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REVIEW ARTICLE

Recombinant factor VIIa: new insights into the mechanism of action through product innovation

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Abstract

Management of bleeding in persons with hemophilia and inhibitors involves treatment with bypassing agents, including recombinant activated factor VII (rFVIIa). Two rFVIIa products are commercially approved for use in the United States and the European Union. Eptacog alfa and eptacog beta share the same amino acid sequence but differ in posttranslational modifications. Although rFVIIa has been used to manage bleeding in persons with hemophilia and inhibitors for over 30 years, its mechanisms of action is still being studied. *In vitro* and *in vivo* studies have suggested that rFVIIa could promote hemostasis by (1) increasing tissue factor-dependent activation of factor (F)X (FX); (2) directly activating FX on the surface of activated platelets; and (3) downregulating protein C anticoagulant activity through binding to the endothelial protein C receptor (EPCR). Studies of rFVIIa and rFVIIa variants in murine models demonstrate that platelet-dependent activity is sufficient for hemostatic efficacy. Dosing levels required in clinical practice are most consistent with a platelet-dependent mechanism of action. However, *in vivo* models also suggest that pathways involving EPCR binding contribute to rFVIIa hemostatic activity. Eptacog beta displays increased platelet- and EPCR-dependent endothelial cell binding compared to eptacog alfa. Thus, the relative

contribution of these mechanisms to the overall hemostatic efficacy of eptacog alfa and eptacog beta may differ. Further research is required to assess the clinical relevance of these differences. A better understanding of the mechanisms by which rFVIIa promotes hemostasis in patients will provide insights when evaluating clinical outcomes of safety and efficacy for innovative bypassing therapies.

KEYWORDS
blood platelets, endothelial protein C receptor, factor VIIa, hemophilia A, hemophilia B, hemostasis, tissue factor

Essentials

- rFVIIa helps stop bleeding in hemophilia patients with inhibitors.
- The majority of rFVIIa activity involves a platelet-dependent FX activation.
- Tissue factor and EPCR binding may contribute to rFVIIa hemostatic activity.
- rFVIIa products, eptacog alfa and eptacog beta, differ in platelet and EPCR binding.

1 | INTRODUCTION

Modern hemophilia treatments reduce bleeding complications, prevent orthopedic impairment, and improve health-related quality of life [1]. For persons with hemophilia without inhibitors, numerous options are available to treat and reduce the incidence of bleeding events, including plasma-derived factor concentrates, standard and extended half-life recombinant products, nonfactor therapies, and gene therapy [2]. Treatment selection can be tailored based on pharmacokinetics, bleeding history, or lifestyle. For breakthrough bleeding, novel extended half-life factor (F)VIII (FVIII) and FIX products have reported rates of single-dose effective bleed control between 83.6% and 97% [3–6].

1.1 | Bypassing agents

The most challenging complication of hemophilia is the development of neutralizing antibodies, or inhibitors, against infused FVIII/FIX. The lifelong risk of inhibitor development is up to 30% for severe hemophilia A and 10% for severe hemophilia B [7,8]. Risk factors include genetic predisposition, intense exposure to factor concentrates, race/ethnicity, and the factor concentrate source. Historically, persons with hemophilia and inhibitors face an increased risk of morbidity secondary to orthopedic complications, reduced quality of life, and increased mortality compared to noninhibitor patients [9–11].

Emicizumab (HEMLIBRA, Hoffman-La Roche) is a humanized bispecific antibody mimetic of activated FVIII (FVIIIa). It shares no structural similarity to FVIII and is not inhibited by anti-FVIII antibodies. However, this therapy is limited to persons with hemophilia A, is only effective for prophylaxis, does not treat breakthrough bleeding, and does not provide sufficient hemostasis for all surgeries and invasive procedures [12]. Thus, for persons with hemophilia and inhibitors, bypassing agents are required to manage and prevent acute bleeding events or for perioperative bleeding control. Until the recombinant activated factor VIIa (rFVIIa) eptacog beta (SEVENFACT/CEVENFACTA, HEMA Biologics, LLC, and LFB SA) was approved by the Food and Drug Administration (FDA) in 2020 for the treatment of bleeding in persons 12 years and older with hemophilia and inhibitors [13], treatment options were limited to plasma-derived activated prothrombin complex concentrate (APCC, FEIBA, Takeda) and the rFVIIa eptacog alfa (NOVOSEVEN RT, Novo Nordisk). APCC was the first FDA-approved bypassing agent for treating bleeding in persons with hemophilia and inhibitors in 1986 [14]. In 1999, eptacog alfa became the first FDA-approved recombinant bypassing agent [15]. An eptacog alfa biosimilar and plasma-derived FVIIa/FX similar products for bypassing are available in other limited geographic regions [16,17].

In a single head-to-head clinical study, the overall efficacy of APCC and eptacog alfa were similar, although inter- and intrasubject responses were variable [18]. The single-dose efficacies of APCC (50-100 U/kg, 36%-52%) and eptacog alfa (90-270 mcg/kg, 10%-59%), when used on-label, also showed significant variability [19–23]. Some bleeding events are refractory to treatment with either single agent APCC or eptacog alfa. In these instances, the sequential use of these products can be effective [18,24,25]. Both APCC and eptacog alfa have a good safety profile, with thrombotic adverse events considered rare when products are used as indicated in the absence of other hemostatic agents [26,27]. However, sequential treatment can increase thrombotic risk and must be carefully monitored [26,28]. In addition, concurrent use of APCC and emicizumab (at ≥100 U/kg/day for ≥24 hours) is associated with venous thrombosis or microangiopathy [29], limiting treatment options for these patients.

