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ORIGINAL ARTICLE

Rational design of a novel engineered factor X with chimeric activation peptide as bypassing agent for hemophilia

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Abstract

Background: While replacement therapy in hemophilia (H)A and B (HB) has provided remarkable improvements, the development of neutralizing antibodies remains a significant complication, with rational engineering of bypassing (BP) molecules being a relevant issue.

Objectives: This study developed a human factor (F)X variant containing a FIX-derived chimeric activation peptide (AP) driving FVIII or FIX deficiency bypass.

Methods: Engineered variants were expressed and purified, and activation/activity properties were evaluated through functional assays, also in plasma with inhibitors from patients with HA and HB.

Results: After screening a panel of recombinant variants, the most promising were purified for functional characterization. Unlike wild-type FX (FX^{WT}), the BP variants FX^{BP4b} and FX^{BP4c} at physiological concentrations effectively normalized the clotting time in HA and HB plasma. Chromogenic assays confirmed that both variants preserved activation via the extrinsic tenase complex or by Russell viper venom X, while the intrinsic activation pathway was impaired, thus favoring their selective dose–dependent FXIa activation. In thrombin generation assays, $FX^{BP4b\text{-}c}$ restored the peak and the endogenous thrombin potential in FX-deficient plasma. Remarkably, the FX^{BP4b} outperformed FX^{WT} in HA and HB plasma and showed a comparable or superior activity in comparison with B-domainless FVIII and FIX. Notably, FX^{BP4b} significantly shortened the clotting time in plasma from patients with high-titer anti-FVIII or anti-FIX inhibitory antibodies, with efficacy levels comparable with those of well-established BP agents.

Conclusion: These results support FX^{BP4b} as a promising and versatile BP agent able to sustain coagulation across multiple hemophilic contexts including inhibitors presence, while maintaining favorable activation profiles.

KEYWORDS
factor X, hemophilia, protein engineering

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1 | INTRODUCTION

Human coagulation factor (F)X, also known as Stuart-Prower factor, is a vitamin K–dependent serine protease synthesized by the liver as a 488-amino acid single-chain precursor. Before secretion, the signal peptide and propeptide, which are essential for targeting the protein to the endoplasmic reticulum and Golgi apparatus for proper post-translational modifications, are cleaved off, together with an Arg-Lys-Arg (RKR) motif. The resulting zymogen is secreted as a 2-chain protein stabilized by a disulfide bond and circulates at a concentration of 8 to 10 μg/mL (135-170 nM), with a half-life of ∼30 to 40 hours [1].

Factor X is a substrate for both the extrinsic (FVIIa/tissue factor [TF]) and intrinsic (FIXa/FVIIIa) tenase complexes, which cleave the peptide bond at Arg194 (R-IVGG sequence; legacy numbering) to generate its active form, FXa, which associates with its cofactor, activated factor V (FVa), to form the prothrombinase complex. This complex is responsible for converting prothrombin (PT) into thrombin, ultimately leading to the transformation of fibrinogen into a stable fibrin clot [1,2]. This process is impaired in hemophilia conditions due to the deficiency of coagulation FVIII (hemophilia [H]A) or FIX (HB). The insufficient intrinsic tenase activity causes bleeding into skeletal muscles and major joints, either spontaneously or following injury/surgery, based upon the disease severity [3].

For decades, replacement therapy with FVIII or FIX proteins has been the main treatment for hemophilia [4]. However, a major limitation is the development of anti-FVIII or FIX inhibitory antibodies, which negatively impact the effectiveness of the infused therapeutic proteins. This and other limitations of replacement therapy have sparked a significant revolution in the treatment landscape of hemophilia over the past years, providing patients with engineered extended-half–life factors [5–15] nonfactor therapies that include FVIII mimetics (ie, emicizumab) and rebalancing inhibitors of anticoagulant pathways (ie, concizumab, marstacimab, and fitusiran) [16–20] and bypassing (BP) agents [21,22]. Gene therapy is also a reality, but it is still evolving, and several challenges related to its long-term safety, affordability, effectiveness, and patient eligibility remain unresolved [23]. BP agents, exemplified by the well-established rFVIIa [22], remain critical for managing bleeding episodes in patients with inhibitors. FXa, for its central role in the coagulation cascade, appears to be an ideal BP agent candidate, whose applicability is, however, hindered by its rapid inactivation via plasma protease inhibitors, resulting in a very short half-life (<2 minutes) [24].

To improve its properties as BP agent, we developed a variant of FX with enhanced activation properties, achieved through the rational engineering of its activation peptide (AP), which allows it to circulate as a zymogen and to be activated by FXIa simultaneously, with the activation of the coagulation cascade upon demand.

2 | MATERIALS AND METHODS

2.1 | Reagents and proteins

For the initial screening phase, all restriction enzymes were from New England Biolabs, pGEM T-easy vector was from Promega, the QuickChange XL Site-Directed Mutagenesis Kit from Agilent Technologies; trypsin-EDTA, fetal bovine serum, and Dulbecco phosphate-buffered saline were from Sigma. Antibiotic-antimycotic, G418 (Geneticin), and Dulbecco modified Eagle medium F-12 were from GIBCO. For FX variants expression and purification phase, cell culture reagents were from GIBCO, puromycin from BD Biosciences-Clontech, and calcium-dependent monoclonal human FX antibody (MAb; 4G3) was obtained from Dr Harold James (University of Texas, Tyler). Bio-Scale CHT5-I Hydroxyapatite was from Bio-Rad Laboratories. Chromogenic FXa substrate was S-2222 (Chromogenix). Synthetic phospholipid vesicles (L-α-phosphatidylcholine/L-α-phosphatidylserine [PCPS]) were from Avanti Polar Lipids) and prepared as previously described [25]. Fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC, reconstituted in 15 mM $CaCl_2$, was obtained from Bachem Bioscience. PT and activated partial thromboplastin time (APTT) reagents were from TriniClot. Pooled normal human plasma (PNP) and FX-, FIX-, and FVIII-deficient plasma samples were obtained from George King Bio-Medical. Actin FS and Innovin (TF) were from Siemens. Recombinant (r)FVIIa was from Novo Nordisk, BeneFIX was from Pfizer, and thrombin, Russell viper venom (RVV)-X, FIXa, FX, and FXIa were from Prolytix. The SQ-FVIII was prepared as described [26].

2.2 | Plasmid construction

The human *F10* cDNA encoding wild-type (WT) FX (FX^{WT}) was amplified using polymerase chain reaction from the pCDNA3-ss-pro-II-FX construct [27,28] with the forward primer 5'-aaaGCTAGCATGGCGCACGTCCGAGGC-3' (*Nhe*I restriction site underlined) and the reverse primers 5'- aaaAAGCTTTCACTTTAATGGAGAGGACGTTATG-3' (*Hind*III restriction site underlined) and the amplicon was then cloned into the pGEM T-easy vector (A1360; Promega kit) through the T/A cloning strategy, used as intermediate step for the variants' production, to avoid the insertion of random mutations in the plasmid subsequently used for the mammalian expression. The FIX/FVIII-BP FX (FX^{BP}) variants were produced starting from FX^{WT} by site-directed mutagenesis using Q5 High-Fidelity DNA Polymerase (M0491S; New England Biolabs) or QuikChange II XL Site-Directed Mutagenesis Kit (200521; Agilent Technologies). The primers for each mutagenesis and its use on the corresponding starting template are summarized in Supplementary Table S1. Expression of FX variants in HEK293 cells were carried out as previously described [29] and secreted protein levels quantified by ELISA with antihuman FX polyclonal antibodies (Cedarlane).

2.3 | Protein purification

Stably transfected Expi293 cells were grown in suspension to a density of $> 5 \times 10^6$ cells/mL, at which point they were pelleted by centrifugation at 500 × *g* and resuspended in fresh culture medium. After 24 to 48 hours, cells were pelleted by centrifugation at 2000 × g, and the media was supplemented with 10 mM benzamidine hydrochloride, 20 mM HEPES pH 7.4, and 5 mM $CaCl_2$. Factor X was captured on a 4G3 immunoaffinity column equilibrated in 20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$ pH 7.4, and eluted from the column with 20 mM HEPES, 150 mM NaCl, 8 mM EDTA pH 7.4. Elution fractions were analyzed by PT clotting activity and Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE), and selected fractions were dialyzed against a 4-mM sodium phosphate buffer, pH 6.8. After dialysis, FX was applied to an Econofit CHT-I hydroxyapatite column (Bio-Rad) and eluted by linear gradient from 4 to 400 mM sodium phosphate, pH 6.8. Fractions were again analyzed by PT and SDS-PAGE, and selected fractions were precipitated with 75% ammonium sulfate and resuspended in 50% glycerol. The final protein preparations were stored at −20 °C.

