare the enzyme kinetic activity of rFIX K5 variants and the rFIX<sub>WT</sub> molecule. Dilution series of either rFIX, rFVIII, or FX were prepared in Tris-BSA buffer. The assay was carried out according to the manufacturer's instructions, with the following modifications: R1 reagent was prepared freshly before each experiment in serial dilutions of either FIX, FVIII, or FX and the other components were kept at constant concentrations. Rate constants based on Michaelis-Menten kinetics (Vmax and Km) were estimated from three independent experiments. Assays were performed with rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, and rFIX<sub>K5R</sub> simultaneously under identical conditions.

### Binding of Recombinant Human Factor IX to Collagen IV

Binding of rFIX molecules to collagen IV was measured by surface plasmon resonance (SPR), using Biacore T-200 (Cytiva Europe GmbH, Germany) and HBS-N (10 mM HEPES, 150 mM NaCl, pH 7.4) as a sample and running buffer. The analysis was performed at 25°C. Human-type IV placental collagen (Sigma, Germany) was immobilized on an active flow cell of a sensor series CM5 chip using an amine coupling chemistry kit (Cytiva) to approximately 3,300 RU. Reference flow cell was treated as blank immobilization. The immobilized collagen IV was washed four times with 10 mM HCl and once with HBS-N before injection of rFIX. Each analyte (human rFIX or human fibronectin) was injected for 180 seconds over reference and active flow cells followed by 180 seconds of dissociation at 30  $\mu$ L/min. Each molecule was analyzed four times. Data were analyzed with Biacore T200 Evaluation Software 3.1.

### Enzyme-Linked Immunosorbent Assay to Determine Factor IX Protein in Plasma Samples

A paired antibody ELISA kit (Cedarlane Labs, Canada) was used to determine FIX antigen concentrations in plasma (FIX:Ag) from HB mice. The plasma was prediluted to at least 1:50 with 0.05 M Tris-buffered sodium chloride containing 0.05% TWEEN20. The FIX protein used in respective *in vivo* studies (injection solution) was used as standard. The lower limit of quantification (LLOQ), linearity, recovery, and matrix effects were determined prior to the analysis of samples.

### Immunofluorescence Staining

Tyramide signal amplification staining was performed in 5  $\mu$ M thick sections from formalin-fixed-paraffin embedded liver tissue using an automated staining system (Ventana Discovery XT, Roche Diagnostics GmbH, Germany). Briefly, tissue sections were incubated with 1  $\mu$ g/mL rabbit anti-human FIX antibody (Abcam, UK) at room temperature for 1 hour followed by incubation with OmniMap goat antirabbit HRP (Roche Diagnostics GmbH, Germany). Sections were subsequently incubated with rhodamine and H<sub>2</sub>O<sub>2</sub> and mounted with DAPI mounting medium for further imaging analysis. Whole section images were acquired with the Zeiss Axio Scan.Z1 (Carl Zeiss Microscopy GmbH, Germany). Image analysis was performed using the ZEN 2.3 lite software. Positively stained areas from at least 20 different viewing fields of each liver cross-section were used for quantification. Liver sections from saline-treated HB mice stained simultaneously under identical conditions served as the negative control. The fluorescence immunostaining is reported as the mean fluorescence intensity (MFI) after correction for background fluorescence signal obtained from negative control sections.

### Experimental Animals

All animals were handled and managed in accordance with animal care protection laws. All studies were approved by the internal animal welfare committee and the governmental animal ethics board (Regional Council of Giessen). HB

mice (B6.129P2-F9tm1Dws/J) were obtained from Charles River Breeding Laboratories, Germany. Mice had access to food pellets and tap water ad libitum and were maintained on a 12 hours light-dark cycle. If possible, 50% females and 50% males were allocated to the experimental group.

### Pharmacokinetics and Liver Sampling in Hemophilia B Mice

FIX proteins (rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, rFIX<sub>K5R</sub>) were administered intravenously at doses of 25 nmol/kg (243–394 IU FIX:C/kg) in HB mice ( $n = 3–5$  per time point). Blood samples were collected from the retro-bulbar sinus at several time points (5 minutes, 2 hours, 6 hours, 24 hours, 48 hours, 72 hours, 5 days, 6 days, 7 days, 10 days, and 14 days) assigned to six different cohorts to restrict blood collection at a maximum of two time points per mouse. At terminal time points (5 minutes, 24 hours, 72 hours, 5 days, and 14 days), blood was sampled via vena cava under deep anesthesia (ketamin 65 mg/kg, xylazin 13 mg/kg, and acepromazin 2 mg/kg i.p.). Blood mixed with sodium citrate was centrifuged at 3,130 g for 10 minutes at room temperature to obtain plasma, and the samples were stored at  $-70^{\circ}\text{C}$  until analysis of FIX antigen and activity levels. Animals were perfused with phosphate buffered saline (PBS) via the heart, and the liver tissues were harvested and stored in 4% paraformaldehyde for evaluation of tissue-bound FIX using immunofluorescence.

### Pharmacokinetic Analysis

A two-compartment model was chosen to model the mean values of dose-normalized plasma FIX activity for all groups. Values below the LLOQ were excluded from pharmacokinetics (PK) analysis. The primary parameters ( $A$ ,  $B$ ,  $\alpha$ , and  $\beta$ ) were computed by weighted least squares estimation, using the respective standard deviations as weights. The terminal elimination rate  $\lambda$  thereby corresponds to the smaller constant of  $\alpha$  and  $\beta$ . The maximum predicted dose-normalized plasma level at time 0 was defined as  $dnC_{max}$ . The area under the plasma concentration curve from zero to the last measured data point ( $AUC_{0-last}$ ) or 24 hours timepoint ( $AUC_{0-24h}$ ) was calculated by numerically integrating the two-compartmental model. Dose-normalized  $AUC_{0-last}$  and  $AUC_{0-24h}$  were defined as  $dnAUC_{0-last}$  and  $dnAUC_{0-24h}$ , respectively. For PK parameters, the statistical analysis was done with the software package Matlab R2020b. The procedure *fmincon* was used for model optimization. Standard deviations for PK parameters were derived by drawing 1,000 samples from the normal distribution for each time point, using the data mean and standard deviation at that time point, and fitting a two-compartmental model to each of those new time courses.

### Tail Clip Bleeding Model

Efficacy of rFIX molecules was evaluated in the tail clip bleeding model following 15 minutes, 24 hours, 72 hours, 7 days, or 14 days postsingle intravenous administration of FIX:C 50 IU/kg in  $10 \pm 2$  weeks old HB mice ( $n = 10$  per group; five females and five males). Blood from the amputated tail (3 mm from the tip) of anesthetized animals was collected

into tubes containing equal volumes (12 mL) of warm isotonic saline ( $37^{\circ}\text{C}$ ). The time of cessation of blood flow over 30 minutes was recorded as bleeding incidence. A quantitative estimate of blood loss was determined by measuring the hemoglobin content of the collected blood using a Hemoglobin Assay Kit (Sigma-Aldrich Chemie GmbH, Germany). The hemoglobin blood concentration in individual animals was determined at the end of the experiment for the calculation of the blood volume. Plasma levels of rFIX antigen and activity were determined in the terminal blood samples.