1.2 | Novel rFVIIa therapies

Historically, inhibitors have posed a barrier to health equity by limiting the opportunity to attain the highest possible level of health. Persons with hemophilia and inhibitors have access to fewer therapeutic choices when compared to those without inhibitors. Attempts have been made to expand rFVIIa treatment options in the case of concerns regarding efficacy, safety, or supply of currently available products. Novel rFVIIa agents with predictable efficacy outcomes and a mechanistic profile that differs from other available products may lead to better hemostasis, improved control of refractory bleeding events, or decreased treatment burden. Two investigational rFVIIa agents with specific amino acid substitutions, vatreptacog alfa (Novo Nordisk) and BAY 86-6150 (Bayer), had increased activity. However, both studies were discontinued due to the formation of antidrug antibodies [19,30]. N7-GP (Novo Nordisk), a glycoPEGylated rFVIIa with an extended half-life designed for prophylaxis, failed in clinical trials due to inadequate efficacy that reasonable increases in dose could not overcome [31]. Marzeptacog alfa (GC Biopharma) has demonstrated increased *in vitro* procoagulant activity and prolonged half-life after subcutaneous administration [32], but clinical studies were terminated.

1.3 | Eptacog beta

Eptacog beta was the first rFVIIa licensed for treating bleeding events in persons with hemophilia and inhibitors in the United States, United Kingdom, Mexico, and European Union following the approval of eptacog alfa over 20 years prior [13]. Eptacog beta has 2 licensed initial dose regimens, as well as a dosing regimen for severe bleeds [13,33]. Based on observed differences in product profiles, eptacog beta is not considered a biosimilar of eptacog alfa by the FDA, which requires a product to be highly similar and have no clinically meaningful differences in safety, purity, and potency from an existing approved treatment [34].

In a phase 3 clinical study, eptacog beta on-demand treatment achieved dose-dependent efficacy for 82% (confidence interval: 72%-91%) and 91% (confidence interval: 84%-98%) of mild/moderate bleeding events at 12 hours after initial infusion using the 75 mcg/kg and 225 mcg/kg initial dose regimens, respectively [13]. Hemostasis was achieved for 84% of bleeding events with a single 225 mcg/kg dose. For the 75 mcg/kg and 225 mcg/kg initial dose regimens 97.6% and 99.5% of bleeding episodes, respectively, did not require the use of alternative bypassing agents [13]. No incidents of thrombosis, neutralizing anti-rFVIIa antibodies, allergic, hypersensitivity, or anaphylactic reactions were observed in adult and pediatric populations [35].

Mechanistic studies of rFVIIa have been ongoing for decades with novel insights generated in recent years. Understanding the pathways through which rFVIIa promotes hemostasis will potentially provide insight into the clinical efficacy and safety of these products. This review describes differences in structure and binding activity of the currently available rFVIIa therapeutic agents and up-to-date knowledge regarding its mechanism of action.

2 | FVIIa STRUCTURE

FVII is a member of the vitamin-K-dependent family of plasma proteins characterized by the posttranslational modification of glutamic acid residues to γ-carboxyglutamic acid (Gla) at well-conserved positions [36,37]. It circulates with a half-life of 4 to 6 hours. FVII undergoes additional posttranslational modifications, including *N*- and *O*-linked glycosylation, and β-hydroxylation (Figure 1) [38,39]. These posttranslational modifications are necessary for secretion and mediating interactions between FVII and tissue factor (TF) or platelet-surface phospholipids [40–42].

FVII contains 4 domains: an N-terminal Gla domain, 2 epidermal growth factor (EGF1 and EGF2)-like domains, and a C-terminal serine protease domain (Figure 1). A single cleavage of FVII at the Arg152-Ile153 peptide bond results in serine protease activation. The active protease (FVIIa) consists of a light and a heavy chain connected by a disulfide bond between Cys135 and Cys262 [37]. The light chain contains the Gla domain that mediates phospholipid membrane binding and EGF1 and EGF2 domains that facilitate interactions between FVIIa and TF, FX, and FIX. The heavy chain contains the serine protease domain responsible for enzymatic activity. Eptacog alfa and eptacog beta share the same amino acid sequence and protein structure as endogenous FVIIa.

2.1 | rFVIIa *N*- and *O*-linked glycans

Protein *N*- and *O*-linked glycosylation involves the covalent attachment of carbohydrate structures to asparagine and serine/threonine residues of a polypeptide, respectively. Glycans influence the physical conformation, solubility, ligand binding, protease activity, immunogenicity, and clearance of coagulation serine proteases [43–45]. For recombinant proteins, the species, cell type, and culture conditions in which the proteins are produced may impact glycosylation patterns [43]. A study of plasma-derived FVII and rFVIIa produced in baby hamster kidney (BHK), Chinese hamster ovary, and human embryonic kidney 293 cells reported each product had different *N*-linked glycan structures [46]. The effects of such differences on protein function cannot be predicted by structure analysis alone and necessitate experimental evaluation [45].

Eptacog alfa and eptacog beta are produced in different organisms and cell types. Eptacog alfa is expressed by cultured BHK cells transfected with the human *F7* cDNA [47]. rFVII is secreted into the media and activated during purification. In contrast, eptacog beta is produced by innovative rPro technology, where transgenic expression of the *F7* cDNA under the β-casein promoter is induced in the New Zealand rabbit mammary gland and secreted into the milk [48,49]. Protease activation occurs during the purification process. Studies of

