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ORIGINAL ARTICLE

Site-directed mutagenesis of tissue factor pathway inhibitor–binding exosite D60A on factor VII results in a new factor VII variant with lower coagulant activity

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Abstract

Background: Recombinant factor (F)VIIa (rFVIIa) has been approved by the US Food and Drug Administration for the treatment of hemophilia A and B with inhibitors and congenital FVII deficiency. Moreover, the investigational uses of rFVIIa are becoming of interest since it can be used to treat various clinical bleeding conditions. However, there is evidence showing that rFVIIa is a potent procoagulant agent that potentially leads to an increased risk of thrombotic complications.

Objectives: To design a new rFVII with lower coagulant activity that could potentially be used as an alternative hemostatic agent aiming to minimize the risk of thrombogenicity.

Methods: D60A was introduced into the *F7* sequence by polymerase chain reaction–based mutagenesis. Wild type (WT) and D60A were generated in human embryonic kidney 293T cells by stable transfection. FVII coagulant activities were determined by amidolytic cleavage of the FVIIa-specific substrate, 2-step FXa generation, thrombin generation (TG), and clot-based assays.

Results: WT and D60A demonstrated similar FVIIa amidolytic activity. However, D60A showed approximately 50% activity on FX activation and significantly longer lag time in the TG assay than that shown by WT. The clotting time produced by D60A spiked in FVII-deficient plasma was significantly prolonged than that of WT. Additionally, the *ex vivo* plasma half-lives of WT and D60A were comparable.

Conclusion: D60A demonstrated lower coagulant activities, most likely due to the weakening of FX binding, leading to impaired FX activation and delayed TG and fibrin formation. Considering that a plasma FVII level of 15% to 25% is adequate for normal hemostasis, D60A is a molecule of interest for future development of an rFVII with a lesser extent of thrombogenicity.

KEYWORDS
hemostasis, recombinant factor VII, TFPI-binding exosite, thrombogenic risk

Essentials

- Treatment with the activated form of factor (F)VII is associated with an increased risk of blood clots.
- D60A is a new FVII with lower clotting activity, most likely due to an impaired binding to FX.
- D60A might be helpful as an alternative treatment for patients with bleeding problems.
- Detailed *in vitro* and *in vivo* studies of D60A clotting activities are warranted.

1 | INTRODUCTION

Factor (F)VII is a vitamin K–dependent coagulation factor that has a pivotal role in the initiation phase of the coagulation cascade [1]. The majority of FVII circulating in plasma is in the zymogen form at a concentration of 500 ng/mL, while the active serine protease FVIIa circulates at a significantly lower concentration of approximately 5 to 50 ng/mL [2]. FVII is synthesized by hepatocytes and secreted into blood circulation as a single chain of 50-kDa zymogen with 406 amino acid residues [3]. FVII must be activated to FVIIa before it initiates the extrinsic pathway of the coagulation cascade by forming a membrane-bound complex with tissue factor (TF) [4]. FVIIa is generated by a single cleavage of the peptide bond between Arg152 and Ile153. The protease FVIIa is composed of light (20 kDa) and heavy (30 kDa) chains. The light chain consists of a gamma-carboxyglutamic acid (Gla) domain and 2 epidermal growth factor–like domains (residues 1-152), while the heavy chain contains the serine protease domain (residues 153-406). The 2 chains are linked together by a single disulfide bond formed between cysteines 135 and 262 [4]. Human FVII contains 10 Gla residues in the N-terminus of the mature protein at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35, which are responsible for the binding of FVII to phospholipid surfaces via Ca^{2+}-dependent interaction [5]. The epidermal growth factor domains are essential for interacting with other proteins of the coagulation cascade [6]. The serine protease domain provides a catalytic triad of amino acids—His193, Asp242, and Ser344—that constitute the catalytic center of FVIIa to increase its catalytic activity when binding with TF [2,7]. Hereditary deficiency of FVII is a rare autosomal recessive coagulation disorder that results in a variable bleeding phenotype in affected individuals ranging from mild to life-threatening bleeding [8]. The disease is caused by mutations in the *F7* gene, which affect the activity and/or levels of FVII in the plasma [9]. Many types of mutations in the *F7* gene have been identified and reported that can affect all of the protein domains [10]. Exon 8 is the largest exon of the *F7* gene, encoding the serine protease catalytic domain of the FVII protein, and harbors a large number of mutations [11].

Recombinant FVIIa (rFVIIa) has been increasingly used in both the US Food and Drug Administration (FDA)–approved and non–FDA-approved indications. The FDA-approved indications include treatment or prevention of bleeding in persons with hemophilia A or B with inhibitors [12,13], patients with congenital FVII deficiency [14–16], and patients with Glanzmann thrombasthenia [17]. Moreover, the investigational uses of rFVIIa are becoming of interest since it can be used to treat various clinical bleeding conditions as a hemostatic agent to promote hemostasis in patients with bleeding who may or may not have coagulation defects [18,19]. The common investigational indications of rFVIIa are excessive bleeding in dengue hemorrhagic fever [20], traumatic bleeding [21,22], perioperative blood loss, postpartum hemorrhage [23,24], spontaneous intracerebral hemorrhage [25], bleeding in liver diseases [26,27], thrombocytopenia [28], and anticoagulant-associated bleeding [29,30].