### Intravital Microscopic Mesentery Arteriole Thrombosis

FIX:C 250 IU/kg was administered to 3-week old male HB mice ( $n = 8$  per group). A rat antimouse platelet antibody (1  $\mu\text{g/g}$  body weight) was injected via the lateral tail vein 24 hours post-rFIX administration. The mesentery was exteriorized gently through a midline abdominal incision, under deep anesthesia. Arterioles (35- to 65- $\mu\text{m}$  diameter) were visualized with a Zeiss Axiovert.Z1 inverted microscope equipped with MRm, a monochrome camera (Carl Zeiss Microscopy GmbH, Germany). The resting blood vessel was recorded for a few seconds, and 18%  $\text{FeCl}_3$  was topically applied on mesenteric arterioles using Whatman filter paper. Images were acquired every second and the thrombus formation of fluorescently labeled platelets was monitored for 40 minutes or until complete occlusion of the vessel. Time to complete occlusion of the vessel, the beginning of first thrombus formation and the size of thrombus at the end of the observation period was recorded.

### Statistical Analysis

All data were plotted and analyzed using GraphPad Prism Version 8 (GraphPad Software, CA, United States) unless stated otherwise. A two-sided  $p$ -value  $< 0.05$  was considered statistically significant.

## Results

### Specific Activity of Recombinant Human Factor IX Proteins

The specific activity of the recombinant FIX proteins (rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, rFIX<sub>K5R</sub>), measured using one-stage clotting assay, is summarized in **Table 1**. The specific activities of the stock solutions were calculated from the measured FIX:C in IU/mL and the protein concentration in mg/mL. The specific

**Table 1** Specific activity of recombinant FIX proteins measured using one stage clotting assay

rFIX proteins	Calculated specific activity (IU/mg)	Time for clot formation (in seconds)
rFIX <sub>WT</sub>	208 $\pm$ 25	38 $\pm$ 1.4
rFIX <sub>K5A</sub>	178 $\pm$ 26	36 $\pm$ 0.1
rFIX <sub>K5R</sub>	238 $\pm$ 37	38 $\pm$ 0.4

Note: Data points are represented as mean  $\pm$  standard deviation.

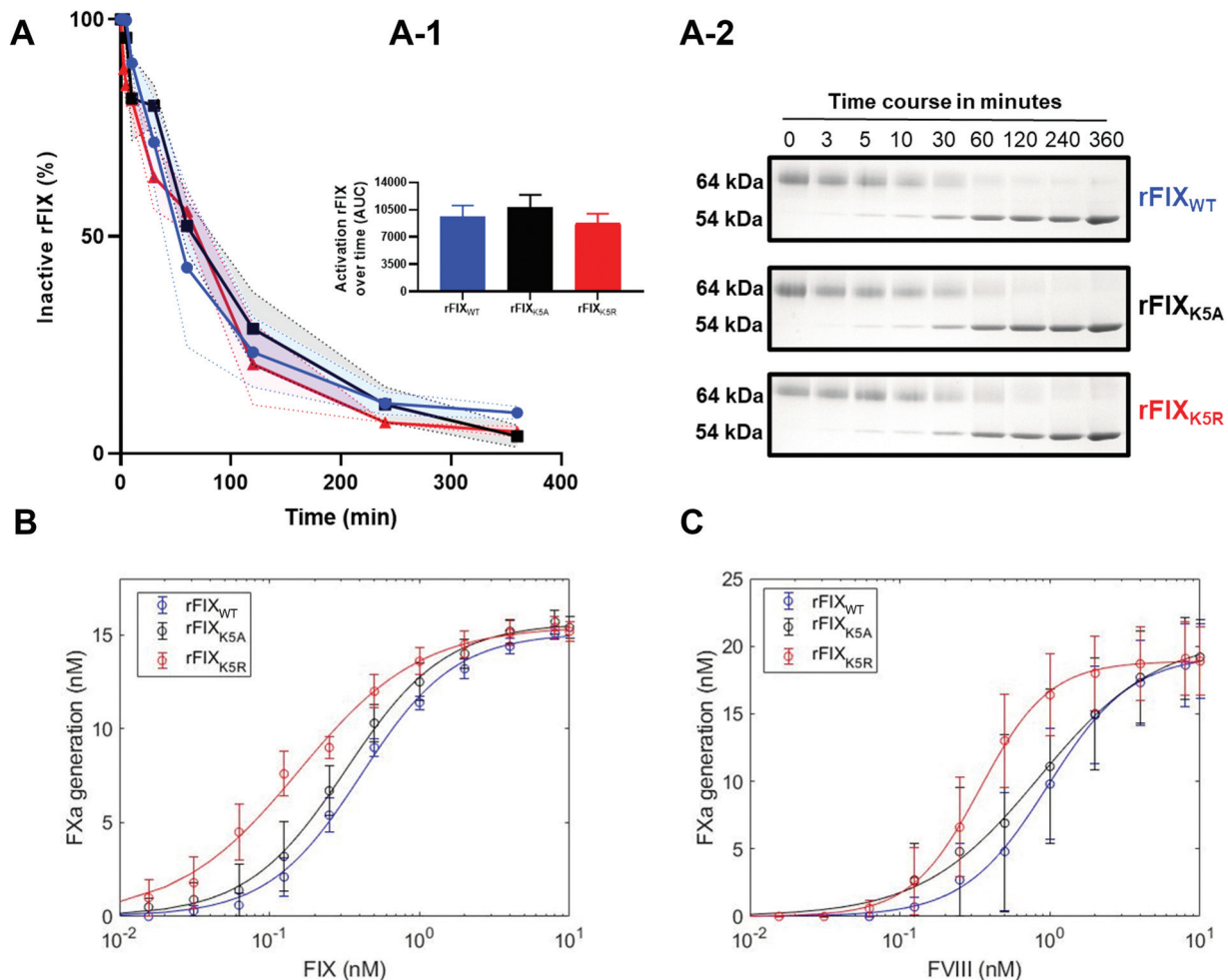
activities of the rFIX proteins were also confirmed experimentally by their ability to restore fibrin clot formation in FIX-deficient plasma (aPTT). The rFIX proteins were spiked at equimolar concentrations in FIX-deficient human plasma and all three rFIX proteins decreased the time for clot formation substantially from  $81 \pm 4$  to  $37 \pm 1$  seconds ( $\blacktriangleright$ Table 1).