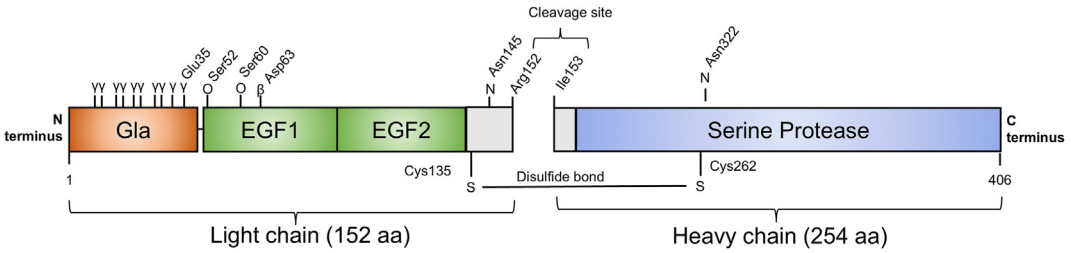

FIGURE 1 The protein domain structure of recombinant activated factor VII (rFVIIa). Factor (F)VII is a vitamin-K-dependent glycoprotein characterized by the posttranslational γ-carboxylation of glutamic acid residues at conserved locations. FVII undergoes several other posttranslational modifications including *N*- and *O*-linked glycosylation and β-hydroxylation. FVII contains 4 domains: an *N*-terminal Gla domain, 2 epidermal growth factor (EGF1 and EGF2)-like domains, and a C-terminal serine protease domain. Activation of FVII involves a single cleavage at the Arg152-Ile153 peptide bond, resulting in the formation of the active serine protease, FVIIa. FVIIa consists of a light and a heavy chain linked by a disulfide bond between Cys135 and Cys262. The light chain contains the Gla domain that mediates phospholipid membrane binding and EGF1 and EGF2 domains that facilitate interactions between FVIIa and TF, FX, and FIX. The heavy chain contains the serine protease domain responsible for enzymatic activity. EGF, epidermal growth factor; aa, amino acids; γ, γ carboxyglutamic acids; S–disulfide bond; O, *O*-linked glycan; N, *N*-linked glycan; β, β hydroxylation.

the *N*-linked glycans of eptacog beta have shown batch-to-batch reproducibility after pooling of raw materials [49].

Eptacog alfa and eptacog beta carry 2 *O*-linked glycans at Ser52 and Ser60 within the EGF1 domain [39,49]. Ser52 is modified in approximately equal proportions by glucose, glucose-xylose, and glucose-(xylose)2, while Ser60 is modified by *O*-linked fucose (Figure 2A) [50,51]. The relative proportion of each structure at Ser52 can vary slightly between products [16,49].

Eptacog alfa and eptacog beta carry 2 *N*-linked glycan structures located at Asn145 adjacent to the EGF2 domain, and Asn322 in the serine protease domain. The primary *N*-linked glycans expressed on eptacog alfa are complex-type, partially sialylated, core-fucosylated biantennary and triantennary structures (Figure 2B) [16]. The *N*-linked glycans on eptacog beta comprise high-mannose, complex, and hybrid-type glycans (Figure 2C) [49]. The majority of structures were biantennary, partially fucosylated, and partially sialylated. The influence of the variances in *N*-linked glycan structures on the functional activity of rFVIIa is not fully understood.

2.2 | rFVIIa γ-carboxylation and β-hydroxylation

The Gla domain of FVII contains 10 glutamic acid residues that undergo vitamin K-dependent γ-carboxylation to form γ-carboxyglutamic acid [37,39]. The negatively charged Gla residues bind calcium ions, locking the Gla domain into a conformation that can tightly bind cell surface phospholipids [52]. BHK-derived rFVIIa is fully γ-carboxylated at the first 9 Gla residues, and partially (~50%) γ-carboxylated at the final residue, Glu35 [47]. Eptacog beta is fully γ-carboxylated at the first 9 Gla residues and not at Glu35 [49]. γ-carboxylation of Glu35 does not alter TF or calcium-binding to rFVIIa [53].

A potential β-hydroxylation site exists at Asp63 in the EGF1 domain of FVII [38]. Additionally, 15% of BHK-derived rFVIIa and 4% of eptacog beta is β-hydroxylated at Asp63 [46,54]. The functional effects of these differences are unknown.

3 | INTRODUCTION TO rFVIIa-MEDIATED PROCOAGULANT ACTIVITY AT THE SITE OF VASCULAR INJURY

The hemostatic response to vascular injury involves primary hemostasis, the formation of a platelet plug to stop the initial bleeding, and secondary hemostasis, the activation of the coagulation cascade to form cross-linked fibrin and stabilize the platelet plug. Secondary hemostasis can be conceptualized as occurring in 3 overlapping phases: initiation, amplification, and propagation [55]. The initiation phase involves the activation of coagulation by TF-FVIIa in response to vessel injury. During amplification, small amounts of thrombin generated in the initiation phase fully activate platelets, increase platelet adhesion at the site of vascular injury, and activate FV, FVIII, and FXI. The propagation phase involves a rapid burst of thrombin generation and fibrin formation driven by the activity of the intrinsic tenase (FIXa/FVIIIa) and prothrombinase enzyme complexes (FXa/FVa) on the activated platelet surface.

Global coagulation functional assays have confirmed the procoagulant activity of rFVIIa in FVIII/FIX-deficient plasma. *Ex vivo* analysis of samples from nonbleeding subjects with hemophilia infused with rFVIIa demonstrated increased peak thrombin and reduced lag time and time to peak as measured by thrombin generation assay and improved clot firmness by rotational thromboelastometry (ROTEM), indicating that rFVIIa can promote secondary hemostasis and the formation of fibrin [56,57]. Fibrin clots produced *ex vivo* with therapeutic levels of rFVIIa in FVIII/FIX-deficient plasmas evaluated by scanning electron microscopy and turbidimetric assays display a more rapid onset of clot formation, increased resistance to lysis, and improved structure compared with clots produced without rFVIIa [58,59]. rFVIIa-mediated thrombin generation increases platelet activation and adhesion in a flow chamber model, contributing to primary hemostasis [60].