Despite the clinical benefits of rFVIIa, there is evidence showing that rFVIIa is a potent procoagulant agent that potentially leads to an increased risk of thrombotic complications [31–36], which are the most serious adverse reactions observed in patients with intracerebral hemorrhage [25], postoperative bleeding [33], and trauma [32,34] receiving rFVIIa. A study based on the FDA's Adverse Event Reporting System reported that of the 144 patients who received rFVIIa, 73 thromboembolic events (52%) occurred within 24 hours, and 30 events within 2 hours after the last dose of rFVIIa [35]. Moreover, medical records of 285 patients who received rFVIIa from 2001 to 2006 were reviewed for evidence of thromboembolic complications. Twenty-seven patients (9.4%) experienced thromboembolic events after administration of rFVIIa. Nine events were thought to be highly related to rFVIIa, and 10 of 14 deaths were, in part, caused by thrombotic complications [36].

In addition, due to the short half-life of rFVIIa, a high dose and multiple infusions are required [37]. Several attempts have been made to improve the properties of FVII and to extend its half-life. However, the problems of immunogenicity and a lack of a dose-response have been reported, resulting in the discontinuation of several molecules [38,39]. Previous studies on the roles of amino acids in the FVIIa protease domain that are important for FX activation by catalysis and substrate recognition of macromolecular substrate FX were R147 and K192 [40,41]. Our previous pathogenic study on R147 and K192 demonstrated a similar FVIIa amidolytic activity toward FVIIa-specific substrate but a 90% to 99% reduction toward FX activation [42]. The D60 position in the FVII protease domain appeared to form a salt-bridge with R20 of the Kunitz-1 domain of TF pathway inhibitor (TFPI) [43]; however, the same amino acid position could also form a hydrogen bond with the glutamine of FX. Moreover, D60N was one of the reported variants with no overt clinical bleeding [44]. Since FVII plasma levels of 15% to 25% of normal concentration are sufficient to maintain normal hemostasis [45–47], selective disruption at this position of FVII could, theoretically, produce a FVII variant with lower coagulant activity that is sufficient to maintain normal hemostasis and could be a molecule of interest for development of a novel therapeutic agent for bleeding disorders with potentially less thrombogenicity. In the present study, we, therefore, investigated the role of D60 in enzymatic and coagulant activities of FVII by site-directed mutagenesis.

2 | METHODS

2.1 | DNA mutagenesis and construction of plasmids expressing the *F7* gene

FVII wild type (WT) was constructed as described previously [48]. Human FVII WT complementary DNA (cDNA) was ligated into the pTG19-T polymerase chain reaction cloning vector (Vivantis Technologies), which was used as a template for mutant FVII D60A cloning. Alanine substitution at position D60 (chymotrypsinogen numbering system) was introduced into the *F7* gene sequence by polymerase chain reaction–based site-directed mutagenesis using a forward primer, 5′-GCGGCCCACTGTTTCGCCAAAATCAAGAACTGG-3′, and a reverse primer, 5′-CCAGTTCTTGATTTTGGCGAAACAGTGGG CCGC-3′ (underlined residues represent the mutated base). The correct clone of the D60A construct was confirmed by full-length FVII cDNA sequencing (Macrogen Inc), cut with *Xho*I and *Bam*HI, and further ligated into the pLVX-puro vector (Clontech Laboratories), which was an expression vector for FVII protein.

2.2 | Cell culture condition

Human embryonic kidney 293T (HEK293T) cells were used for FVII protein expression via stable transfection. Cells were cultured in Dulbecco's Modified Eagle Medium high glucose medium (HyClone) containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine (HyClone), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (HyClone). Cells were grown at 37 °C with 5% CO_2 and 95% humidity.

2.3 | Expression of FVII in mammalian cells

HEK293T cells expressing FVII WT and D60A were transfected with the pLVX-puro plasmid containing FVII cDNA using FuGENE6 transfection reagent (Promega) for 48 hours. Transfected cells were selected with puromycin (2 μg/mL) in a complete medium for 14 days. Individual puromycin-resistant colonies were selected for testing FVII protein expression in culture supernatants by Western blotting. Cells stably expressing FVII were cultured in serum-free media containing 10 μg/mL of vitamin K and 1× insulin-transferrin-selenium for 24 hours. Cell culture supernatants were harvested, and cell debris was removed from the supernatants by centrifugation at 3000 × *g* for 10 minutes at 4 °C. The supernatants were stored in aliquots at −80 °C.

2.4 | Western blot analysis

One microgram of total proteins was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions using 2-mercaptoethanol (Bio-Rad) and run at 100 V for 1.5 hours. The proteins were transferred to the polyvinylidene difluoride membrane (Thermo Fisher Scientific) at 200 mA for 2 hours. The membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20 at 4 °C overnight. Subsequently, the membrane was probed with a rabbit monoclonal anti-FVII (1:10,000; Abcam) for 3 hours at room temperature (RT) and further probed with peroxidase-conjugated goat anti-rabbit immunoglobulin G H&L (1:20,000; Abcam) for 1 hour at RT. Proteins were detected by the enhanced chemiluminescence substrate (Bio-Rad) and visualized using the ChemiDoc MP Imaging System (Bio-Rad).