### Recombinant Human Factor IX Activation and Simulation of the Tenase Complex

The kinetics of activation of rFIX<sub>K5A</sub>, rFIX<sub>K5R</sub>, and rFIX<sub>WT</sub> proteins by FXIa were similar and are shown in  $\blacktriangleright$ Fig. 1A. The area under the curve (AUC) values from  $\blacktriangleright$ Fig. 1A are plotted in  $\blacktriangleright$ Fig. 1A-1 and a representative SDS-PAGE picture for each protein is shown in  $\blacktriangleright$ Fig. 1A-2. These data indicate that the substitution of the amino acid at position 5 from lysine to arginine (rFIX<sub>K5R</sub>) or from lysine to alanine (rFIX<sub>K5A</sub>) does not lead to major differences in the activation by FXIa. We then compared the proteolytic activity of activated rFIX<sub>WT</sub>,

rFIX<sub>K5A</sub>, and rFIX<sub>K5R</sub> proteins to its physiological substrate FX. The rate of FXa generation was determined using a chromogenic method for the *in vitro* quantitative determination of FIX activity. A standard curve of known FXa concentrations and FXa substrate Sx-11 (Biophen) was used to determine the concentration of FXa from the intrinsic Xase enzyme kinetic assay ( $\blacktriangleright$ Supplementary Figure S1, available in the online version).

The enzyme kinetics of FX activation were investigated in the presence of constant amounts of FVIII and increasing concentrations of rFIX ranging from 0 to 10 nM. The velocity of the rFIXa-FVIIIa-FX reaction increases and reaches saturation at approximately 15 nM ( $\blacktriangleright$ Fig. 1B). Under the mentioned conditions, rFIX<sub>K5R</sub> was slightly more efficient ( $K_m$ ) in activating FX at lower concentrations and displayed a approximately twofold lower  $K_m$  compared to rFIX<sub>WT</sub> and rFIX<sub>K5A</sub> proteins as depicted in  $\blacktriangleright$ Table 2. In the presence of increasing concentrations of cofactor rFVIII ( $\blacktriangleright$ Fig. 1C), saturation was reached at approximately 19 nM in all rFIX



**Fig. 1** Activation and activity of rFIX<sub>WT</sub> (blue), rFIX<sub>K5A</sub> (black), and rFIX<sub>K5R</sub> (red). (A) The rate of activation of rFIX was determined by SDS PAGE and the percentage of noncleaved, inactive rFIX was determined over time in relation to the base value (100%) at timepoint 0. (A-1) The area under the curve (AUC) from the graph A was plotted as bar graphs and (A-2) a representative image of a nonreducing SDS-PAGE gel depicts the cleavage of zymogen rFIX (ca. 64 kDa) into activated rFIX with light and heavy chain held together by disulfide bonds (ca. 54 kDa). Each timepoint (0, 3, 5, 10, 30, 60, 90, 120, 180, and 360 minutes) was loaded in lanes 1 to 10. (B) The fitted curves of FXa generation at increasing FIX concentration or (C) at increasing rFVIII concentrations, while maintaining all other factors constant. The data were fit to the Michaelis–Menten model using R software. Each data point represents the mean value of three independent experiments, and the error bars show mean  $\pm$  standard error of mean.

**Table 2** In vitro enzyme kinetics of activation of FX in the tenase complex

Enzyme	Increasing concentration of rFIX			Increasing concentration of rFVIII		
	$V_{max}$ ( $\text{min}^{-1}$ )	$K_m$ (nM)	Hill coefficient	$V_{max}$ ( $\text{min}^{-1}$ )	$K_m$ (nM)	Hill coefficient
rFIX <sub>WT</sub>	15.2 ± 0.51	0.30 ± 0.02	1.35 ± 0.16	18.7 ± 2.93	1.51 ± 0.62	2.13 ± 0.28
rFIX <sub>K5A</sub>	15.9 ± 0.82	0.26 ± 0.06	1.22 ± 0.16	19.2 ± 2.89	1.70 ± 0.92	2.10 ± 0.15
rFIX <sub>K5R</sub>	15.6 ± 0.76	0.15 ± 0.02	1.05 ± 0.13	18.7 ± 2.60	0.13 ± 0.06	2.23 ± 0.23

Note: Data points are represented as mean ± standard error of mean.

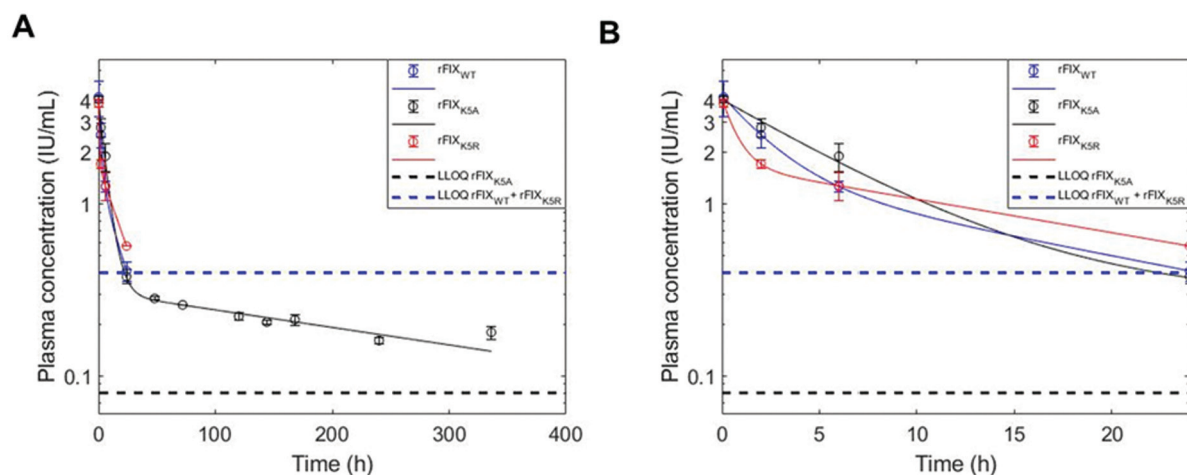
proteins. Similar to the observation with increasing concentrations of rFIX, rFIX<sub>K5R</sub> was more efficient in activating FX at lower concentrations ( $K_m$ ) of rFVIII compared to rFIX<sub>WT</sub> and rFIX<sub>K5A</sub> (►Table 2). All rFIX proteins exhibit hill coefficients greater than 1 (►Table 2), indicating positive cooperativity in which binding of one protein facilitates binding of subsequent proteins at other sites.