It is commonly accepted that rFVIIa mediates its hemostatic effects by increasing thrombin generation, however, the contributing

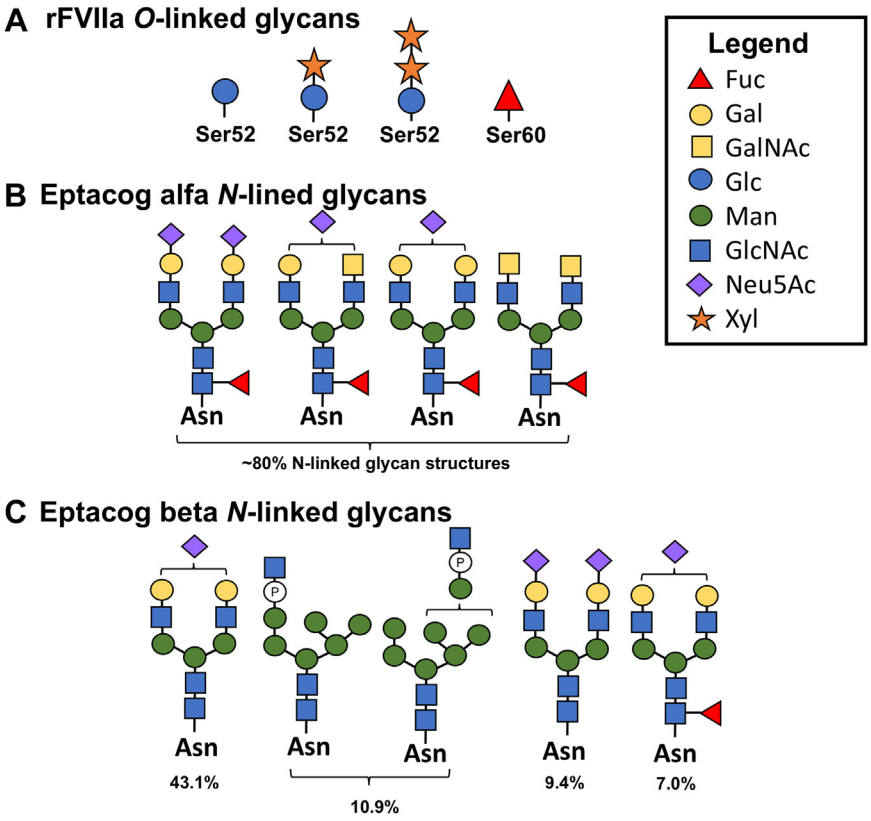

FIGURE 2 *O*- and *N*-linked glycans of recombinant activated factor VII (rFVIIa). (A) rFVIIa carries similar *O*-linked glycans [16,49,50]. (B) Eptacog alfa contains complex-type, partially sialylated, core-fucosylated biantennary and triantennary *N*-linked glycans [16]. (C) Eptacog beta contains high-mannose, complex, and hybrid-type partially fucosylated biantennary *N*-linked glycans [49]. The *N*-linked glycans of eptacog alfa and eptacog beta differ based on the presence of GalNAc-containing glycans and increased core-fucosylation on eptacog alfa, and the presence of high-mannose glycans expressed on eptacog beta [16,49]. Ser, serine; Asn, asparagine; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine; Neu5Ac, acetylneuraminic acid (sialic acid); Xyl, xylose.

pathways are the subject of continued investigation and debate. rFVIIa has been reported to exert its procoagulant effects through the initiation (TF) and propagation (tenase) phases of coagulation in hemophilia [61,62]. Additionally, recent evidence suggests that rFVIIa interactions with the endothelial protein C receptor (EPCR) can downregulate protein C (PC) activation and promote the formation of procoagulant endothelial extracellular vesicles [62,63].

3.1 | TF-rFVIIa extrinsic tenase activity

Endogenous FVII circulates in plasma in both an active (FVIIa, ~1%) and inactive forms (FVII, ~99%) [37,64]. TF, a transmembrane protein, is normally expressed on cells outside the vasculature [65]. FVII and FVIIa bind TF with similar high affinity following vascular injury [66]. Once FVII binds to cell-associated TF, it is rapidly converted to FVIIa by limited proteolysis [67]. Perivascular TF can exist in a complex with FVII/FVIIa even in the absence of injury [68], where it can rapidly activate coagulation upon exposure to the blood. Binding to TF enhances the enzymatic activity of FVIIa by up to 10^6-fold [37,69].

Cell-associated FVIIa/TF complexes ("extrinsic tenase") activate FX to FXa and FIX to FIXa (Figure 3) [70]. The FXa remains on the surface of the TF-bearing cell and, in the presence of its cofactor FVa, converts small amounts of prothrombin to thrombin. This small amount of thrombin activates platelets, FVIII, FV, and FXI in the amplification step of hemostasis but is not sufficient to form a durable hemostatic fibrin clot [55]. Activated platelets provide the major procoagulant membrane surface for hemostatic thrombin generation. Following platelet activation, FVIIIa, FVa, and FXIa remain associated with the activated platelet surface. FIXa can diffuse from TF-bearing cells to adjacent platelets, as well as being formed directly on the activated platelet surface by FXIa. On activated platelets, FIXa and FVIIIa form the "intrinsic tenase" which efficiently converts FX to FXa [37]. The FXa binds to FVa and supports large-scale thrombin generation on platelet surfaces during the propagation phase of hemostasis [55].

In hemophilia, FVIIIa/FIXa activity is absent, FX cannot be activated directly on platelets, and the platelet surface thrombin burst does not occur. Most of the FXa activated by FVIIa/TF is inhibited in the fluid phase by plasma protease inhibitors antithrombin and tissue factor pathway inhibitor (TFPI), limiting its diffusion to the platelet surface. This reduces the ability of FVIIa/TF on nearby cells to supply sufficient FXa to support a normal burst of thrombin generation on platelet surfaces, thereby limiting the formation of fibrin and impairing stable clot formation in response to vessel injury.