2.5 | Measurement of secreted FVII protein levels

The levels of secreted FVII protein were determined by enzyme-linked immunosorbent assay using the Human FVII Enzyme-Linked Immunosorbent Assay Kit (Abcam) according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm using a Varioskan Flash microplate reader (Thermo Fisher Scientific). The secreted FVII levels were analyzed after comparison with a standard curve.

2.6 | Determination of FVII coagulant activities

2.6.1 | FVII activation by FXa in the presence of TF

FVII (0.5 nM) was incubated with FXa (60 nM; Abcam) and lipidated TF (2 nM; Siemens) in a reaction buffer (20-mM HEPES/150-mM NaCl, pH 7.4) at 37 °C. The reaction mixture was sampled at 0, 1, 5, 10, 20, and 30 minutes to visualize FVII activation by Western bot analysis as described above.

2.6.2 | FVIIa amidolytic activity by hydrolysis of peptidyl substrate

To activate FVII to FVIIa, FVII (15 nM) was incubated with FXa (60 nM) in a reaction buffer (20-mM HEPES/150-mM NaCl, pH 7.4) at 37 °C for 30 minutes, followed by the addition of rivaroxaban (120 nM; Bayer Pharma AG) to inhibit any residual activity of FXa at 37 °C for 30 minutes. Subsequently, FVIIa amidolytic activity was measured by adding FVIIa-specific peptidyl substrate (0.8 mM; Sigma-Aldrich) immediately before measuring the absorbance at a wavelength of 405 nm. The actual absorbance for FVIIa-specific amidolytic activity was calculated by subtracting the optical density of the total reaction from the optical density of FXa + rivaroxaban.

2.6.3 | Two-step FXa generation by FVII with TF

FVII-mediated FX activation was examined using the FVII Human Chromogenic Activity Kit (Abcam) according to the manufacturer's instructions. WT and D60A (0.5 nM) were added to the wells and incubated for 2 hours at RT. Subsequently, the assay mixture (recombinant

human TF and FX) was added to each well and incubated at 37 °C for 30 minutes. FVII-mediated FX activation was determined by adding FXa substrate to each well. The absorbance was measured at a wavelength of 405 nm for 30 minutes and compared to a standard curve.

2.6.4 | Thrombin generation assay

The ability of FVII to support TF-triggered thrombin generation (TG) was examined as described previously [42]. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems), and TG curves over time were generated using the Thrombinoscope software (Thrombinoscope B.V.). Four parameters (lag time, time to peak, peak thrombin, and endogenous thrombin potential) were derived from the TG curves.

2.6.5 | Clotting assay

FVII coagulant activity was performed by a modified prothrombin time (PT) assay. FVII (5 nM) was incubated with FVII-deficient plasma (Siemens) for 5 minutes, and the clotting time was initiated by adding human placental thromboplastin (Siemens) into the reaction mixture. The semiautomated coagulation analyzer CA-104 (Sysmex Europe GmbH) was used to record the time to clot formation.

2.6.6 | Determination of *ex vivo* plasma half-life of FVII

FVII (5 nM) was incubated with FVII-deficient plasma for 0 to 6 hours at 37 °C prior to sampling into the modified PT assay. The residual coagulant activity of FVII was calculated as FVII concentrations using a standard curve and subsequently converted to percentage of residual FVII activity and plotted against the incubation time.

2.7 | Statistical analysis

Data are presented as mean ± SEM (n = 3), and the comparison between groups was performed using unpaired Student's t-test. The statistical significance was considered when $P < .05$. The figures and statistical analyses were prepared and analyzed using GraphPad Prism 5.0 (GraphPad Software Inc).

3 | RESULTS

3.1 | The zymogen form of FVII was detected in cells transfected with FVII

Alanine was successfully substituted into the *F7* sequence at the D60 position on the FVII protease domain. After stable transfection, the expression of FVII in culture supernatants was examined by Western blotting. Nontransfected HEK293T cells (as a negative control) did not express FVII. HEK293T cells transfected with WT and D60A expressed the FVII zymogen form, as shown by a single band at a molecular weight of 55 kDa (Figure 1). rFVIIa demonstrated a single band corresponding to the light chain of protease FVIIa with a molecular weight of 20 kDa.