Protein concentrations were determined using the following molecular weights and extinction coefficients ($E_{0.1\%}$ 280 nm): FX^{WT}, 58.800 and 1.16; FX^{BP4b} and FX^{BP4c}, 54.000 and 1.16; BeneFIX, 55.000 and 1.32; SQ-FVIII, 165 000 and 1.16; FXIa, 160 000 and 1.34; FXa, 45 300 and 1.16; FVIIa, 50.000 and 1.39; FII, 36.000 and 1.83; and RVV-X, 93.000 and 1.18, respectively; all listed reagent or protein concentrations are final concentrations for experimental conditions.

2.4 | SDS-PAGE analysis

For SDS-PAGE, proteins (4 μg/well) were run under both reducing and nonreducing conditions using 4% to 12% gradient NuPage gels (Invitrogen), followed by staining with Coomassie Blue R-250. As a molecular weight standard, SeeBlue Plus2 Pre-Stained (Invitrogen) was used. The gel was analyzed using the Image Studio Lite software (Licor).

2.5 | Clotting assays

Factor X procoagulant activity was measured by PT in FX-deficient plasma and by APTT using FVIII- and FIX-deficient plasma. In a typical PT assay, 50 μL of FX-deficient plasma was mixed with 50 μL of sample (diluted in HEPES-buffered saline + 0.1% [w/v] bovine serum albumin), followed by a 60-second incubation at 37 °C, and coagulation was then activated after the addition of 100 μL of PT reagent (TriniClot PT Excel; TCoag). BP activity of FX variants was evaluated by APTT assay, in both HA and HB plasma; 50 μL of HA or HB plasma were incubated with 50 μL of the sample (prepared as earlier) and 50 μL of APTT reagent (Triniclot Automated APTT; TCoag), followed by 180-second incubation at 37 °C, and coagulation was initiated with 25 mM $CaCl_2$. Time to clot was measured using a Start4 coagulation instrument from Diagnostica Stago.

2.6 | *In vitro* activation of FX variants

2.6.1 | Extrinsic Xase activation

Increasing concentrations of FX variants (0-2000 nM) were incubated with 0.1 nM rFVIIa, 10% v/v Innovin (B4212-100; Siemens), and 20 μM PCPS in reaction buffer (20 mM HEPES, 150 mM NaCl, 0.1% PEG-8000, 5 mM $CaCl_2$, pH 7.4). Aliquots for time course were removed every 30 seconds for 3 minutes and quenched by a 1:10 dilution of samples in quenching buffer (20 mM HEPES, 150 mM NaCl, 50 mM EDTA, pH 7.4). Quenched samples were further diluted 1:40 in quenching buffer, and FXa concentration was determined using a chromogenic FXa substrate (S-2222; S820316; Chromogenix) at the final concentration of 250 μM, by monitoring the initial steady state increase in absorbance at 405 nm using the SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices) and then evaluated by interpolating the data in an FXa standard curve.

2.6.2 | Intrinsic Xase activation

FVIIIa was generated by mixing SQ-FVIII at a 1:1 ratio with thrombin in assay buffer (with 20 μM PCPS) for 1 minute at 25 °C, after which, thrombin was quenched with a 2-fold excess of hirudin. Factor VIIIa was rapidly added into a Xase mixture with 40-fold excess concentration of FIXa and 40 μM PCPS, along with 2× Xase master mix incubated with increasing concentrations (0-2000 nM) of FX variants. The final concentrations of the intrinsic Xase components were 20 nM FIXa, 0.5 nM FVIIIa, and 20 μM PCPS. The FX activation time course and FXa chromogenic measurements are the same as described earlier.

2.6.3 | RVV-X activation

FX variants at 0.25 mg/mL were incubated with 2.5 μg/mL of RVV-X in reaction buffer at 37 °C for 1 hour. The samples were quenched through a 1:10 dilution of samples in quenching buffer, and in the final reaction, samples were diluted 1:40 in quenching buffer. The evaluation of FXa generation was made as described earlier.

2.6.4 | Factor XIa activation

Activation via FXIa was carried out dose dependently by incubation of increasing concentrations of FX variants (0-10 μM) with 25 nM FXIa or FX variants (2 μM) with increasing concentrations of FXIa (0-50 nM), for 1 hour at 37 °C [30], after which FXa enzyme activity was determined by the addition of S-2222 (250 μM) and evaluation of FXa generation as earlier.

2.7 | Thrombin generation assay

Assays were performed in black microtiter plates (Nunc F16 Maxisorp) using 40 μL of plasma (FX-deficient, HA or HB, or PNP from George King Bio-Medical), 20 μL of sample (FX variants, SQ-FVIII, BeneFIX, or buffer control, at the concentrations indicated earlier), 10 μL of Actin FS (1:50 dilution), and 50 μL of the fluorogenic substrate Z-Gly-Gly-Arg-AMC (0.5 mM final) in 15 mM $CaCl_2$. Fluorescence was recorded for 90 minutes at 37 °C (λ_ex, 360 nm; λ_em, 460 nm) using a SpectraMax M5 plate reader (Molecular Devices). Thrombin concentrations were calculated from a calibration curve (Technothrombin calibrator), and thrombin generation parameters (lag time, peak, and endogenous thrombin potential [ETP]) were derived from the resulting curves.

2.8 | Patient samples

Coagulant assays using samples from patients with inhibitors were carried out in accordance with the Declaration of Helsinki principles. Written permission for research purposes was obtained. Inclusion criteria were patients >18 years manifesting with severe HA and HB (FVIII/FIX activity levels <1%) and inhibitor titer >5 Bethesda Units (BU)/mL. The inhibitor titer for the selected patients with HA and HB was 211 and 88 BU/mL, respectively. Samples were collected and processed at the Haemophilia Centre of the Careggi Hospital during scheduled follow-ups as part of routine control for the evaluation of inhibitor titers. In particular, blood was collected in sodium citrate buffer and processed to plasma by centrifugation at 3300 × *g* for 5 minutes before storage at −80 °C. Plasma FX antigen levels (patient with HA, 10.1±1.8 μg/mL; patient with HB, 10.2 ± 3.5 μg/mL) were evaluated by ELISA, as indicated earlier for FX variants in media.

2.9 | T- and B-cell peptide epitope prediction

Potential T-cell epitopes were predicted using the NetMHC4.1 tool, with 9-mer peptides against representative human leukocyte antigen supertypes [31]. The default rank thresholds for strong (0.5) and weak (2.0) binders were used. Potential B-cell peptide epitopes were predicted using the Immune Epitope Database (IEDB) Bepipred Linear Epitope Prediction (BepiPred) 2.0, with a threshold of 0.5 [32].

2.10 | Statistical analysis

Statistical differences were analyzed by 2-tailed unpaired Student's *t*-tests in GraphPad Prism (version 10.5.0; GraphPad Software), with $P < .05$ defined as statistically significant and a 95% CI.

3 | RESULTS

3.1 | Rational engineering of FXIa-activatable FX variants

A stepwise engineering approach was used to modify the FX-AP and generate a panel of variants to be assessed as BP agents in HA or HB conditions. The strategy was based on partial or complete substitution of the FX-AP with a portion of FIX-AP, thereby enabling activation via FXIa and circumventing intrinsic tenase deficiency, the primary driver of the hemophilic condition. The key regions essential for the maturation and catalytic activity of FX remained unaltered to preserve critical functional elements, specifically: the RKR tripeptide and its adjacent SV residues, the recognition site for FX processing, and the TR-IVGG sequences, crucial for efficient postcleavage activation, were maintained (Figure 1).

Factor X variants were systematically optimized by AP engineering, and functional properties were evaluated following 3 sequential rounds of optimization (Table). The first-generation variants ($FX^{BP1\text{-}4}$) were made to evaluate the ideal FIX cleavage site sequence. Specifically, FX^{BP1} and FX^{BP2} incorporated the FIX-AP cleavage motif at R145–A146 (SQTSK), while FX^{BP3} and FX^{BP4} incorporated the alternative cleavage motif at R180–V181 (STQSFNDF), each tested either with retention or removal of the native FX-AP. This design allowed us to dissect how distinct FIX-derived cleavage contexts influence the activation efficiency of engineered FX variants under hemophilic conditions. Among these, FX^{BP4} exhibited the most promising profile and was further engineered to generate the second-generation variants ($FX^{BP4a\text{-}c}$), incorporating additional upstream residues from the selected FIX-AP sequence. Encouraged by the improvement of the functional profile of $FX^{BP4b\text{-}c}$, we applied a similar strategy to develop third-generation variants ($FX^{BP4d\text{-}e}$). However, these later modifications did not yield further improvements, confirming $FX^{BP4b\text{-}c}$ as the most effective candidates.