**Pharmacokinetics Profile and Tissue Levels of Recombinant Human Factor IX in Hemophilia B Mice**  
HB mice were injected with equimolar doses (25 nmol/kg) of rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, and rFIX<sub>K5R</sub> via the lateral tail vein. Plasma levels of rFIX activity were used for PK evaluation and liver samples were used for FIX immunostaining. The plasma concentrations of individual rFIX proteins are shown in ►Fig. 2A and B. At the early time points, that is, up to 24 hours postadministration only rFIX<sub>K5A</sub> had a linear elimination phase, while rFIX<sub>WT</sub> and rFIX<sub>K5R</sub> were cleared faster at initial time points suggesting a faster distribution into the extravascular compartment (►Fig. 2B). The total exposure was evidently higher for rFIX<sub>K5A</sub> which exhibited a  $\text{dnAUC}_{0-\text{last}}$  of  $0.442 \pm 0.025 \text{ IU/mL}^* \text{h/IU}$ , followed by lower and comparable  $\text{AUC}_{0-\text{last}}$  of rFIX<sub>WT</sub> ( $0.069 \pm 0.023 \text{ IU/mL}^* \text{h/IU}$ ) and rFIX<sub>K5R</sub> ( $0.056 \pm 0.031 \text{ IU/mL}^* \text{h/IU}$ , ►Table 3). rFIX<sub>WT</sub> and rFIX<sub>K5R</sub> had an LLOQ of 0.4 IU/mL limiting their detection only until 24 hours, whereas rFIX<sub>K5A</sub> had an LLOQ of 0.1 IU/mL and was detectable until 336 hours. Given the differ-

ences in LLOQ of the proteins, the  $\text{dnAUC}_{0-24\text{h}}$  was evaluated for better comparison of the proteins. As shown in ►Table 3, rFIX<sub>K5A</sub> had nearly twofold higher  $\text{dnAUC}_{0-24\text{h}}$  ( $0.112 \pm 0.007 \text{ IU/mL}^* \text{h/IU}$ ) compared to rFIX<sub>K5R</sub> ( $0.056 \pm 0.031 \text{ IU/mL}^* \text{h/IU}$ ) and rFIX<sub>WT</sub> ( $0.069 \pm 0.023 \text{ IU/mL}^* \text{h/IU}$ ). The predicted dose normalized maximum concentrations ( $\text{dnC}_{\text{max}}$ ) were comparable between rFIX<sub>WT</sub> ( $0.013 \pm 0.003 \text{ IU/mL/IU}$ ), rFIX<sub>K5R</sub> ( $0.013 \pm 0.001 \text{ IU/mL/IU}$ ), and slightly higher for rFIX<sub>K5A</sub> ( $0.018 \pm 0.001 \text{ IU/mL/IU}$ ). The sampled livers were processed, and the quantification of the FIX immunostaining in at least six independent sections is shown in ►Fig. 3A. At 15 minutes following intravenous injection, all three rFIX proteins were detected in the liver with similar MFI. However, at 24 hours following intravenous injection, only rFIX<sub>K5R</sub> was present in sufficient amounts to detect a strong and robust signal, while faint signals were detected in the rFIX<sub>K5A</sub> and rFIX<sub>WT</sub> groups. The representative image in ►Fig. 3B shows the distribution of FIX in the liver. ►Fig. 3C shows a higher magnification of FIX staining in the liver at different time points for all three rFIX proteins.

### Colocalization and Binding of Recombinant Human Factor IX and Collagen IV

Immunofluorescence colocalization staining of rFIX and collagen IV in the liver of HB mice treated with rFIX exhibited a high degree of spatial overlap because of the similar distribution of FIX and collagen IV, a ubiquitously expressed



**Fig. 2** HB mice were injected with equimolar doses (25 nmol/kg) of rFIX<sub>WT</sub> (blue), rFIX<sub>K5A</sub> (black), and rFIX<sub>K5R</sub> (red) via the lateral tail vein. Pharmacokinetic profiles from two-compartmental modeling of rFIX<sub>WT</sub> with a lower limit of quantification (LLOQ) at 0.4 IU/mL, rFIX<sub>K5A</sub> with a LLOQ at 0.1 IU/mL, and rFIX<sub>K5R</sub> with LLOQ at 0.4 IU/mL were determined by measuring FIX activity levels at various time points. (A) Overlay of curves from rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, and rFIX<sub>K5R</sub> plasma levels up to 336 hours after injection. (B) Zoom-in of curves up to 24 hours after injection.

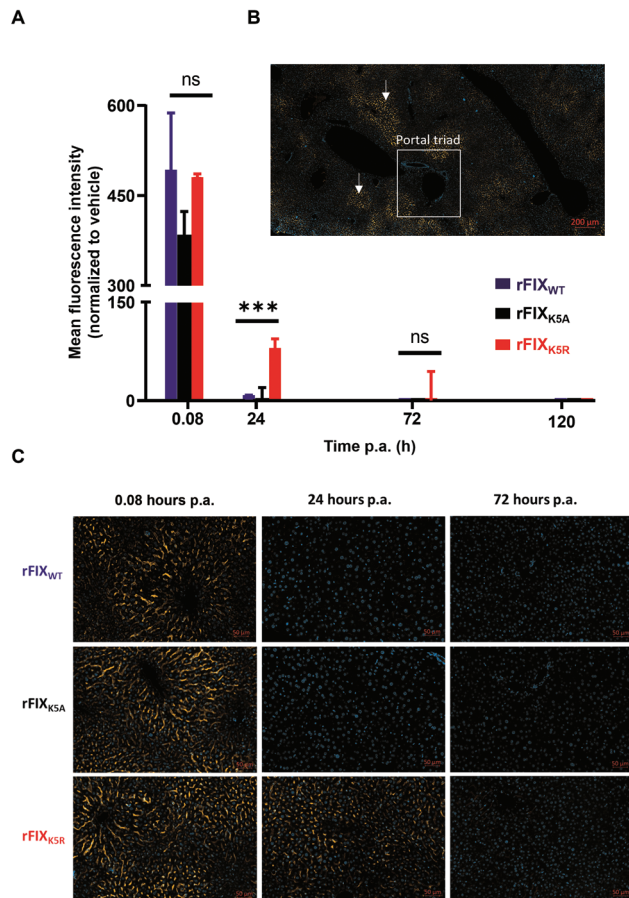
**Table 3** Pharmacokinetic profile of rFIX proteins

Groups (IU/kg)	Activity dose <sup>a</sup> (IU/kg)	dnAUC <sub>0-last</sub> (IU/mL <sup>*</sup> h/IU)	dnAUC <sub>0-24h</sub> (IU/mL <sup>*</sup> h/IU)	dnC <sub>max</sub> (IU/mL/IU)
rFIX <sub>WT</sub>	338	0.069 ± 0.023	0.069 ± 0.023	0.013 ± 0.003
rFIX <sub>K5A</sub>	233	0.442 ± 0.025	0.112 ± 0.007	0.018 ± 0.001
rFIX <sub>K5R</sub>	313	0.056 ± 0.031	0.056 ± 0.031	0.013 ± 0.001

Note: HB mice were injected with equimolar doses (25 nmol/kg) of rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, and rFIX<sub>K5R</sub> via the lateral tail vein. Doses were normalized (dn) based on FIX activity. Pharmacokinetic parameters of rFIX proteins were determined using two-compartmental modeling.