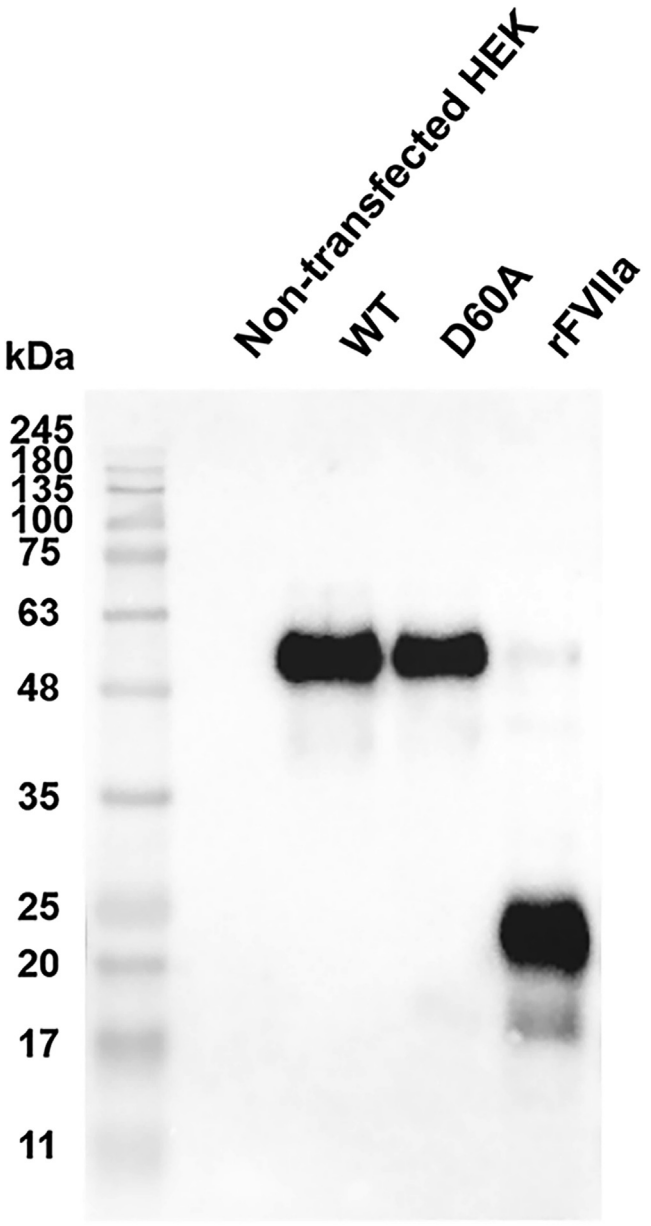

FIGURE 1 Western blot of wild type (WT) and D60A in culture media supernatant. Protein samples (1 μg of total protein) were run on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Supernatant from nontransfected human embryonic kidney (HEK) 293T cells (1 μg of total protein) was used as a negative control. Recombinant factor (F)VIIa (rFVIIa) (100 ng; Novo Nordisk) was used as a positive control. The zymogen FVII and the light chain of the protease FVIIa appeared as a single band with a molecular weight of approximately 55 and 20 kDa, respectively.

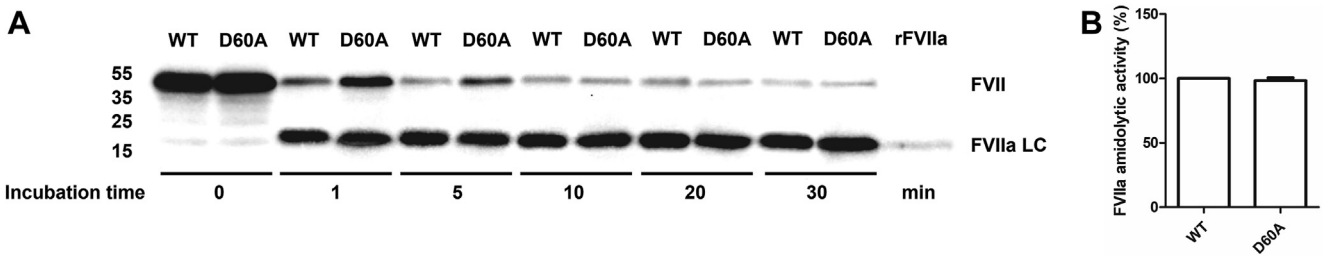

FIGURE 2 Factor (F)VII activation by FXa with tissue factor determined by Western blot analysis and FVIIa amidolytic activity determined by the hydrolysis of FVII-specific peptidyl substrate. (A) After incubation of FVII (0.5 nM) with FXa (60 nM) and lipidated tissue factor (2 nM) for 0, 1, 5, 10, 20, and 30 minutes at 37 °C, the enzymatic reactions were sampled and quenched with the reducing sample buffer, and Western blot was performed with a rabbit anti-FVII monoclonal antibody (1:10,000). (B) After activation of FVII (15 nM) by FXa (60 nM) for 30 minutes at 37 °C, any residual FXa activity was subsequently inhibited by rivaroxaban (120 nM). FVIIa amidolytic activity was quantified by amidolytic cleavage of FVIIa-specific substrate (0.8 mM). Data are presented as the percentage of activity (mean ± SEM, n = 3) compared with wild type (WT). FVIIa LC, light chain of protease factor VIIa; rFVIIa, recombinant factor VIIa.

3.2 | Cells expressing D60A showed decreased levels of FVII secretion

Cells transfected with WT and D60A secreted 3031.56 ± 93.06 ng/mL and 1550.32 ± 73.50 ng/mL of FVII, respectively. This result shows that D60A-expressing cells significantly reduced FVII protein production compared with WT-expressing cells. The concentrations of FVII from each construct were used to further determine FVII coagulant activities.