To characterize the engineered variants, we performed a dual screening approach: (1) PT assays in FX-deficient plasma (Figure 2A) to assess preservation of normal FX activity; and (2) APTT assays in HA plasma (Figure 2B and Table) to evaluate their BP potential. This strategy allowed us to simultaneously verify that (1) all variants retained a normal specific activity (activity/antigen ratio), close to 1 ($FX^{BP4b,4c}$) or slightly increased (∼1.7 for $FX^{BP2,BP3,BP4d,BP4e}$; ∼2.5 for $FX^{BP1,BP4,BP4a}$) compared with FX^{WT}; (2) whereas all FX^{BP} variants led to a reduction in clotting time in APTT assays in human FVIII-deficient plasma, FX^{WT} was ineffective (154.1 ± 7.6 seconds) and comparable with the negative control (mock medium, 154.1 ± 6.2 seconds). The FX^{BP4b} (105.2 ± 7.7 seconds) and FX^{BP4c} (105.6 ± 5.9 seconds) were the most active in terms of BP properties, with significantly shortened coagulation times ($P < .0001$) compared with controls. Before purification, we evaluated the coagulant activity of

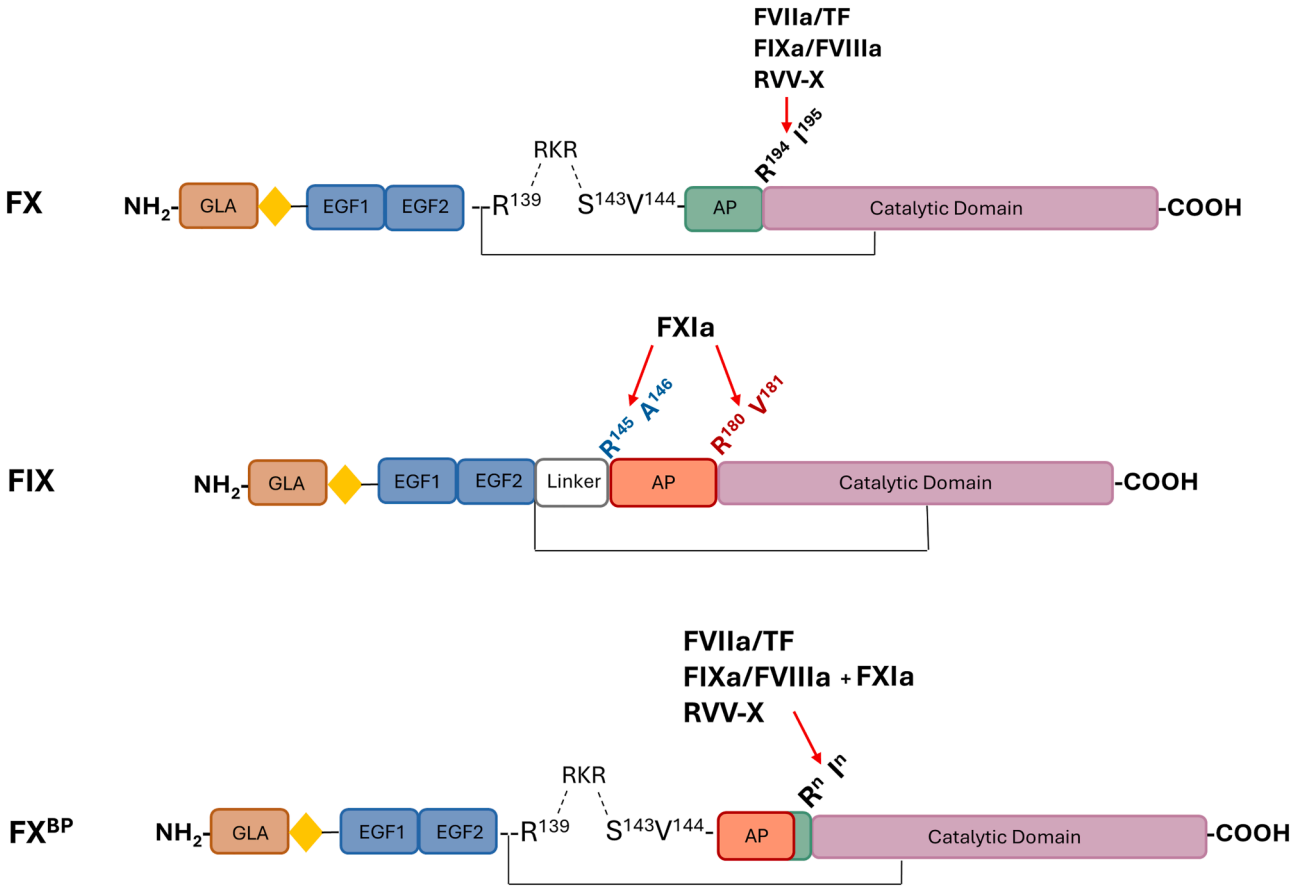


FIGURE 1 Schematic diagrams of factor (F)X, FIX, and FX^{BP} protein structures. The panel of FX^{BP} designed molecules contains a partial or a total substitution of the original AP with 1 of the 2 FIX-derived proteolytic cleavage sites, allowing direct activation of FX^{BP} via FXIa, in addition to the canonical one. R^n I^n indicates the new FX cleavage site. GLA domain, vermillion; aromatic stack, yellow; EGF1 and EGF2 domains, blue; FIX-linker, white; FX-AP, blue green; FIX-AP, red; catalytic domain, pale violet. AP, activation peptide; BP, bypassing; EGF, epidermal growth factor-like domain; GLA, γ-carboxyglutamic acid domain; RVV-X, Russell viper venom; TF, tissue factor.

both variants at higher concentrations, collecting media from HEK293 stable clones. In these assays, both FX^{BP4b} and FX^{BP4c} confirmed their ability to shorten clotting times both in HA and HB plasma in a dose-dependent manner, reaching activity levels comparable to those of normal plasma (Figure 2C, D).

3.2 | Purification and characterization of the FX^{BP4b-c} variants

Factor X^{BP4b}, FX^{BP4c}, and FX^{WT} recombinant proteins were stably expressed in Expi293 cells and purified from conditioned media by immunoaffinity and hydroxyapatite chromatography. Purified FX^{BP} variants together with FX^{WT} and plasma-derived (pd)FX were analyzed under reducing and nonreducing conditions by SDS-PAGE before and after activation by RVV-X (Supplementary Figure S1). The 2 FX^{BP} proteins migrated under nonreducing conditions as a single band with an apparent molecular weight of ~55 kDa, according to the modifications made on their AP, while FX^{WT} and pdFX, containing the authentic AP, showed a molecular weight of ~ 60 kDa. Under reducing conditions, pdFX and FX migrated as 2 bands at ~55 kDa and 20 kDa, reflecting heavy and light chains of FX, respectively. Noticeably, although the relative position of FX^{BP} zymogen corresponded to protein forms with a smaller size, as expected by AP engineering, the FXa heavy and light chain forms generated after activation displayed a molecular weight overlapping with that of pdFX or FX^{WT}. These results confirmed the correct processing of FX variants to their mature/activated forms, a requirement for downstream functional characterization.

3.3 | Evaluation of *in vitro* activation of FX^{BP4b-c} variants

We investigated whether modifications in the AP affect the activation profile of FX^{BP} variants. As shown in Figure 3A, FX^{BP4b} and FX^{BP4c} were efficiently converted to FXa by the nonphysiological RVV-X activator (FX^{BP4b}, 89.1% ± 7.1%; FX^{BP4c}, 98.9% ± 5.0%) and the extrinsic Xase complex (FX^{BP4b}, 93.8% ± 17.7%; FX^{BP4c}, 94.8% ± 2.9%), as also observed in dose-dependent assays (Figure 3B),

TABLE Summary of amino acid sequences of FX^{BP} variants.