Note: Data points are represented as mean ± standard deviation.

<sup>a</sup>Activity dose was calculated based on the activity measured in injection solution.



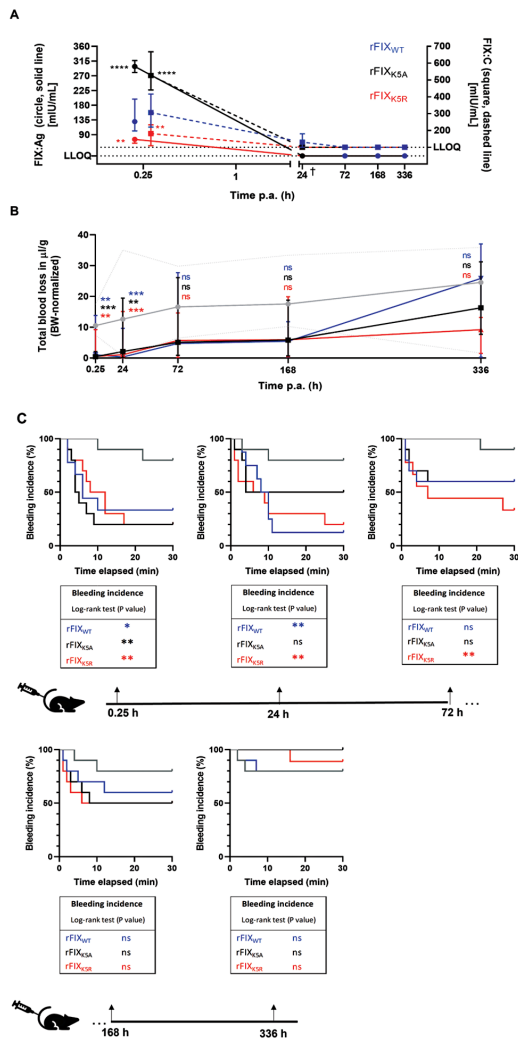
**Fig. 3** Immunofluorescence staining of liver sections following intravenous administration of rFIX (25 nmol/kg) in HB mice that were previously perfused to remove blood from the circulation. (A) Quantitative analysis of FIX positive liver sections collected at 0.08 hours (15 minutes), 24 hours, 72 hours, and 120 hours after treatment with rFIX. Each bar represents the median ± 95% CI. The blue, black, and red bars represent rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, rFIX<sub>K5R</sub>, respectively. All three proteins were compared at each time point using 1-way ANOVA test to determine the *p*-value, (ns) not significant, (\*\*\*) *p* < 0.0001 and p.a., postadministration. *n* = 8 to 10 animals per group. (B) Representative image (scale bar: 200 μm) from a liver section stained for DAPI (blue) and FIX (yellow) highlighting the portal triad and FIX positive regions in the pericentral zone (arrows). (C) Higher magnification images (scale bar: 50 μm) of FIX-stained livers used for the quantification depicted in the graph 3A.

extracellular matrix protein. Interestingly, some areas surrounding hepatic vascular structures were restricted to collagen IV, but no FIX signal was detected at the same

location, as highlighted by the white arrows in **►Supplementary Figure S2A** (available in the online version). The respective controls demonstrating the specificity of primary and secondary antibodies are shown in **►Supplementary Figure S2B** (available in the online version). The specificity of the primary antibody against Collagen IV, according to the manufacturer, exhibits <10% cross-reactivity with collagen type I, II, III, V, and VI. The staining pattern of Collagen IV was in agreement with the expected target distribution. In SPR, no direct binding of rFIX proteins to human collagen IV could be detected (**►Supplementary Figure S3**, available in the online version), which is in conflict with previously published results from Cheung et al in 1996 based on a radiolabeled competition assay with cultured endothelial cell matrix.<sup>12</sup> Although no binding of rFIX proteins to collagen IV could be detected in SPR, a clear binding of the positive control, fibronectin, was observed.

### Efficacy of Recombinant Human Factor IX in Tail Clip Bleeding Model

As shown in **►Fig. 4A**, rFIX antigen and activity were detectable only in samples collected after the observation period from animals examined at 15 minutes posttreatment. rFIX protein was either very low or not detectable in circulation after 24 hours, as expected for standard half-life rFIX proteins. Hence, hemostatic efficacy observed at 24 hours and beyond is potentially attributed to extravascular FIX. Consistent with our previous observation in the PK profile of the molecules, the plasma concentrations of rFIX<sub>K5A</sub> at 15 minutes time point were significantly higher than rFIX<sub>WT</sub>, while rFIX<sub>K5R</sub> was significantly lower than rFIX<sub>WT</sub> protein. Treatment of HB mice with rFIX proteins protected mice from bleeding at 15 minutes postadministration (blood loss in vehicle group: approximately 10 μL/g of body weight vs. rFIX-treated groups: approximately 1 μL/g of body weight). As depicted in **►Fig. 4B**, all three rFIX-treated groups had significantly lower blood loss until 24 hours compared to the vehicle group. Though not statistically significant beyond 24 hours, reduced blood loss was observed until 7 days (168 hours) in all rFIX-treated groups compared to the vehicle group. However, at 14 days (336 hours) only the rFIX<sub>K5R</sub> group demonstrated 50% lower blood loss compared to the vehicle group (vehicle group: approximately 18 μL/g of bodyweight vs. rFIX<sub>K5R</sub> approximately 9 μL/g of body weight). For statistical analysis, the set of rFIX molecules (rFIX<sub>WT</sub>,