3.3 | D60A demonstrated a delayed FVII activation but similar FVIIa amidolytic activity compared with those of WT

Both WT and D60A were activated to FVIIa after incubation of the zymogen FVII with FXa and TF at 37 °C, as shown by the presence of bands corresponding to the light chain of FVIIa at a molecular weight of 20 kDa during 30 minutes of incubation (Figure 2A). Interestingly, the band intensity of the D60A zymogen was apparently denser than that of the WT zymogen at the 1- and 5-minute time points (Figure 2A), suggesting that FXa-dependent D60A activation occurred at a slower rate than that of WT. However, both WT and D60A demonstrated a comparable band intensity of residual FVII zymogen after 10 minutes of incubation, indicating a nearly complete FVII activation within 30 minutes. Therefore, the 30-minute incubation was used for FVII activation in the FVIIa amidolytic activity. In the FVIIa-specific amidolytic activity assay, D60A exhibited an activity similar to that of WT (Figure 2B).

3.4 | D60A decreased FX activation

The ability of FVII to activate FX to FXa was investigated by the cleavage of FXa chromogenic substrate. WT showed a higher FVII activity (0.8090 ± 0.0948 IU/mL), whereas D60A demonstrated approximately 50% reduction in activity compared to WT on FX activation (0.3749 ± 0.0575 IU/mL) (Figure 3).

3.5 | D60A delayed plasma TG

Both WT and D60A (1 nM) supported TF-triggered TG in FVII-deficient plasma (Figure 4) and demonstrated similar patterns in mean time to peak (11.59 ± 0.49 minutes vs 12.46 ± 0.22 minutes),

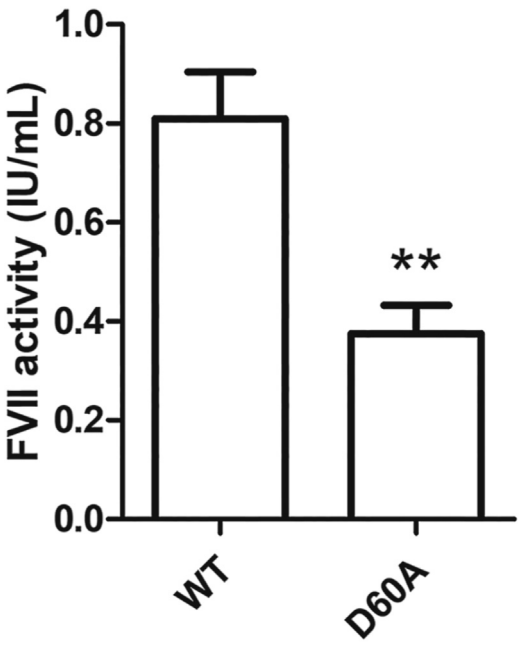

FIGURE 3 Factor (F)VII–mediated FX activation determined by the cleavage of FXa chromogenic substrate (2-step FXa generation). FVII (0.5 nM) was added to each well to determine the coagulant activity. The assay mixture containing tissue factor and FX was added and incubated at 37 °C for 30 minutes, and the FVII-mediated FX activation was measured by adding FXa chromogenic substrate. Data are presented in FVII activity (mean ± SEM, n = 3). $^{**}P < .01$ for comparison between wild type (WT) and D60A.

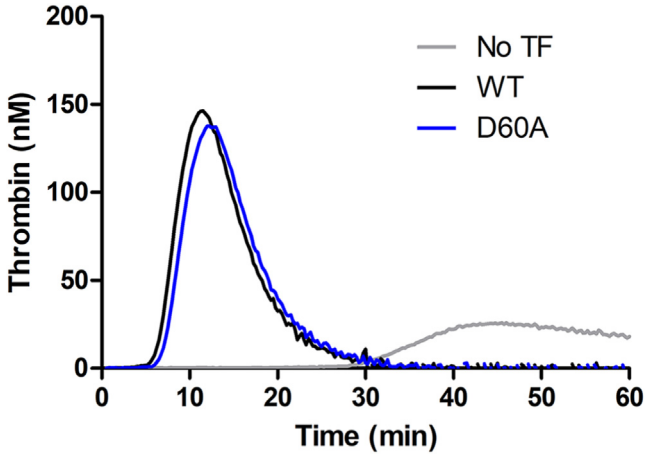

FIGURE 4 Effect of D60A on plasma thrombin generation in factor (F)VII–deficient plasma. Thrombin generation curves demonstrated the ability of both wild type (WT) and D60A to support tissue factor (TF)–triggered thrombin generation in FVII-deficient plasma. The lag time of D60A was significantly longer than that of WT.

peak thrombin (149.00 ± 10.89 nM vs 138.90 ± 5.10 nM), and endogenous thrombin potential (1380.00 ± 29.18 nM·min vs 1342.00 ± 23.42 nM·min). However, D60A showed a significantly longer lag time (7.42 ± 0.08 minutes) than that of WT (6.71 ± 0.18 minutes) (Table 1).

3.6 | D60A demonstrated a decrease in coagulant activity

The ability of FVII (5 nM) to generate fibrin clots in FVII-deficient plasma was examined by a modified PT assay. WT could shorten the clotting time from 54.23 ± 0.58 seconds (negative control, no rFVII) to 18.60 ± 0.39 seconds, whereas the clotting time produced by D60A under the same condition was 23.88 ± 0.44 seconds (Table 2).