AP type	Name	Sequence
FX^{WT}	AP^{FX}	…RKR|SVAQATSSSGEAPDSITWKPYDAADLDPTENPFDLLDFNQTQPERGDNNLTR|IVGG…
FIX^{WT}	AP^{FIX}	…SV**SQTSK**LTR|AETVFPDVDYVNSTE**AETILDNITQSTQSFNDF**TR|VVGG…
First generation	FX^{BP1}	…RKR|SV**SQTSK**LTR|IVGG…
	FX^{BP2}	…RKR|SV-AP^{FX}-**SQTSK**LTR|IVGG…
	FX^{BP3}	…RKR|SV-AP^{FX}-**STQSFNDF**TR|IVGG…
	FX^{BP4}	…RKR|SV**STQSFNDF**TR|IVGG…
Second generation	FX^{BP4a}	…RKR|SV**TQSTQSFNDF**TR|IVGG…
	FX^{BP4b}	…RKR|SV**NITQSTQSFNDF**TR|IVGG…
	FX^{BP4c}	…RKR|SV**LDNITQSTQSFNDF**TR|IVGG…
Third generation	FX^{BP4d}	…RKR|SV**TILDNITQSTQSFNDF**TR|IVGG…
	FX^{BP4e}	…RKR|SV**AETILDNITQSTQSFNDF**TR|IVGG…

Sequences are shown aligned to the wild-type (WT) factor (F)X sequence (FX^{WT}). The blue highlight indicates the sequence context of the first cleavage site of activation peptide $(AP)^{FIX}$ (consensus STQSK). The red highlight indicates the second engineered sequence (STQSFNDFT), with upstream extensions shown in green (TQ), violet (NI), dark blue (LD), orange (TI), and purple (AE). FX^{BP2} and FX^{BP3} constructs contain the native FX-AP (denoted by AP^{FX}). In all other constructs (FX^{BP1}, FX^{BP4}, and deriving variants) the AP^{FX} has been removed.

indicating that AP modifications do not impair activation via these pathways. In contrast, activation by the intrinsic Xase complex was moderately impaired for FX^{BP4b} and FX^{BP4c} (57.1% ± 9.9% and 80% ± 13.7%, respectively) compared with FX^{WT} (Figure 3A). Interestingly, this trend was reversed upon FXIa-mediated activation (Figure 3C). The FX^{BP} variants, particularly FX^{BP4b}, displayed an enhanced dose-dependent FXIa activation profile compared with FX^{WT} (Figure 3D). The ability of FX variants to be activated by FXIa was also assessed in the absence of phospholipids to avoid autoactivation or degradation due to FXa appearance [33,34]. Furthermore, self-incubation of FX variants did not generate FXa, confirming that the improved properties of our variants were not a consequence of autoactivation by traces of FXa in our preparations (Supplementary Figure S2). These findings demonstrated that FX-AP modifications enable robust FXIa-mediated activation, potentially contributing to their improved activation profile associated with the chimeric AP.

3.4 | Coagulant and thrombin generation activity of FX^{BP4b-c} in HB and HA plasma

The addition of FX^{WT} did not affect APTT coagulation times in HA (Figure 4A) or HB plasma (Figure 4B). In contrast, the addition of FX^{BP4b} and FX^{BP4c} to HA (FX^{BP4b}, 29.6 ± 0.14 seconds; FX^{BP4c}, 29.5 ± 0.42 seconds) or HB (FX^{BP4b}, 28.9 ± 0.21 seconds; FX^{BP4c}, 36.1 ± 0.21 seconds) plasma fully corrected the APTT clotting times to that obtained for PNP (35.9 ± 1.2 seconds) with physiological FX concentrations (10 μg/mL). Strikingly, the dose-response curves for the FX^{BP} variants were nearly identical to the curves generated by serial dilutions of the standard replacement therapies, SQ-FVIII (41 ± 0.28 seconds at 100 ng/mL) and BeneFIX (36.4 ± 0.21 seconds at 2.5 μg/mL), as well as with PNP. This indicates that the engineered FX variants can improve coagulation with an efficacy equivalent to physiological FVIII or FIX levels (Figure 4). To extend these findings in HA or HB conditions, we performed thrombin generation assays (TGAs). As expected, the addition of SQ-FVIII to HA plasma (Figure 5A; Supplementary Table S2A) or BeneFIX to HB plasma (Figure 5C; Supplementary Table S2B), resulted in a dose-dependent increase in peak thrombin and ETP, with a corresponding shortening of lag time. At full reconstitution (100% of physiological concentration), SQ-FVIII restored thrombin generation (peak, 706.5 ± 54.5 nM; ETP, 5309 ± 398 min·nM IIa) like PNP (peak, 734.4 ± 86.6 nM; ETP, 4966 ± 435 min·nM IIa), with a lag time of 6.8 ± 1.2 minutes. In comparison, HB plasma alone generated a low thrombin peak (147.4 ± 14.3 nM) and low ETP (3797 ± 406 min·nM IIa), with a prolonged lag time (19.3 ± 1.5 minutes). At 30%, BeneFIX restored a peak of 647.5 ± 76.1 nM and an ETP of 5110 ± 456 min·nM IIa, with a lag time of 10.0 ± 1.2 minutes. Even at the lowest concentration tested (0.1%), we still observed a significant improvement over the HB baseline (Figure 5C).

Interestingly, the FX^{BP} variants showed appreciable thrombin generation in both settings. In HA plasma, FX^{BP4c} at 30 μg/mL generated a peak of 360.6 ± 27.8 nM and ETP of 4737 ± 385 min·nM IIa, with a lag time of 6.8 ± 0.3 minutes, while FX^{BP4b} induced a comparable peak (330.7 ± 30.0 nM) and ETP (4733 ± 416 min·nM IIa), with an identical lag time. Notably, these lag times were significantly shorter than those observed for any dose of SQ-FVIII < 30%, suggesting a distinct BP profile (Figure 5B). At physiological concentrations (10 μg/mL), FX^{BP4c} maintained residual activity, comparable with that of SQ-FVIII at 10% to 30%. Similarly, FX^{BP4b} at 10 μg/mL reached a peak of 268.4 ± 22.4 nM and ETP of 4543 ± 403 min·nM IIa with a slightly shorter lag time (8.0 ± 0.0 minutes) (Figure 5B; Supplementary Table S2B).

In HB plasma, at 30 μg/mL, FX^{BP4c} yielded a peak of 224.0 ± 80.9 nM and ETP of 3605 ± 1119 min·nM IIa, whereas FX^{BP4b} reached a peak of 195.1 ± 62.1 nM and ETP of 3600 ± 994 min·nM IIa. Lag times for both variants were shortened to ∼9.5 to 9.8 minutes,

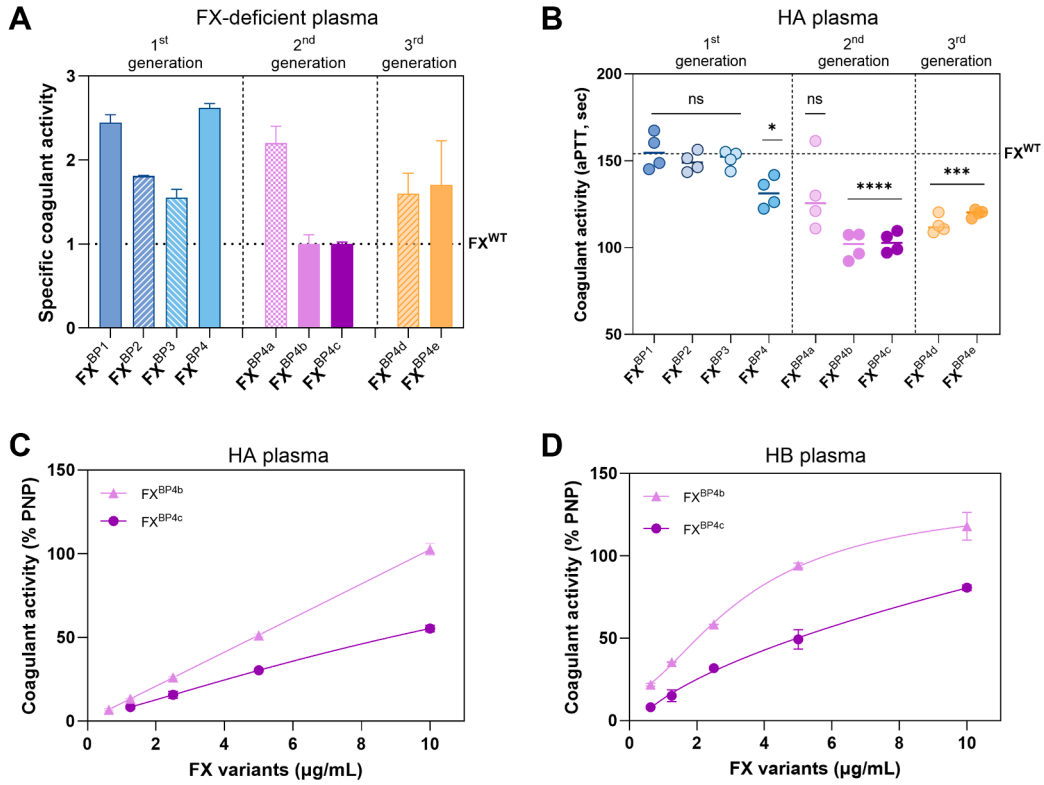


FIGURE 2 Analysis of factor (F)X^{BP} clotting activity. Specific coagulant activity (PT-based assays; n = 3) in FX-deficient plasma (A), coagulant activity (activated partial thromboplastin time [APTT]–based assays; n = 4) in hemophilia (H)A plasma (B), and dose-dependent coagulant activity (APTT-based assays; n = 4) in HA (C) or HB (D) plasma of FX^{BP} variants in conditioned media quantified by ELISA. Specific activity in (A) refers to activity/antigen ratio of FX^{BP} levels (%) compared with FX^{WT} (dotted line), resulting in adimensional numbers ranging from 0 to 1. Activity is expressed as seconds (panel B; 500 ng/mL FX, with FX^{WT} as reference, as indicated by the dotted line) or relative to the activity measured from serial dilutions of pooled normal plasma (PNP; panels C and D, %PNP). Data represent mean ± SD. $^{**}P < .01$; $^{***}P < .005$; $^{****}P < .0001$. BP, bypassing; WT, wild type.