In the presence of inhibitors, infused rFVIIa was initially thought to bypass the impaired intrinsic (FVIII/FIX) tenase complex by increasing FXa generation through the extrinsic tenase (TF) pathway. Some *in vitro* thrombin generation studies and mathematical models have suggested that most rFVIIa procoagulant activity involves TF [71–73]. However, clinical studies clearly demonstrate that massively supraphysiologic levels of rFVIIa (~250-fold above endogenous FVIIa levels) are required to achieve hemostatic efficacy in hemophilia with inhibitors [13,15]. This clinical observation casts doubt on a TF-mediated mode of action, since the concentration of FVIIa required

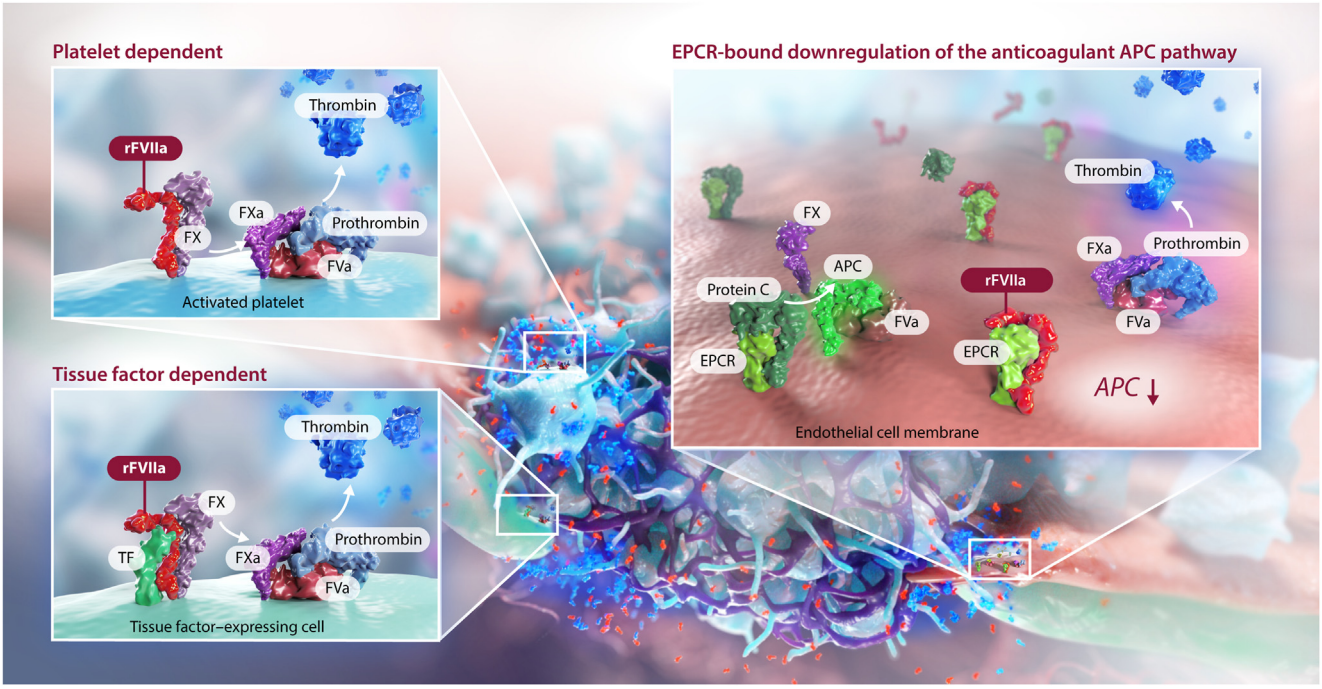

FIGURE 3 Mechanisms of action of recombinant activated factor VII (rFVIIa). rFVIIa has been proposed to exert hemostatic efficacy through tissue factor (TF)-dependent, platelet-dependent, and endothelial cell protein C receptor (EPCR)-dependent pathways. Upon vascular injury, circulating rFVIIa binds TF expressed by subendothelial cells and activates the coagulation cascade by converting coagulation factor (F)X (FX) to FXa. FXa, along with FVa and prothrombin form the prothrombinase complex on active platelets, generating small amounts of thrombin. Thrombin amplifies the activation of coagulation by fully activating platelets that had been partially activated through exposure to collagen at the site of vessel injury, and by activating FV, FVIII, and FXI, leading to amplification of coagulation. Platelet-bound rFVIIa can directly activate FX, enhancing prothrombinase activity and leading to a burst of thrombin generation and fibrin formation. The binding of rFVIIa to EPCR can attenuate the formation of activated protein C by the thrombin-thrombomodulin complex, thereby impairing the inactivation of FVa and FVIIIa and enhancing thrombin generation. EPCR, endothelial cell protein C receptor; rFVIIa, recombinant activated factor VII; TF, tissue factor.

for hemostatic efficacy is approximately 2 orders of magnitude greater than the Kd for TF binding to FVIIa, which is in the low nanomolar or subnanomolar range [74].

Another proposed TF-dependent mechanism suggested that endogenous FVII can inhibit TF-mediated FVIIa activity and that higher levels of rFVIIa can reverse this effect [71,73,75]. Reversal of this "zymogen inhibition" is maximal at 10 nM rFVIIa [71], again well below the level of rFVIIa required for hemostatic efficacy in clinical settings. Autoactivation of zymogen FVII bound to TF has been reported to be sufficient to offset the inhibitory effects of endogenous FVII [76]. "Zymogen inhibition" has only been demonstrated in biochemical experiments using purified TF relipidated into phospholipid vesicles and is not observed when cell-associated TF is used [77]. Recent studies of rFVIIa variants with modified TF-dependent activity (described in Section 3.2) have additionally cast doubt on the TF-dependent contribution to the hemostatic activity of rFVIIa.

3.1.1 | TF-binding activity of eptacog alfa and eptacog beta

Previous studies have suggested that FVII *N*-linked glycans can modulate the binding activity to TF [41]. However, eptacog alfa and eptacog beta bind to soluble and full-length TF with similar affinity (Table) [78,79]. Studies using recombinant TF suggest that both products display a similar ability to activate FIX and FX substrates and generate thrombin in hemophilia A plasmas. To date, the relative activity of eptacog alfa and eptacog beta on the surface of TF-expressing cells has not been reported.