TABLE 1 TG parameters derived from the TG curves produced by recombinant factor VII in factor VII–deficient plasma.

TG parameters	WT	D60A	*P* value
Lag time (min)	6.71 ± 0.18	7.42 ± 0.08[a]	.02
Time to peak (min)	11.59 ± 0.49	12.46 ± 0.22	.178
Peak thrombin (nM)	149.00 ± 10.89	138.90 ± 5.10	.4486
ETP (nM·min)	1380.00 ± 29.18	1342.00 ± 23.42	.3669

The mean lag time of D60A was significantly prolonged than that of WT. The mean time to peak, peak thrombin, and ETP of D60A were similar to those of WT. Data are expressed as mean ± SEM (n = 3).

ETP, endogenous thrombin potential; TG, thrombin generation; WT, wild type.

[a]$P < .05$ for the comparison between WT and D60A.

TABLE 2 Coagulant activity of rFVII determined by modified prothrombin time assay.

rFVII	Clotting time (s)	FVII activity (ng/mL)
No rFVII	54.23 ± 0.58[a]	ND
WT	18.60 ± 0.39	88.04 ± 2.92
D60A	23.88 ± 0.44[a]	56.26 ± 2.15[a]

After incubation of factor (F)VII (5 nM) in FVII-deficient plasma for 5 minutes, coagulant activity was initiated by human placental thromboplastin. Clotting time of FVII variants was converted to FVII activity using a standard curve. Data are expressed as mean ± SEM (n = 3).

ND, not detectable; rFVII; recombinant factor VII; WT, wild type.

[a]$P < .001$ for comparison between WT and D60A.

3.7 | D60A demonstrated a similar *ex vivo* half-life to WT in FVII-deficient plasma

After incubating WT and D60A in FVII-deficient plasma for 0 to 6 hours, their *ex vivo* half-lives in FVII-deficient plasma were not different (Figure 5).

4 | DISCUSSION

rFVIIa has been used in various clinical bleeding conditions with some success but potentially leads to thrombotic risks [31–36]. In this study, we designed the new FVII molecule with a lower coagulant activity aiming to minimize thrombotic complications. The D60 position located on the FVII protease domain was selected and substituted with alanine because the position resides on the surface of FVII and is not involved in the catalytic loop [49]. In addition, alanine is a small nonpolar and neutral amino acid; substitution with alanine eliminates

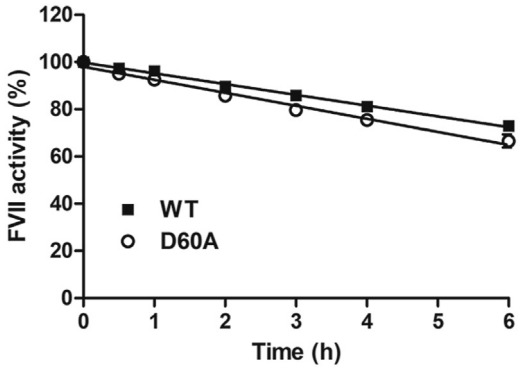

FIGURE 5 Effect of D60A on the *ex vivo* half-life in human factor (F)VII–deficient plasma. A standard curve was generated in FVII-deficient plasma supplemented with human recombinant FVII (0-200 ng/mL) in the modified prothrombin time assay. Similarly, both wild type (WT) and D60A (5 nM) in FVII-deficient plasma were incubated at 37 °C for 0 to 6 hours prior to determination of residual FVII activity in the modified prothrombin time assay. Clotting time at each time point was calculated to the residual activity using the standard curve and plotted against incubation time. The results are expressed as mean ± SEM (n = 3).