indicating rapid initiation of thrombin generation (Figure 5D). Interestingly, at 10 μg/mL, both FX^{BP4c} and FX^{BP4b} maintained comparable thrombin peaks (144.3 ± 26.9 and 129.8 ± 44.8 nM, respectively) and ETP (3218 ± 529 and 3005 ± 811 min·nM IIa), with similar lag times (~10.3-10.5 minutes) (Figure 5D; Supplementary Table S3B). Notably, in both settings, FX^{WT} maintained a comparable profile with that of the plasma supplemented with only buffer (Figure 5B, D). TGA was also assessed in PNP supplemented with FX^{BP} variants and initiated with 1 pM TF, which revealed thrombin generation curves overlapping with that of PNP alone, thus ruling out an appreciable additive effect (Supplementary Figure S3).

3.5 | APTT-based assessment of the FX^{BP4b} variant in HA and HB patient plasma with inhibitors

To explore the coagulant potential of FX^{BP4b} in the presence of anti-FIX or anti-FVIII inhibitors, we performed APTT assays in plasmas from 2 patients with severe HB (p.R252X mutation) or HA (IVS22 inversion) and documented inhibitory titers (211 and 88 BU/mL, respectively). These samples were tested in parallel with FX^{WT} (as a negative control), BeneFIX, NovoSeven, and FEIBA, each at 2 concentrations, to benchmark the response of the FX^{BP4b} variant, chosen because of its better functional profile compared with the FX^{BP4c} variant. Although the FX^{WT} remained ineffective, the FX^{BP4b} consistently reduced APTT values in both contexts, achieving comparable or superior results in comparison with NovoSeven and FEIBA, particularly at 30 μg/mL (Figure 6).

4 | DISCUSSION

Over the past 2 decades, nonreplacement therapies such as FVIII mimetics and rebalancing agents have represented a turning point in hemophilia treatment [4]. Although data on long-term joint preservation remain limited, some agents have already demonstrated efficacy and safety in infants and neonates, with reliable prevention of spontaneous bleeds and intracranial hemorrhage during the first year of life [35]. Nevertheless, in patients undergoing these therapies, breakthrough bleedings may still occur, and for these events, as well as for invasive procedures, adjunctive treatment is required, particularly in the presence of inhibitors [20]. In these cases, the clinical utility of

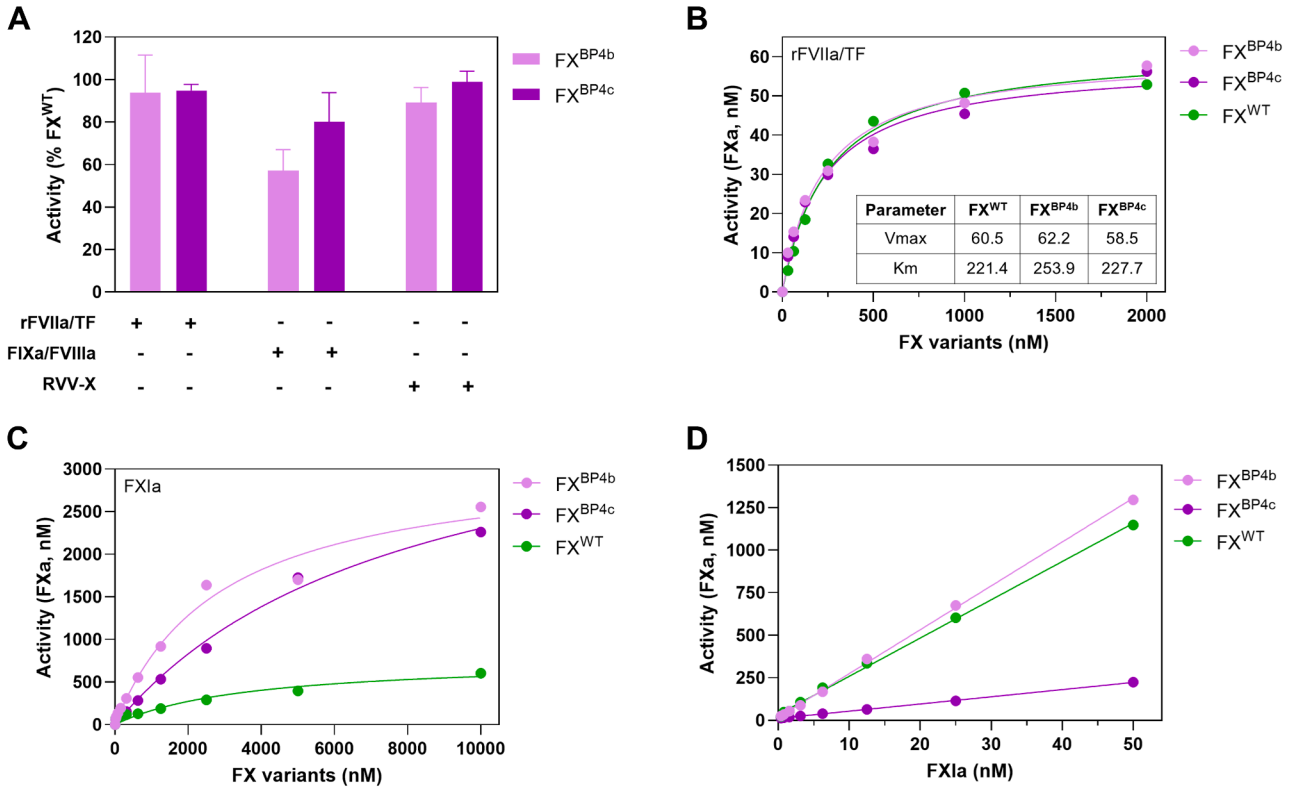


FIGURE 3 Activation of factor (F)X variants evaluated through chromogenic assays. Factor Xa generation following activation of FX^{BP4b} and FX^{BP4c} and FX^{WT} by the extrinsic (rFVIIa/TF) pathway in kinetic experiments (A), by physiological (extrinsic, rFVIIa/TF; intrinsic, FIXa/FVIIIa) or nonphysiological (RVV-X) pathways (B) and by FXIa (25 nM) in FX (C) or FXIa (D) assayed in a concentration-dependent manner. Data are expressed mean ± SD as concentration of FXa generated (panels A, C, and D; $n = 3$) or as percentage relative to FX^{WT} ($n = 8$). BP, bypassing; RVV, Russell viper venom; TF, tissue factor; rF, recombinant factor; WT, wild type.

NovoSeven, a cornerstone of BP therapy, is limited by its short plasma half-life (~2-3 hours), which necessitates frequent intravenous dosing to maintain hemostasis. This pharmacokinetic profile makes it suboptimal for prophylaxis and imposes a significant burden, particularly in the management of major bleeding episodes [36].

These limitations underscore the demand for next-generation therapies featuring enhanced pharmacokinetic properties with a prolonged half-life, to achieve durable hemostatic protection. Due to its crucial role at the crossroad of the blood coagulation cascade, FXa, an ideal candidate for use as a BP agent in hemophilia, has been explored by different approaches, in particular by using an active form of FX combined with phospholipids [37] or PT [38]. Despite their demonstrated efficacy *in vivo*, FXa-based strategies bear a significant thrombogenic risk [39,40]. Moreover, their applicability is

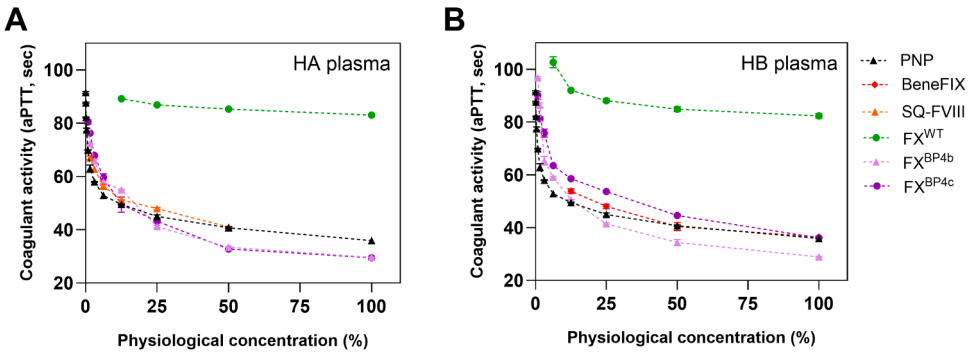


FIGURE 4 Clotting activity of purified factor (F)X variants in activated partial thromboplastin time (APTT)–based assay. Coagulant activity of FX^{BP} variants evaluated by APTT-based assays in hemophilia (H)A (A; $n = 3$) and HB (B, $n = 3$) plasma. The data plotted represent serial dilutions of each sample: 100% refers to 200 ng/mL SQ-FVIII, 5 μg/mL BeneFIX, and 10 μg/mL FX. BP, bypassing; PNP, pooled normal plasma; WT, wild type.