3.2 | rFVIIa-tenase activity on activated platelets

Bom and Bertina were the first to report that FVIIa can activate FX on negatively charged phospholipid surfaces independent of TF [80], and Rao and Rapaport suggested that this interaction might be responsible for the hemostatic activity of rFVIIa in hemophilia [81]. Subsequently, therapeutic levels of rFVIIa were found to bind the surface of activated platelets and activate FIX or FX in the presence of physiological levels of coagulation factors [82–84]. FXa on the platelet surface forms the prothrombinase complex with FVa, rapidly generating a burst of thrombin at levels sufficient to promote fibrin formation and a hemostatic clot (Figure 3). Since activated platelets localize to the site of vessel injury, rFVIIa activation of FX is confined to the growing clot. The low-affinity binding of the rFVIIa Gla domain to platelet phosphatidylserine has been hypothesized to mediate this interaction [40],

TABLE The structure, function, and binding activity of eptacog alfa and eptacog beta.

	Eptacog alfa	Eptacog beta
Protein production, structure, and posttranslational modifications		
Expression	Baby hamster kidney (BHK) cells [15]	New Zealand rabbits mammary gland [13]
Primary sequence identical to plasma-derived FVII	Yes [79]	Yes [78]
O-linked glycans identical to plasma-derived FVII	Yes [78]	Yes [49]
N-linked glycans	Partially sialylated, core-fucosylated, complex-type, biantennary, and triantennary structures[16]	Partially sialylated, partially fucosylated, high-mannose, complex, and hybrid-type biantennary structures [49]
γ-carboxylation	~50% γ-carboxylated at Glu35 [47]	0% γ-carboxylated at Glu35 [49]
Functional assays		
Active site titration (moles active site/moles protein)	0.7 [78]	0.7 [78]
aPTT (relative)	—	Identical to eptacog alfa [54]
Thrombin generation (relative)	—	Identical to eptacog alfa [54]
Activation of FIX (k_{cat}/K_m)	3.9 s^{-1}/0.6 μM [54]	3.3 s^{-1}/0.44 μM [54]
Activation of FX (k_{cat}/K_m)	10.1 s^{-1}/0.26 μM [54]	10.5 s^{-1}/0.32 μM [54]
Binding activities		
Soluble TF binding (K_d)	0.8 nM [78]	0.9 nM [78]
Activated platelet binding (K_d)	0.94 μM [78]	1.2 μM [78]
Activated platelet binding at saturation (B_{max}) (relative)	—	Approximately 40% higher than eptacog alfa [78]
Soluble EPCR binding (K_d)	36.9 nM [78]	40.6 nM [78]
HUVEC binding (relative)	—	Approximately 25% to 40% higher than eptacog alfa [78]
Inactivation by antithrombin (apparent second-order rate constant)	5.7×10^3 M^{-1} s^{-1} [78]	5.9×10^3 M^{-1} s^{-1} [78]

aPTT, activated partial thromboplastin time; B_{max}, maximum specific binding; EPCR, endothelial protein C receptor; FVII, factor VII; HUVEC, human umbilical vein endothelial cells; k_{cat}, catalytic constant; K_d, dissociation constant; K_m, Michaelis-Menten constant; TF, tissue factor.

which may also involve platelet-expressed glycoprotein Ib-alpha (GPIbα) and EPCR [85,86].

Platelet-dependent rFVIIa activation of FX has also been investigated in animal models. Holmberg et al. [87] generated rFVIIa variants with increased platelet-dependent activity, but similar TF-dependent activity compared to eptacog alfa. Using a severe murine tail vein bleeding model in antibody-induced hemophilia A mice, the most potent of these variants reduced blood loss by 91%, as compared with eptacog alfa which reduced blood loss by 69%. Thus, an increase in TF-independent activity correlated with an increase in preclinical efficacy. Conversely, a chimeric murine FIX/FVIIa molecule (combining the Gla and EGF1 domains of FIX with the EGF2 and catalytic domains of FVII) is unable to bind TF, yet exhibited equal hemostatic efficacy to wild-type murine rFVIIa in hemophilia B mice [88]. This study demonstrated that, at least in hemophilic mice, TF-dependent activity was not necessary for the hemostatic effect of rFVIIa.

Keshava et al. [89] examined the influence of TF levels on the therapeutic effectiveness of rFVIIa. Using the saphenous vein bleeding assay in an antibody-induced model of hemophilia A, no significant differences in rFVIIa hemostatic effects were observed in mice expressing low levels of human TF (1%-20%) compared to mice with normal levels of human TF, and wild-type mice. More recently, rFVIIa-mediated thrombin generation in response to a vessel injury was examined in hemophilic mice with normal platelet levels compared to thrombocytopenic mice [90]. rFVIIa increased thrombin-antithrombin complexes levels in both groups of mice, although the increase was greater in mice with normal platelet levels. Overall, these data support platelet-dependent FX activation as a dominant mechanism by which rFVIIa promotes hemostasis in mouse hemophilia models.

The contribution of platelet-dependent tenase activity to the hemostatic efficacy of rFVIIa in human studies has not been directly tested. The finding that the efficacy of rFVIIa increased with dose escalation to levels of 100 nM or more tends to support a platelet-dependent mechanism in humans. Studies demonstrating interindividual variability of isolated human platelets in supporting tenase and prothrombinase activities may explain the interindividual variability in

hemostatic response to rFVIIa [91]. Vatreptacog alfa, an engineered rFVIIa with 3 amino acid changes (V158D/E296V/M298Q) designed to enhance platelet-dependent tenase activity, was developed as a therapeutic agent [92]. *In vitro* studies demonstrated that vatreptacog alfa displayed a ~30-fold increase in FX activation and increased platelet-dependent thrombin generation *in vitro* as compared with wild-type rFVIIa [92]. In phase 3 clinical studies, vatreptacog alfa demonstrated non-inferiority of effective bleed control at 12 hours and better secondary efficacy compared to eptacog alfa [19]. However, the study was terminated early due to the development of antidrug antibodies before the apparent greater potency of vatreptacog alfa could be fully evaluated [79]. While not ruling out a TF-dependent effect, the current evidence suggests that a platelet surface mechanism plays the major role in rFVIIa efficacy in persons with hemophilia.