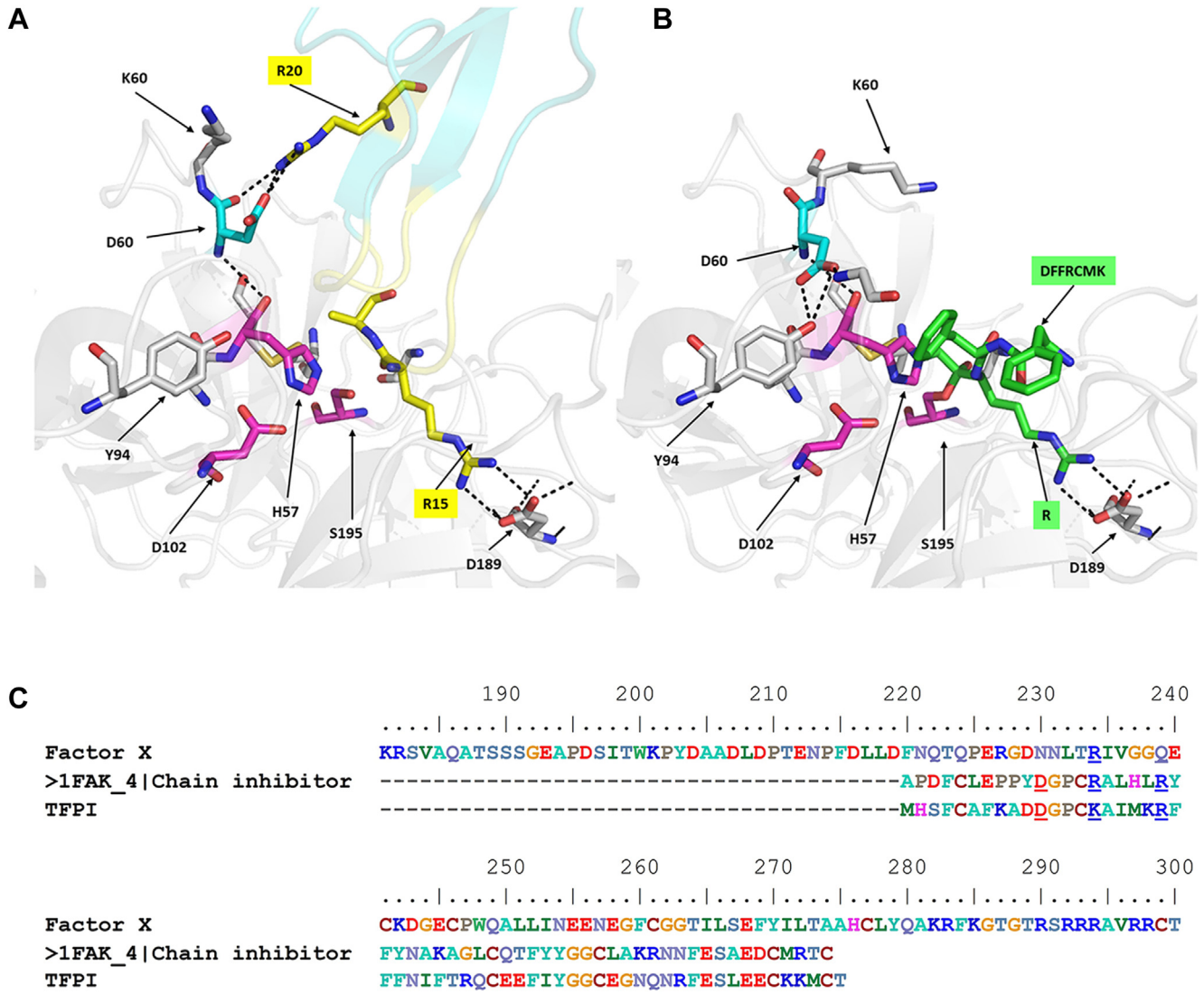

FIGURE 6 The recognition of substrate and inhibitors by factor (F)VIIa. (A) The binding of tissue factor pathway inhibitor (TFPI) to the substrate-binding site in FVIIa adapted from Protein Data Bank ID: 1FAK [53]. D60 is shown as cyan sticks. The Kunitz-1 domain of TFPI is shown as yellow sticks. The catalytic triads in the active site are shown in magenta. Other residues within the substrate-binding pocket are shown in gray. Hydrogen bonds are shown as black dotted lines. (B) The binding of small peptide inhibitor dansyl-Phe-Phe-Arg chloromethy ketone (DFFR-CMK) (Protein Data Bank ID: 1DAN) to the substrate-binding site in FVIIa. Small peptide inhibitor DFFRCMK is shown in green. (C) Sequence alignment of FX (natural substrate), BPTI mutant (from 1FAK), and TFPI. Amino acids interacting with the substrate-binding site in FVIIa are underlined.

the side-chain interactions without altering the main-chain conformation or causing significant electrostatic or steric effects [50–52]. D60N was reported as a natural variant with no pathological effect [44]. Therefore, a single alanine substitution on the surface of FVII should, theoretically, preserve the FVII protein structure with minimally altered protease function.

Recently, our group reported that mutation of TFPI-binding exosites (R147A and K192A) of FVII caused bleeding phenotypes in FVII deficiency, most likely due to the impairment of FX activation [42]. These amino acids reside within the substrate-binding pocket of FVII involved in the binding with both FX and TFPI, which could form the hydrogen bonds with asparagine of FX and recognize macromolecular substrate FX before FX activation [41,42]. In addition to R147 and K192 of FVII, we further analyzed the D60 of FVIIa through structural analysis and sequence comparison (Figure 6) [53]. D60 potentially forms the hydrogen bond with Q239 (Q20) of FX before FX activation at the equivalent site to R20 of the Kunitz-1 domain of TFPI (Figure 6A) but forms the hydrogen bond with Y272 (Y94) when the substrate is absent (Figure 6B). The Q239 or R20 are the fifth amino acids downstream of the cleavage site (Figure 6C). Therefore, it does not interact with the chromogenic substrate and would provide less contribution to substrate specificity. In fact, the substrate-binding pocket of FVIIa can be accessed by peptides with variation in sequences, implying that they may not be the key player in providing substrate specificity. In addition, the peptide inhibitor dansyl-Phe-Phe-Arg chloromethyl ketone (DFFR-CMK) (Protein Data Bank ID: 1DAN)

can access the substrate-binding pocket of FVIIa, although it possesses 2 hydrophobic amino acids at P2 and P3 (Figure 6B), while FX contains smaller aliphatic amino acids (Figure 6C). Most of the amino acids in the FX loop are nonpolar amino acids that cannot provide specific interaction with the substrate-binding pocket. Hence, D60 may be involved in positioning P2' to P4' within the substrate-binding pocket when FX is the substrate. Therefore, alanine substitution at this position should also result in an intact protease function with decreased FX activation (due to impaired binding) and hypothetically lead to the novel FVII variant with lower coagulant activity.