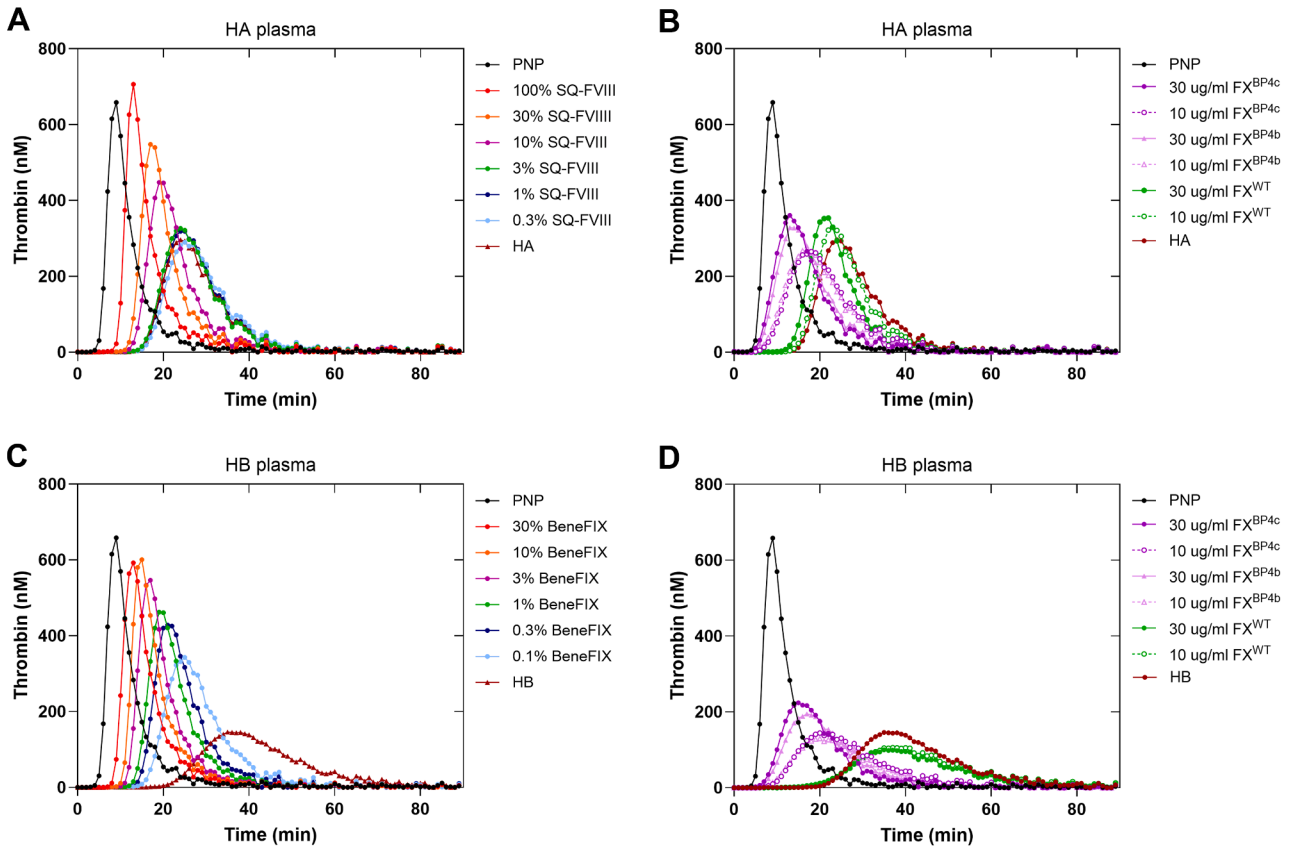


FIGURE 5 Thrombin generation profiles in hemophilia (H)A or HB human plasma. Thrombin generation curves in HA plasma supplemented with serial dilutions of SQ-FVIII (A) or FX^{WT} and FX^{BP} variants (B), and in HB plasma supplemented with serial dilutions of BeneFIX (C) or FX^{WT} and FX^{BP} variants (D). Thrombin generation curves are representative of 3 independent experiments. Reactions were initiated with 1% Actin FS. Results are compared with HA or HB plasma added with buffer or pooled normal plasma (PNP): 100% refers to 200 ng/mL SQ-FVIII and 5 µg/mL BeneFIX. BP, bypassing; WT, wild type.

limited due to the rapid inactivation of FXa by circulating protease inhibitors, resulting in a short half-life (<1-2 minutes) [41]. To address these issues, 2 FXa mutants (FXa^{I16L} and FXa^{V17A}) [42] with zymogen-like properties were developed [25,43,44]. Functional studies demonstrated that these variants have partial resistance to protease inhibitors (eg, antithrombin and TF pathway inhibitor), leading to a prolonged half-life in plasma, and FXa^{I16L} was highly effective in promoting thrombin generation in hemophilic plasma with or without inhibitors. These findings prompted *in vivo* studies in mice, where this variant has been demonstrated to be highly effective

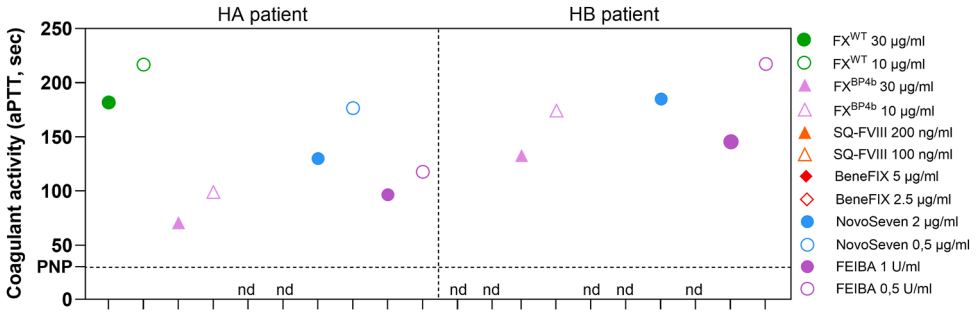


FIGURE 6 Activated partial thromboplastin time (APTT) clotting times in plasma from patients with severe hemophilia and inhibitors. Each dot represents the clotting time (seconds) measured by APTT assays in plasma from 2 patients with inhibitors, hemophilia (H)A1 (intron 22 inversion, 88 BU/mL) and HB1 (p.R252X mutation, 211 BU/mL), after incubation with 1 of the tested treatments at 2 different concentrations. Treatments include the following: (10 and 30 µg/mL) FX^{WT} or FX^{BP4b}, rFVIIa (2 and 0.5 µg/mL NovoSeven), activated prothrombin complex concentrate (1 and 0.5 U/mL; FEIBA), and SQ-FVIII (200 and 100 ng/mL) as controls. nd, out of range, referring to coagulation times longer than 250 seconds. BP, bypassing; WT, wild type.

in acute bleeding settings [44,45], was able to reverse the effects of FXa DOACs *in vitro* and mitigated DOAC-associated bleeding *in vivo* [46]. Despite the promising results during clinical development, the half-life remained short, making it more suitable for acute bleeding than for prophylactic treatment [47–49]. Alternative strategies have focused on redirecting the activation pathway of FX by engineering its AP. One such approach was described by Louvain-Quintard et al. [50] and later revised by Abache et al. [51], who introduced specific substitutions within the FX-AP, enabling its cleavage by thrombin. Another attempt was made by Ebert et al. [52], who developed FX variants that could be selectively activated by FXIa. These strategies collectively illustrate the feasibility and therapeutic promise of redirecting FX activation through targeted modification of its AP. Our work follows a conceptually similar rationale but diverges in key aspects of the molecular design. In comparison with previous reports and patents (ie, WO 98/38317, WO 98/38318, and WO2006128668A2), our FX^{BP} variants were designed and sequentially modified to be devoid of the original AP and sequentially chimeric with that deriving from FIX, and thus conferring activatability by FXIa, along with preserved amino acid contexts in FX-specific processing sites (RKR and SV). Specifically, we explored the incorporation of FIX-derived AP sequences—either alone or in combination with the native FX sequence into the activation site of FX, aiming to generate variants activatable by FXIa but retaining distinct activation profiles and pharmacologic features. Among a panel of variants, we identified the 2 most promising, FX^{BP4b} and FX^{BP4c}. Unlike WT FX, which is strictly activated by FXa-generating complexes (FVIIIa/FIXa, FVIIa/TF, and RVV-X enzyme), FX^{BP} variants can also be activated directly by FXIa. This design mimics the redundancy of physiological coagulation, providing multiple routes for activation and improving the chances of thrombin generation in the absence of FVIII or FIX. Importantly, no spontaneous activity was observed for FX^{BP} variants in the absence of the activator FXIa, and their addition to PNP did not influence thrombin generation curves, suggesting a favorable safety profile.