**Fig. 4** Comparison of the hemostatic efficacy at different timepoints postadministration (p.a.) of rFIX proteins (FIX:C 50 IU/kg) in a tail clip bleeding model. rFIX<sub>WT</sub> is depicted in blue, rFIX<sub>K5A</sub> in black, rFIX<sub>K5R</sub> in red, and animals injected with saline were referred to as the control vehicle group (*shaded in gray*). Each data point represents the median with 95% confidence intervals represented by the error bars of  $n = 8$  to 10 animals (five females and five males) per group. (A) FIX antigen levels (solid lines), and activity levels (*dashed lines*) were measured from blood samples collected at the end of each experiment. Antigen levels were not measured in the rFIX<sub>WT</sub> group at 24 hours (†). The statistical significance of the treatment groups with the variants (*black and red*) were compared to the rFIX<sub>WT</sub> group (*blue*) at 0.25 hours, using a 1-way ANOVA-test followed by Dunnett's post hoc test, and were reported as the adjusted  $p$ -values, (\*\*\*)  $p < 0.01$  (\*\*\*\*)  $p < 0.0001$ , for FIX:Ag and FIX:C levels. The results for vehicle group were below the assay's lower limit of quantification (LLOQ) at each time point. (B) The total blood loss ( $\mu\text{L}$ ) normalized by body weight (BW) in gram is depicted in a curve over time. A data-driven cutoff was calculated based on historical data from 695 vehicle animals (*shaded in light gray*). The statistical comparison of the treatment groups to the vehicle group was done using a 1-way ANOVA-test followed by Dunnett's post hoc test, and results of the statistical analyses were reported as adjusted  $p$ -values. (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.001$ , (\*\*\*\*)  $p < 0.0001$ , and (ns) not significant against the vehicle group. (C) Bleeding time observed over 30 minutes is plotted as a Kaplan–Meier curve for each treatment group. The statistical comparison of the rFIX treatment groups to the vehicle group was performed using a Log-rank (Mantel–Cox) test and the  $p$ -values were summarized in the table for each timepoint. ANOVA, analysis of variance.

rFIX<sub>K5A</sub>, and rFIX<sub>K5R</sub>) was compared to the vehicle group at each time point as shown in ► **Supplementary Fig. S4** (available in the online version). These results clearly show that rFIX<sub>K5R</sub> has prolonged hemostatic efficacy even when no FIX levels were detectable in plasma after 24 hours. The time of cessation of blood flow over 30 minutes is plotted in a Kaplan–Meier curve as the incidence of bleeding for each time point of analysis. As expected, all three rFIX proteins significantly reduced the bleeding time at 15 minutes post-administration (► **Fig. 4C**). Interestingly, rFIX<sub>K5A</sub> had increased bleeding incidence after 24 hours (► **Fig. 4C**). At 72 hours postadministration, only rFIX<sub>K5R</sub>-treated groups had significantly reduced bleeding incidence compared to the vehicle group (► **Fig. 4C**). In general, our results demonstrate that rFIX<sub>K5R</sub> conveys the longest protection against bleeding independently of plasma exposure. While rFIX<sub>K5A</sub> is highly efficacious at 15 minutes postadministration, the *in vivo* efficacy decreases rapidly following reduced plasma levels.

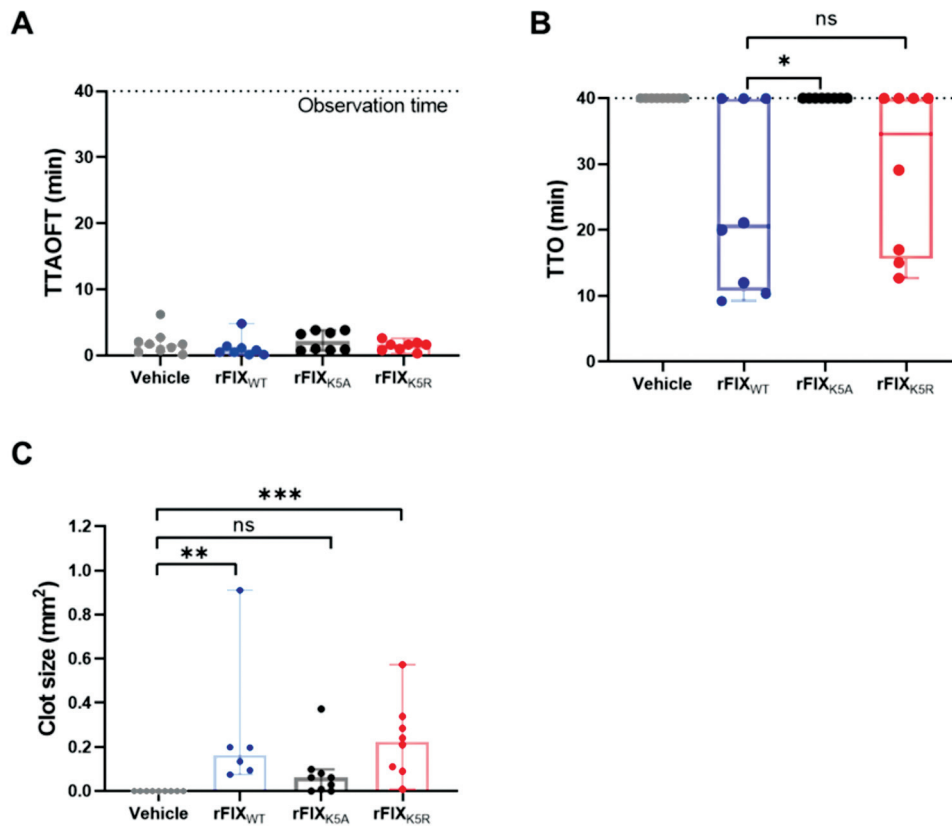
### Efficacy of Recombinant Human Factor IX in Mesenteric Thrombosis Model

The clot formation was investigated *in vivo* in an arterial thrombosis model in HB mice using FeCl<sub>3</sub> as shown in representative videos (► **Supplementary Videos 1–4**, available in the online version). The model was established using C57BL/6J and FXI ko mice as positive and negative controls, respectively (► **Supplementary Figure S5A**, available in the online version). Based on our dose-response experiment with rFIX<sub>WT</sub>, the dose of 250 IU/kg was selected for further experiments (► **Supplementary Figure S5B**, available in the online version). Thrombosis was induced in HB mice after 24 hours of treatment with PBS (vehicle control), rFIX<sub>WT</sub>, rFIX<sub>K5A</sub>, or rFIX<sub>K5R</sub>. The time of appearance of the first thrombi did not differ between groups (► **Fig. 5A**). The time to occlusion was significantly different between rFIX<sub>WT</sub> and rFIX<sub>K5A</sub> groups (► **Fig. 5B**). While some animals in the rFIX<sub>WT</sub>- and rFIX<sub>K5R</sub>-treated groups had fully occluded blood vessels, none of the vehicle and rFIX<sub>K5A</sub>-treated animals had occluded vessels. Consistent with these results, the rFIX<sub>K5A</sub>-treated group had a smaller clot size, while the rFIX<sub>WT</sub>- and rFIX<sub>K5R</sub>-treated groups developed larger stable clots leading to vessel occlusion (► **Fig. 5C**).

### Supplementary Video 1

Representative video of thrombus formation 24 hours after intravenous injection of 250 IU/kg rFIX<sub>K5R</sub> in HB mice. Online content including video sequences viewable at: <https://www.thieme-connect.com/products/ejournals/html/10.1055/a-2090-9739>.