After successful alanine substitution at D60, we further evaluated the FVII enzymatic and coagulant activities of D60A. WT and D60A showed comparable amidolytic activity toward hydrolysis of FVIIa-specific chromogenic substrate, indicating that the protease activity of D60A remained intact. As expected, the FXa-dependent activation of D60A occurred at a slower rate than that of WT, and vice versa; FVIIa-mediated FX activation of D60A demonstrated approximately 50% activity compared to WT. In the TG assay, D60A produced a significantly longer lag time than that of WT. Moreover, the clotting time produced by D60A was significantly prolonged than that of WT. Therefore, D60A demonstrated approximately 50% reduction in activity to activate FX, as demonstrated by the 2-step FXa generation, leading to delayed TG and, ultimately, prolonged clotting time. These observations suggest that alanine substitution at D60 may impair FX binding.

D60 of FVII is a position that is important for interaction with R20 of the Kunitz-1 domain of TFPI [43]. TFPI is a major physiological inhibitor of the extrinsic pathway and is responsible for the inhibition of FVIIa [54]. Therefore, we postulated that the D60A may disrupt the interaction between FVII and the Kunitz-1 domain of TFPI and, subsequently, could be less inhibited by the TFPI, resulting in a prolonged half-life of the FVII molecule. A prolonged plasma half-life of procoagulant protein could be potentially thrombogenic [55]. Therefore, the effect of D60A on the *ex vivo* half-life of rFVII in human FVII-deficient plasma was determined. Our results demonstrated that WT and D60A exhibited no difference in their half-lives in FVII-deficient plasma (>6 hours). Therefore, this novel D60A with a normal *ex vivo* plasma half-life should not, theoretically, carry a high risk of thrombotic complications after the patient's administration.

Several bioengineered extended half-life rFVIIa products, including the glycoPEGylated rFVIIa [56], the rFVIIa albumin fusion protein [57], the FVII variants with insertion of activation peptides of coagulation factors [58], and the modified rFVIIa protein [38], have been developed with questions on the theoretical risk for thrombosis [37]. On the contrary, a short-acting FVIIa CT-001 molecule has been developed to overcome the thrombogenicity of rFVIIa as a safe and effective approach for the treatment of acute bleeding. The CT-001 is a new rFVIIa engineering 4 amino acids (P10Q, K32E, T106N, and V253N) developed from BAY 86-6150 (P10Q, K32E, A34E, R36E, T106N, and V253N) [59]. It has been reported that neutralizing antibodies were detected in 10% of individuals after multiple exposures to BAY 86-6150 [60]. However, the CT-001 was not tested for immunogenicity, but it has been shown that its faster clearance *in vitro* may lower immunogenicity [60]. Genetic modification of proteins can cause unwanted immunogenicity by generating neutralizing antibodies in patients [61,62]. Moreover, vatreptacog alfa, a rFVIIa molecule engineering 3 amino acids (V158D, E296 V, and V M298Q) [63], has reported immunogenicity in approximately 11% of persons with hemophilia A or B with inhibitors in the phase III clinical trial, resulting in the discontinuation of this molecule [64,65]. Therefore, we speculate that the D60A in this study, engineering only 1 amino acid, may minimize unwanted immunogenicity.

In summary, the results from our preliminary study demonstrated that the D60A molecule exerted a lower coagulant activity and did not prolong the *ex vivo* half-life. Given that FVII plasma levels of 15% to 25% (approximately 0.075-0.125 μg/mL) are considered adequate for normal hemostasis [45–47], we expect that the D60A (with ∼50% coagulant activity) can effectively rescue bleeding phenotypes in various bleeding disorders and may potentially minimize the risk of thrombotic complications, which are the most serious adverse reactions observed in patients receiving rFVIIa. Taken together, our results suggest that the D60A is a FVII variant of interest that might be used for the treatment or prevention of bleeding episodes as an alternative therapeutic agent aiming to minimize thrombogenicity. Further in-depth *in vitro* and *in vivo* studies of coagulant activities of D60A are warranted.

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AUTHOR CONTRIBUTIONS

K.S. performed the experiments, interpreted data, and wrote the manuscript; V.K. performed the factor VII activation experiment; P.P. performed the molecular model for the recognition of substrate and inhibitors by factor VIIa and helped in data interpretation; N.S., P.A., and A.C. reviewed the manuscript; S.H. supervised and supported the overall project; and P.T. designed the overall research, discussed and interpreted data, and revised the manuscript.

RELATIONSHIP DISCLOSURE

N.S. reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events for Novo Nordisk, Pfizer, and Takeda and support for attending meetings and/or travel from Novo Nordisk. A.C. reports honoraria for speakers bureau from Grifols, Roche, Takeda, and Novo Nordisk. There are no other competing interests to disclose.

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