Both variants demonstrated the ability to exert BP procoagulant activity *in vitro* across a range of conditions: in APTT-based assays, $FX^{BP4b\text{-}c}$ shortened clotting times significantly in both HA and HB, displaying a dose-dependent response, comparable with that of PNP or SQ-FVIII and BeneFIX, respectively. In TGA, $FX^{BP4b\text{-}c}$ partially improved peak thrombin levels and ETP, with a major impact on lag times, in both HA and HB plasma. Notably, FX^{BP4b} also shortened APTT clotting times in inhibitor-positive HA or HB patient plasmas, restoring near-physiological values, with a performance comparable or superior to rFVIIa or FEIBA.

Although the engineered variant is designed as a zymogen with chimeric AP and ensuring an activation-driven BP property, the thrombotic potential of FX-based hemostatic therapies has to be considered. In the present study, while FX^{BP} variants markedly reduced clotting times and shortened the initiation phase in TGA assays, they did not normalize peak height or total thrombin generation in FVIII/FIX-deficient plasma. This points toward a limited thrombogenicity potential, in turn suggesting a potentially favorable efficacy–safety balance. Moreover, the T- and B-cell peptide epitope analysis indicated an overall number of predicted MHC-binding peptides comparable with that of FX^{WT} and sequence similarities in the predicted B-cell epitopes, thus suggesting a limited overall immunogenic potential of the FX^{BP} variants (Supplementary Table S4). Dedicated studies in *in vivo* models will be necessary to further define the efficacy and safety profile of FX^{BP} variants.

In conclusion, we provide the proof-of-concept that a novel multiactivatable FX variant is effective in HA and HB conditions even in the presence of high-titer inhibitors, suggesting that FX^{BP4b} could represent an attractive therapeutic.

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AUTHOR CONTRIBUTIONS

A.B., M.P., and R.M.C. conceived the study and designed research. R.T. created the expression vectors. R.T., E.T., and M.F.T. performed expression studies to screen FX variants for bypassing activity. R.T. and M.W.B. optimized expression protocols for the Expi system, purified and characterized the best performing FX variants. G.C. collected and processed plasma samples. R.T., R.M.C., M.P., and A.B. analyzed data and wrote the manuscript. F.B. contributed with critical revision of the manuscript. All authors read and approved the final version of the paper.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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REFERENCES

[1] Camire RM. Blood coagulation factor X: molecular biology, inherited disease, and engineered therapeutics. *J Thromb Thrombolysis*. 2021;52:383–90.
[2] Krishnaswamy S. The transition of prothrombin to thrombin. *J Thromb Haemost*. 2013;11(Suppl 1):265–76.
[3] Mannucci PM, Tuddenham EG. The hemophilias—from royal genes to gene therapy. *N Engl J Med*. 2001;344:1773–9.
[4] Mannucci PM. Hemophilia treatment innovation: 50 years of progress and more to come. *J Thromb Haemost*. 2023;21:403–12.
[5] Metzner HJ, Pipe SW, Weimer T, Schulte S. Extending the pharmacokinetic half-life of coagulation factors by fusion to recombinant albumin. *Thromb Haemost*. 2013;110:931–9.
[6] Powell JS, Pasi KJ, Ragni MV, Ozelo MC, Valentino LA, Mahlangu JN, Josephson NC, Perry D, Manco-Johnson MJ, Apte S, Baker RI, Chan GC, Novitzky N, Wong RS, Krassova S, Allen G, Jiang H, Innes A, Li S, Cristiano LM, et al. Phase 3 study of recombinant factor IX Fc fusion protein in hemophilia B. *N Engl J Med*. 2013;369:2313–23.

[7] Konkle BA, Stasyshyn O, Chowdary P, Bevan DH, Mant T, Shima M, Engl W, Dyck-Jones J, Fuerlinger M, Patrone L, Ewenstein B, Abbuehl B. Pegylated, full-length, recombinant factor VIII for prophylactic and on-demand treatment of severe hemophilia A. *Blood*. 2025;126:1078–85.

[8] Klamroth R, Simpson M, von Depka-Prondzinski M, Gill JC, Morfini M, Powell JS, Santagostino E, Davis J, Huth-Kühne A, Leissinger C, Neumeister P, Bensen-Kennedy D, Feussner A, Limsakun T, Zhou M, Veldman A, St Ledger K, Blackman N, Pabinger I. Comparative pharmacokinetics of rVIII-SingleChain and octocog alfa (Advate(®) in patients with severe haemophilia A. *Haemophilia*. 2016;22:730–8.

[9] Nolan B, Mahlangu J, Perry D, Young G, Liesner R, Konkle B, Rangarajan S, Brown S, Hanabusa H, Pasi KJ, Pabinger I, Jackson S, Cristiano LM, Li X, Pierce GF, Allen G. Long-term safety and efficacy of recombinant factor VIII Fc fusion protein (rFVIIIFc) in subjects with haemophilia A. *Haemophilia*. 2016;22:72–80.

[10] Santagostino E, Martinowitz U, Lissitchkov T, Pan-Petesch B, Hanabusa H, Oldenburg J, Boggio L, Negrier C, Pabinger I, von Depka Prondzinski M, Altisent C, Castaman G, Yamamoto K, Álvarez-Roman MT, Voigt C, Blackman N, Jacobs I, PROLONG-9FP Investigators Study Group. Long-acting recombinant coagulation factor IX albumin fusion protein (rIX-FP) in hemophilia B: results of a phase 3 trial. *Blood*. 2016;127:1761–9.

[11] Baumann A. PEGylated biologics in haemophilia treatment: current understanding of their long-term safety. *Haemophilia*. 2020;26:e11–3.

[12] Bern M, Nilsen J, Ferrarese M, Sand KMK, Gjølberg TT, Lode HE, Davidson RJ, Camire RM, Bækkevold ES, Foss S, Grevys A, Dalhus B, Wilson J, Høydahl LS, Christianson GJ, Roopenian DC, Schlothauer T, Michaelsen TE, Moe MC, Lombardi S, et al. An engineered human albumin enhances half-life and transmucosal delivery when fused to protein-based biologics. *Sci Transl Med*. 2020;12:eabb0580. https://doi.org/10.1126/scitranslmed.abb0580

[13] Lombardi S, Aaen KH, Nilsen J, Ferrarese M, Gjølberg TT, Bernardi F, Pinotti M, Andersen JT, Branchini A. Fusion of engineered albumin with factor IX Padua extends half-life and improves coagulant activity. *Br J Haematol*. 2021;194:453–62.

[14] Dargaud Y, Leuci A, Ruiz AR, Lacroix-Desmazes S. Efanesoctocog alfa: the renaissance of factor VIII replacement therapy. *Haematologica*. 2024;109:2436–44.

[15] Aaen KH, Testa MF, Nilsen J, Tarantino R, Canepari C, Benedusi M, Benjakul S, Nyquist-Andersen M, Herigstad ML, Cantore A, Valacchi G, Sandlie I, Bernardi F, Pinotti M, Branchini A, Andersen JT. Tailored collagen binding of albumin-fused hyperactive coagulation factor IX dictates in vivo distribution and functional properties. *Nat Commun*. 2025;16:8433.

[16] Young G, Srivastava A, Kavakli K, Ross C, Sathar J, You CW, Tran H, Sun J, Wu R, Poloskey S, Qiu Z, Kichou S, Andersson S, Mei B, Rangarajan S. Efficacy and safety of fitusiran prophylaxis in people with haemophilia A or haemophilia B with inhibitors (ATLAS-INH): a multicentre, open-label, randomised phase 3 trial. *Lancet*. 2023;401:1427–37.