**Fig. 5** The efficacy of rFIX proteins (FIX:C 250 IU/kg) to induce thrombus formation in HB male mice 24 hours after treatment (postadministration, p.a.) was visualized over 40 minutes under the microscope in the mesenteric thrombosis model. The saline group is referred to as the vehicle group. If not stated otherwise, data are represented as scatter dot plot of individual animal values, median bars with  $\pm$  95% confidence intervals and  $n = 6-8$  animals per group (*male only*). (A) The time to appearance of first thrombi (TTAOFT) ( $> 10 \mu\text{m}$ ) and (B) the time to occlusion (TTO) of the artery are shown in minutes (min). The data set is represented in a box-plot diagram. (C) Quantification of the maximum thrombus area in the injured vessels. The clot size was divided by the total area of the injured vessel. The statistical comparison of groups was performed using a Kruskal–Wallis test followed by Dunn’s post hoc test and is reported as adjusted  $p$ -values. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.001$ , and (ns) not significant against the vehicle group.

### Supplementary Video 2

Representative video of thrombus formation 24 hours after intravenous injection of 250 IU/kg rFIX<sub>K5A</sub> in HB mice. Online content including video sequences viewable at: <https://www.thieme-connect.com/products/ejournals/html/10.1055/a-2090-9739>.

### Supplementary Video 4

Representative video of thrombus formation 24 hours after intravenous injection of buffer in HB mice. Online content including video sequences viewable at: <https://www.thieme-connect.com/products/ejournals/html/10.1055/a-2090-9739>.

### Supplementary Video 3

Representative video of thrombus formation 24 hours after intravenous injection of 250 IU/kg rFIX<sub>WT</sub> in HB mice. Online content including video sequences viewable at: <https://www.thieme-connect.com/products/ejournals/html/10.1055/a-2090-9739>.

## Discussion

The *in vivo* efficacy of extravascular rFIX was investigated experimentally in animal models of hemostasis and thrombosis using mutant rFIX proteins with enhanced (rFIX<sub>K5R</sub>) or reduced (rFIX<sub>K5A</sub>) binding to the extracellular matrix as demonstrated by Cheung et al.<sup>12</sup> Compared to the rFIX<sub>WT</sub> molecule, no difference was detected in the activation of these mutant rFIX proteins by FXIa (**Fig. 1A, 1A-1, and 1A-2**)

suggesting that arginine or alanine at position 5 do not affect the binding of rFIX to FXIa, neither is the proteolytic conversion to the fully activated two-chain serine protease, namely FIXa, affected. All proteins decreased the aPTT from FIX-deficient human plasma similarly (from ca.  $81 \pm 4$  seconds to  $37 \pm 1$  seconds) suggesting that all three rFIX molecules are able to restore clot formation *in vitro* despite differences in specific activity (►Table 1). The activity of FIX was evaluated using a chromogenic assay that stimulates the assembly of the intrinsic tenase complex and the resultant generation of FXa. The proteolytic activity of FIXa toward FX increases at least 105-fold upon FVIIIa cofactor binding.<sup>14</sup> Modified FXa generation assay with serial dilutions of either FIX or FVIII showed that rFIX<sub>K5R</sub> was more efficient in activating FX compared to rFIX<sub>WT</sub> and rFIX<sub>K5A</sub> (►Fig. 1B and C), while *in vitro* efficacy experiments showed a comparable reduction of aPTT in human FIX deficient plasma (►Table 2), as previously reported.<sup>9</sup> This effect is most likely explained by inherent variations between the chromogenic and aPTT assays.<sup>15</sup> The intrinsic tenase of FIXa and FVIIIa assemble on negatively charged phospholipid surface. *In vivo*, this surface is mainly provided by activated platelets. *In vitro*, the source of phospholipids can differ. They can be either obtained synthetically, as in the case of the chromogenic assay, or from animals or from plants, as in the aPTT reagent. It was demonstrated in a similar chromogenic assay that a faster rate of FX activation can be the result of higher binding to FVIIIa.<sup>16</sup> Because the Gla domain regulates binding to activated membranes, a higher binding affinity of rFIX<sub>K5R</sub> to the phospholipids cannot be discarded even though a basic residue, like lysine, is not presented at position 5 in the Gla domain of other proteins. This suggests that a positive charge at residue 5 is not required for binding to activated membranes. Arginine, instead of lysine, at position 5 may influence the binding affinity because it makes the largest number of hydrogen bonds<sup>17</sup> and the position 5 is in close proximity to the membrane surface forming direct interactions with negative head groups of phospholipids. Further studies to assess the binding of rFIX<sub>K5R</sub> to activated platelets or FVIII would be highly informative. In general, the *in vitro* data confirmed the integrity and functionality of the generated proteins.

The PK evaluation of equimolar doses of rFIX<sub>WT</sub>, rFIX<sub>K5R</sub>, and rFIX<sub>K5A</sub> proteins in HB mice demonstrated a biphasic clearance curve for all tested rFIX molecules (►Fig. 2A–B). The plasma levels of rFIX<sub>K5A</sub> were cleared slower than rFIX<sub>K5R</sub> and rFIX<sub>WT</sub> during the initial phase (10 hours), which can be visualized as a log-linear progression across the initial 24 hours (►Fig. 2B). The rFIX<sub>K5R</sub> on the other hand behaved like the rFIX<sub>WT</sub> but exhibited a faster distribution from blood within the first 2 hours postadministration implying binding to extravascular (tissue) proteins. This observation was reflected in the high AUC values for rFIX<sub>K5A</sub> compared to rFIX<sub>WT</sub> and rFIX<sub>K5R</sub> (►Table 3). rFIX antigen levels were evaluated in this PK study, and results showed a similar AUC pattern (data not shown here). These *in vivo* results are in agreement with Gui and colleagues who showed that mutation at position 5 is a determinant for the PK/PD profile in HB mice.<sup>18</sup> In general, the plasma levels of rFIX<sub>K5A</sub> were

consistently higher at early time points, regardless of whether activity or antigen was measured, in line with lower uptake in the extravascular compartment.