3.2.1 | Platelet binding activity of eptacog alfa and eptacog beta

Comparative studies have shown that both eptacog alfa and eptacog beta bind to activated platelets in the presence of calcium in a dose-dependent manner [78]. Consistent with other studies that suggest rFVIIa binds with low affinity to platelet surface constituents such as phosphatidylserine, eptacog alfa and eptacog beta bind to activated platelets with a K_d of 0.94 μM and 1.2 μM, respectively (Table) [78]. Importantly, the maximum binding (B_{max}) at saturation was approximately 40% higher for eptacog beta as compared with eptacog alfa, suggesting that platelets may have a larger number of binding sites available for eptacog beta than eptacog alfa.

3.3 | EPCR-binding activity of rFVIIa

EPCR was first characterized as the cell surface receptor for the zymogen PC and activated PC (APC) [93]. Binding of the PC Gla domain to EPCR localizes PC to the endothelial cell surface, where it is subsequently activated by thrombomodulin-bound-thrombin, forming APC. APC, an endogenous anticoagulant, downregulates thrombin generation by inactivating FVa and FVIIIa [93]. Therapeutic levels of rFVIIa bind to EPCR with an affinity comparable to PC (~40 nM) [94]. This interaction is mediated by the Gla domain of rFVIIa, which shares strong homology with the Gla domain of PC [95]. The binding of rFVIIa to EPCR impairs binding of PC to EPCR and reduces APC formation by approximately 40% (Figure 3) [94,95].

Mouse models have provided insight into the influence of rFVIIa-EPCR binding on APC generation. Infusion of high-dose active site-inhibited rFVIIa (which binds EPCR and TF but does not activate FX) increased PC plasma levels in EPCR-overexpressing mice, suggesting that therapeutic levels of rFVIIa can displace EPCR-bound PC [96]. A bioengineered murine FVIIa variant with enhanced murine EPCR-binding activity exhibited increased hemostatic efficacy in an EPCR-dependent manner compared with wild-type murine FVIIa [97]. EPCR-overexpressing mice with antibody-induced hemophilia A treated with rFVIIa had significantly increased blood loss and time to achieve hemostasis compared with mice expressing normal EPCR levels [98]. In addition, antibody-induced hemophilia A mice who received high-dose active site-inhibited rFVIIa, and *F8* knockout mice who received an anti-EPCR blocking antibody required less rFVIIa to achieve hemostasis than controls [98,99]. Collectively, these *in vivo* animal models suggest that rFVIIa-EPCR binding results in the displacement of PC from ECPR and impairs the activation of the anticoagulant APC, thereby enhancing thrombin generation and hemostatic clot formation.

While the evidence that EPCR contributes to the mechanism of action of rFVIIa is limited to *in vitro* data and animal models, the direct inhibition of APC can regulate bleeding in preclinical and clinical studies [100]. The recombinant serine protease inhibitor SerpinPC, which specifically inhibits APC, enhances *in vitro* thrombin generation and thrombus formation in hemophilia B mice [101]. Preliminary results from a phase 2A clinical study demonstrate that prophylactic use of SerpinPC reduces annualized bleeding rates in persons with hemophilia when compared with a pre-exposure observation period [102].

3.3.1 | Binding of eptacog alfa and eptacog beta to EPCR

Surface plasmon resonance binding analyses have demonstrated that both eptacog alfa and eptacog beta bind soluble EPCR with similar affinity (Table) [78]. However, a human umbilical vein endothelial cell (HUVEC)-based cell surface binding assay, which assessed 2 doses of rFVIIa to represent a range of therapeutic plasma concentrations, demonstrated that eptacog beta displayed a 25% to 40% increase in binding activity compared to eptacog alfa [78]. Quantitative ELISA analysis of HUVEC lysates confirmed approximately 40% more eptacog beta molecules were bound per cell than eptacog alfa [78]. The binding of eptacog alfa and eptacog beta to HUVECs was attenuated by ~50% by a 10-fold excess of PC or soluble EPCR [78]. Together, these data suggest eptacog alfa and eptacog beta have different EPCR-dependent endothelial cell binding activities, requiring further study to better understand the potential clinical significance.

3.4 | Endocytosis of rFVIIa by EPCR

Ligand binding to EPCR induces receptor-mediated endocytosis of the complex through a caveolar- and dynamin-dependent pathway [103]. This process returns EPCR and its ligands to the cell surface through recycling endosomes. Endocytosis of rFVIIa by EPCR also transports rFVIIa from the apical to the basal side of the endothelial cell [103]. In mouse models, rFVIIa is transported and redistributed to extravascular tissues such as the kidney, liver, and bone joints (calcified cartilage) in an EPCR-dependent manner [104]. Both BHK-derived FVIIa and eptacog beta accumulate and are retained in the bone and joints of hemophilic mice [104,105]. rFVIIa stored in extravascular tissues remains functionally active for periods of up to least 7 days [106,107]. The redistribution of infused rFVIIa in human tissues has

not been described. However, rFVIIa endocytosis has been hypothesized to provide extended protection from joint bleeding [106].