[17] Young G. Nonfactor therapies for hemophilia. *Hemasphere*. 2023;7:e911. https://doi.org/10.1097/HS9.0000000000000911

[18] Alcedo Andrade PE, Mannucci PM, Kessler CM. Emicizumab: the hemophilia A game-changer. *Haematologica*. 2024;109:1334–47.

[19] Sambe T, Miwa T, Yoneyama K, Ishida T, Yamazaki T, Shima M. A first-in-human study of NXT007, a next-generation, activated factor VIII-mimetic bispecific antibody, in healthy participants. *J Thromb Haemost*. 2025;23:3098–110.

[20] Van Thillo Q, Hermans C. Rebalancing agents in hemophilia: knowns, unknowns, and uncertainties. *Haematologica*. 2025;110:2902–12.

[21] Aledort LM. Factor VIII inhibitor bypassing activity (FEIBA)—addressing safety issues. *Haemophilia*. 2008;14:39–43.

[22] Shima M. Current status and future prospects of activated recombinant coagulation factor VIIa, NovoSeven®, in the treatment of haemophilia and rare bleeding disorders. *Ann Hematol*. 2024;103:2647–58.

[23] Herzog RW, Kaczmarek R, High KA. Gene therapy for hemophilia—from basic science to first approvals of "one-and-done" therapies. *Mol Ther*. 2025;33:2015–34.

[24] Camire RM. Bioengineering factor Xa to treat bleeding. *Thromb Res*. 2016;141(Suppl 2):S31–3.

[25] Bunce MW, Toso R, Camire RM. Zymogen-like factor Xa variants restore thrombin generation and effectively bypass the intrinsic pathway in vitro. *Blood*. 2011;117:290–8.

[26] Nguyen GN, George LA, Siner JI, Davidson RJ, Zander CB, Zheng XL, Arruda VR, Camire RM, Sabatino DE. Novel factor VIII variants with a modified furin cleavage site improve the efficacy of gene therapy for hemophilia A. *J Thromb Haemost*. 2017;15:110–21.

[27] Camire RM, Larson PJ, Stafford DW, High KA. Enhanced gamma-carboxylation of recombinant factor X using a chimeric construct containing the prothrombin propeptide. *Biochemistry*. 2000;39:14322–9.

[28] Ferrarese M, Pignani S, Lombardi S, Balestra D, Bernardi F, Pinotti M, Branchini A. The carboxyl-terminal region of human coagulation factor X as a natural linker for fusion strategies. *Thromb Res*. 2019;173:4–11.

[29] Branchini A, Baroni M, Burini F, Puzzo F, Nicolosi F, Mari R, Gemmati D, Bernardi F, Pinotti M. The carboxyl-terminal region is NOT essential for secreted and functional levels of coagulation factor X. *J Thromb Haemost*. 2015;13:1468–74.

[30] Matafonov A, Cheng Q, Geng Y, Verhamme IM, Umunakwe O, Tucker EI, Sun MF, Serebrov V, Gruber A, Gailani D. Evidence for factor IX-independent roles for factor XIa in blood coagulation. *J Thromb Haemost*. 2013;11:2118–27.

[31] Reynisson B, Alvarez B, Paul S, Peters B, Nielsen M. NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. *Nucleic Acids Res*. 2020;48:W449–54.

[32] Jespersen MC, Peters B, Nielsen M, Marcatili P. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res*. 2017;45:W24–9.

[33] Mertens K, Bertina RM. Pathways in the activation of human coagulation factor X. *Biochem J*. 1980;185:647–58.

[34] Jesty J, Spencer AK, Nakashima Y, Nemerson Y, Konigsberg W. The activation of coagulation factor X. Identity of cleavage sites in the alternative activation pathways and characterization of the COOH-terminal peptide. *J Biol Chem*. 1975;250:4497–504.

[35] Pipe SW, Collins P, Dhalluin C, Kenet G, Schmitt C, Buri M, Jiménez-Yuste V, Peyvandi F, Young G, Oldenburg J, Mancuso ME, Kavakli K, Kiialainen A, Deb S, Niggli M, Chang T, Lehle M, Fijnvandraat K. Emicizumab prophylaxis in infants with hemophilia A (HAVEN 7): primary analysis of a phase 3b open-label trial. *Blood*. 2024;143:1355–64.

[36] Erhardtsen E. Pharmacokinetics of recombinant activated factor VII (rFVIIa). *Semin Thromb Hemost*. 2000;26:385–91.

[37] Giles AR, Mann KG, Nesheim ME. A combination of factor Xa and phosphatidylcholine-phosphatidylserine vesicles bypasses factor VIII in vivo. *Br J Haematol*. 1988;69:491–7.

[38] Himmelspach M, Richter G, Muhr E, Varadi K, Turecek PL, Dorner F, Schwarz HP, Schlokat U. A fully recombinant partial prothrombin complex effectively bypasses fVIII in vitro and in vivo. *Thromb Haemost*. 2002;88:1003–11.

[39] Giles AR, Johnston M, Hoogendoorn H, Blajchman M, Hirsh J. The thrombogenicity of prothrombin complex concentrates: I. The

relationship between in vitro characteristics and in vivo thrombogenicity in rabbits. *Thromb Res*. 1980;17:353–66.

[40] Tans G, Janssen-Claessen T, Hemker HC, Zwaal RF, Rosing J. Meizothrombin formation during factor Xa-catalyzed prothrombin activation. Formation in a purified system and in plasma. *J Biol Chem*. 1991;266:21864–73.

[41] Gitel SN, Medina VM, Wessler S. Inhibition of human activated factor X by antithrombin III and alpha 1-proteinase inhibitor in human plasma. *J Biol Chem*. 1984;259:6890–5.

[42] Bode W, Mayr I, Baumann U, Huber R, Stone SR, Hofsteenge J. The refined 1.9 A crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J*. 1989;8:3467–75.

[43] Toso R, Zhu H, Camire RM. The conformational switch from the factor X zymogen to protease state mediates exosite expression and prothrombinase assembly. *J Biol Chem*. 2008;283:18627–35.

[44] Ivanciu L, Camire RM. Hemostatic agents of broad applicability produced by selective tuning of factor Xa zymogenicity. *Blood*. 2015;126:94–102.

[45] Ivanciu L, Toso R, Margaritis P, Pavani G, Kim H, Schlachterman A, Liu JH, Clerin V, Pittman DD, Rose-Miranda R, Shields KM, Erbe DV, Tobin JF, Arruda VR, Camire RM. A zymogen-like factor Xa variant corrects the coagulation defect in hemophilia. *Nat Biotechnol*. 2011;29:1028–33.

[46] Thalji NK, Ivanciu L, Davidson R, Gimotty PA, Krishnaswamy S, Camire RM. A rapid pro-hemostatic approach to overcome direct oral anticoagulants. *Nat Med*. 2016;22:924–32.

[47] Parng C, Markiewicz V, Chen J, Leary B, Duriga N, Dyleski L, Caiazzo T, Bolt M, Joyce A, Gorovits B, Pittman DD, Webster R. Preclinical pharmacokinetics, pharmacodynamics, tissue distribution, and interspecies scaling of recombinant human coagulation factor XaI16L. *J Pharm Sci*. 2017;106:2136–43.

[48] Parsons-Rich D, Hua F, Li G, Kantaridis C, Pittman DD, Arkin S. Phase 1 dose-escalating study to evaluate the safety, pharmacokinetics, and pharmacodynamics of a recombinant factor Xa variant (FXaI16L). *J Thromb Haemost*. 2017;15:931–7.

[49] Silva Blas Y, Diringer MN, Lo B, Masjuan J, Pérez de la Ossa N, Cardinal M, Yong F, Zhu T, Li G, Arkin S. Phase 1b study to evaluate safety, tolerability, and maximum tolerated dose of PF-05230907 for intracerebral hemorrhage. *Stroke*. 2021;52:294–8.

[50] Louvain-Quintard VB, Bianchini EP, Calmel-Tareau C, Tagzirt M, Le Bonniec BF. Thrombin-activable factor X re-establishes an intrinsic amplification in tenase-deficient plasmas. *J Biol Chem*. 2005;280:41352–9.

[51] Abache T, Fontayne A, Grenier D, Jacque E, Longue A, Dezetter AS, Souilliart B, Chevreux G, Bataille D, Chtourou S, Plantier JL. A mutated factor X activatable by thrombin corrects bleedings in vivo in a rabbit model of antibody-induced hemophilia A. *Haematologica*. 2020;105:2335–40.

[52] Ebert M, Raquet E, Schweisgut S, Schmidt PM, Weimer T. Identification of a modified coagulation factor X with enhanced activation properties as potential hemostatic agent. *Blood Cells Mol Dis*. 2021;89:102570. https://doi.org/10.1016/j.bcmd.2021.102570

SUPPLEMENTARY MATERIAL

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