FIX staining in the liver at different time points after treatment with rFIX showed positive staining for all three rFIX proteins after 0.08 hours (15 minutes). However, only rFIX<sub>K5R</sub>-treated liver tissues showed the presence of FIX up to 24 hours after treatment (►Fig. 3A and C). Further, the histological analysis revealed that all three administered rFIX proteins were localized mainly in close proximity to the central veins (►Fig. 3B) suggesting a spatial selectivity of unknown molecular basis.<sup>7</sup> As expected, FIX-positive staining localized primarily in the extrahepatocytic space (outside hepatocytes). Based on published data postulating collagen IV as the binding partner,<sup>12,19–21</sup> we performed costaining of FIX and collagen IV in liver sections of rFIX-treated HB mice. FIX staining overlapped mostly with Collagen IV-stained regions in the liver, but there were certain liver vascular beds stained specifically for collagen IV with no detectable FIX in that region suggesting that collagen IV alone does not lead to FIX deposition (►Supplementary Figure S2, available in the online version). These results are consistent with previous observation by Cooley et al, who also reported a FIX selectivity of unknown molecular basis shown by liver histology.<sup>7</sup> The colocalization of collagen IV and FIX staining does not provide proof of direct interaction between these two proteins. Hence, the direct binding of rFIX to collagen IV was evaluated using SPR. The positive control fibronectin had a strong binding to collagen IV, whereas none of the human rFIX proteins bound to immobilized human collagen IV (►Supplementary Figure S3, available in the online version). In order to address potential species difference that might explain our *in vivo* observation of the binding of rFIX<sub>K5R</sub> to murine liver tissue, direct binding of different human rFIX proteins to murine collagen IV was evaluated in SPR. Similar to our observation with human collagen IV, rFIX variants did not bind to murine collagen IV in SPR (data not shown). These results do not confirm the previous observations that reported collagen IV as an extravascular binding partner for human FIX, and direct binding by similar interaction assay has not been shown so far. In the previous studies, potential contamination by other proteins cannot be totally excluded.<sup>12,19</sup> Collectively, these results indicate that collagen IV alone is not the binding partner of FIX, and detailed investigation using state-of the art technologies is required in future studies to determine the extravascular binding partner of FIX.

Our *in vivo* results in a tail clip bleeding model showed that all rFIX proteins significantly reduced blood loss in HB mice until 24 hours after treatment (►Fig. 4B and ►Supplementary Figure S4 [available in the online version]). Though not statistically significant, treatment with rFIX proteins reduced blood loss by approximately 65% compared to vehicle (5–6  $\mu\text{L/g}$  of bodyweight vs. approximately 17  $\mu\text{L/g}$  of bodyweight) at day 7 even in the absence of detectable levels of plasma FIX (►Fig. 4A). The blood loss was reduced by 50% even after 14 days of treatment with rFIX<sub>K5R</sub> (►Fig. 4B). In addition to blood loss, rFIX treatment of HB mice

15 minutes prior to transection of the tail also reduced bleeding time observed over 30 minutes (shown as incidence of bleeding, ►Fig. 4C), as expected. At 24 hours the efficacy of rFIX<sub>K5A</sub> decreased, with approximately 50% of the animals treated with rFIX<sub>K5A</sub> failed to stop bleeding, while rFIX<sub>WT</sub> and rFIX<sub>K5R</sub> groups were relevantly protected from bleeding (►Fig. 4C, 24 hours). The rFIX<sub>K5R</sub>-treated group exhibited the longest efficacy in reducing bleeding time until 72 hours (►Fig. 4C). These results suggest that a noncirculating reservoir of rFIX<sub>K5R</sub> may convey additional protection in this model since neither activity nor antigen levels of rFIX<sub>K5R</sub> were detectable in the plasma at this time point (►Fig. 4A). The differences in duration of efficacy between blood loss (24 hours, ►Fig. 4B) and bleeding incidence (72 hours, ►Fig. 4C) for the rFIX<sub>K5R</sub>-treated group could be due to the fact that blood incidence relies on complete cessation of blood flow were as the blood loss measures the volume of blood lost.

The efficacy of rFIX proteins was determined in a mesenteric artery thrombus model. We observed that the time to appearance of first thrombi was not different between vehicle and rFIX-treated groups (►Fig. 5A), and the time to occlusion of the artery was reduced in rFIX<sub>WT</sub>- and rFIX<sub>K5R</sub>-treated groups (►Fig. 5B). None of rFIX<sub>K5A</sub>-treated animals had occluded arteries indicating a lack of efficacy of rFIX<sub>K5A</sub> in this model. Consistent with time to occlusion, the size of the clots formed was significantly higher in rFIX<sub>WT</sub>- and rFIX<sub>K5R</sub>-treated animals compared to the vehicle group (►Fig. 5C). rFIX<sub>K5A</sub>-treated animals also had smaller clots in the vessels which explains the observed lack of occluded arteries (►Fig. 5C). This observation can be explained by decreased binding of rFIX<sub>K5A</sub> to locally available extravascular components leading to poorer efficacy. In our studies, using two mutant rFIX proteins with postulated enhanced or reduced binding affinity to the extravascular space, we were able to demonstrate that increased extravascular distribution of the rFIX<sub>K5R</sub> variant was associated with prolonged hemostatic efficacy even in the absence of detectable plasma FIX. In the saphenous vein bleeding model, it was also shown that the half-life extended rFIX products with Fc or albumin fusion have different efficacy associated with extravascular distribution.<sup>6</sup> It is plausible that the Fc and albumin fusion proteins have different binding partners which results in differential distribution in the extravascular space. It needs to be evaluated whether this observed finding in saphenous vein bleeding can be reproduced in other animal models as well.

In contrast to published data,<sup>12</sup> we were unable to demonstrate direct binding of collagen IV to rFIX using SPR. Hence, further investigations are required to identify binding partner(s) of zymogen FIX in the extravascular compartment. Despite this evidence in preclinical models, the relevance of extravascular FIX in the clinical setting is unclear. To date, plasma levels of FIX activity remain to be the major factor to determine the frequency of dosing in HB individuals. Additional studies are required to identify the relevance of extravascular FIX in a clinical setting.

### What is known about this topic?

- Binding of FIX to extravascular space can be modulated by aminoacid substitution at the N-terminus of FIX at position 5.
- Extravascular FIX has the potential to support hemostasis in the absence of measurable plasma FIX activity.
- Evidence on hemostatic efficacy of extravascular FIX relied primarily on a saphenous vein bleeding model.

### What does this paper add?

- rFIX protein with enhanced binding to components of extravascular space has prolonged tissue retention.
- Extravascular binding of FIX impacts its pharmacokinetic profile.
- Efficacy of extravascular FIX was demonstrated using tail clip bleeding and FeCl<sub>3</sub>-induced thrombosis models.

### Authors' Contribution

The manuscript has been read and approved for submission by all authors. S.K.M., H.P., M.M., P.C., S.P., and P.P. contributed to the design and/or implementation of the research, to the analysis of the results and to the writing of the manuscript. M.M. performed the pharmacokinetic evaluations. T.K. planned and carried out SPR experiments. E.H., M.B., M.W.N., and M.B. provided critical feedback and helped shape the final manuscript.

### Funding

The work was funded by CSL Behring Innovation GmbH.

### Conflict of Interest

All authors, except Michael Bacher are employees (and potentially shareholders) of CSL Behring Innovation GmbH.

### Acknowledgment

The authors are grateful to Benjamin Kühnemuth, Jennifer Marciniak, Julia Riedesel, Katharina Iliev, Peter Niebl, Tanja Viertelhausen, and Waltraud Seyfert-Brandt (all CSL Behring Innovation GmbH) for their valuable contribution.

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