3.5 | rFVIIa-mediated release of endothelial extracellular vesicles

Endothelial extracellular vesicles (EEVs), membrane-bound vesicles that are constitutively released at low levels from endothelial cells, regulate cell-cell communication and contribute to both thrombosis and hemostasis [108]. The binding of rFVIIa to EPCR can facilitate proteolytic cleavage of protease-activated receptor-1 (PAR1) [109] at Arg41, activating intracellular signaling pathways [110,111]. PAR1 signaling through rFVIIa-EPCR increases the release of EEVs (predominantly microparticles) from cultured endothelial cells and in mouse models [63]. These EEVs express surface EPCR and E-cadherin but are devoid of TF and rFVIIa [63]. rFVIIa-EPCR-released EEVs support EPCR-mediated APC generation and increased phosphatidylserine-dependent tenase and prothrombinase activity when compared with constitutively released EEVs.

Using an *in vivo* saphenous vein bleeding model, infusion of rFVIIa-induced HUVEC- and murine plasma-derived EEVs supported hemostasis in hemophilic mice that received a subtherapeutic dose of rFVIIa and in wild-type platelet-depleted mice [63]. Studies of plasma samples from 10 persons with hemophilia who received a single dose of eptacog alfa (90 μg/kg) showed a marked increase in EEV-production 0.5 and 6 hours after rFVIIa infusion [112]. However, only EEVs circulating 6 hours after infusion supported increased tenase activity in the presence of rFVIIa, and to date the impact of EEVs on the clinical activity of rFVIIa is unclear.

3.6 | Anti-inflammatory and barrier-protective effects of rFVIIa

rFVIIa-EPCR-mediated PAR-1 signaling can also have anti-inflammatory and barrier-protective effects in both *in vitro* and *in vivo* models [109,110,113]. For example, rFVIIa-EPCR-induced EEVs carry the anti-inflammatory microRNA miR10a, which can be taken up by monocytes/macrophages and endothelial cells, promoting an anti-inflammatory phenotype and reducing vascular permeability [111,112]. Overall, these early data suggest that rFVIIa may reduce vascular inflammation associated with hemarthrosis. This could have implications for vascular leakage and remodeling, development of synovitis, and the risk of rebleeding in the injured joint, as well as the development of cardiovascular disease in older individuals [114]. Further research is required to determine if such effects are seen in humans.

4 | REGULATION OF rFVIIa ACTIVITY

The activity of rFVIIa is tightly regulated by anticoagulant mechanisms that prevent dysregulated thrombin generation and fibrin formation. Even at therapeutic doses, circulating rFVIIa displays weak enzymatic activity without TF or platelet-surface phosphatidylserine cofactor activity [115]. As platelet activation occurs mainly at the site of vessel injury, rFVIIa platelet-dependent activity is also localized to the site of clot formation [82]. Activation of coagulation by rFVIIa can also be regulated by endogenous protease inhibitors and anticoagulant pathways. TFPI directly inhibits FXa, and also forms a complex with FVIIa/TF/FXa, thereby limiting extrinsic tenase activity [116]. rFVIIa activity is also inhibited, though very slowly compared with other plasma proteases, by direct binding to antithrombin and, to a much lesser extent, α2-macroglobulin [117].

The activity of rFVIIa is also regulated by its clearance from the plasma through pathways that involve binding to antithrombin and endocytic receptors expressed by cells in the liver, spleen, and kidney [117–122]. *N*-linked glycans are important determinants of coagulation factor half-life [44]. Although head-to-head pharmacokinetic studies of eptacog alfa and eptacog beta have not been performed, levels of both products return to baseline 8 to 12 hours post administration [56,123], suggesting that the differences in glycosylation do not substantially influence clearance. The inactivation rate of eptacog beta by antithrombin is similar to eptacog alfa (Table) indicating that both products may be cleared in part through common pathways [78].

5 | CONCLUSIONS

The contributions of TF- and platelet-dependent pathways to the hemostatic activity of rFVIIa have long been recognized, and our understanding of the rFVIIa mechanism of action has recently expanded to include EPCR-dependent pathways. Data support the dominance of the platelet-dependent pathway in driving the majority of rFVIIa-induced procoagulant activity. Attempts have been made to improve the clinical efficacy of rFVIIa by enhancing its platelet-dependent activity. However, this approach has been hampered by issues with the immunogenicity of modified FVIIa molecules.

Regulatory approval of eptacog beta marked a major milestone in increasing treatment choices for persons with hemophilia and inhibitors. Eptacog beta has increased platelet and EPCR-dependent HUVEC binding activities at therapeutic concentrations compared with eptacog alfa, which could suggest that the relative contribution of these mechanisms to the hemostatic activity of these products may differ. These observations may be related to the methods of recombinant protein production, resulting in differences in the *N*-linked glycans expressed on each product and influencing functional binding activity.

Emerging real-world experience suggests eptacog beta is safe and effective in multiple settings, including for the treatment of acute bleeding events refractory to eptacog alfa [124]. Additional confirmatory real-world experience is needed. Eptacog beta is available in 3 licensed dosing regimens, expanding treatment options that can be tailored to meet individual patient needs. For the 225 μg/kg initial dose regimen, observations of rapid clinical response, with most bleeding events resolved within 3 hours [33] linked to the single-dose

efficacy, where 84% of bleeding events can be treated with one infusion, can improve healthcare equity by decreasing treatment burden. In addition, eptacog beta has a favorable safety profile and has not been associated with reports of thrombosis or immune response.

Future laboratory investigations are needed to confirm the initial observations of eptacog beta platelet- and EPCR-binding activity and evaluate its procoagulant influence using functional activity assays. Computational approaches, previously used to develop structural models for membrane-bound coagulation factors and coagulation factor complexes, may complement experimental methods and enhance our understanding of how the posttranslational differences between eptacog alfa and eptacog beta may impact their biochemical properties [125]. Additional clinical investigations may provide insights into how the enhanced platelet- and EPCR-binding activity of eptacog beta relates to observations of its clinical efficacy.

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DATA AVAILABILITY

Not applicable as no new data were generated for this report.

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