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ORIGINAL ARTICLE

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In vitro characterization of MOD-5014, a novel long-acting carboxy-terminal peptide (CTP)-modified activated FVII

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Introduction: Recombinant FVIIa (rFVIIa) is an effective treatment for haemophilia through frequent administration. However, the short half-life of rFVIIa decreases its prophylactic ability to reduce bleeding. Carboxy-terminal peptide (CTP)-modified FVIIa (MOD-5014) is a long-acting rFVIIa developed for on-demand treatment of haemophilia using either an intravenous or subcutaneous injection with the aim of less frequent administrations, as well as for prophylactic use.

Aim: The comprehensive evaluation of the activity MOD-5014 vs commercially available rhFVIIa, as well as their interaction with cofactors and inhibitors.

Methods: The in vitro characterization included clotting activity, affinity by surface plasmon resonance, cleavage of synthetic substrates, thrombin generation (TG) and rotation thromboelastometry.

Results: Reduced specific activity was obtained for MOD-5014 compared to rhFVIIa, while both compounds demonstrated comparable affinity to tissue factor (TF). MOD-5014 showed reduced TG when spiked at a similar concentration as rhFVIIa, suggesting that an increased concentration might be needed in a clinical setting to provide initial haemostatic effect. MOD-5014 demonstrated a slightly lower affinity for binding to activated platelets and slightly lower proteolytic activity on the platelet surface, possibly as the fusion of CTP has the potential to sterically hinder binding to both the platelet membrane and to protein substrates. Both compounds showed a similar dose-dependent stimulatory effect on clot formation, and both showed a similar deactivation pattern following incubation with TF pathway inhibitor (TFPI), antithrombin and heparin.

Conclusion: The comparable in vitro activity of MOD-5014 and rhFVIIa paves the way for in vivo pharmacology evaluations of MOD-5014 in preparation for clinical studies.

KEYWORDS

aFVII therapy, coagulation, haemorrhagic disorders

1 | INTRODUCTION

Haemophilia is a group of inherited haemorrhagic disorders caused by defects in, or the absence of, critical factors in the coagulation cascade, which severely compromise thrombin generation and fibrin clot formation. This leads to spontaneous bleeding episodes, most commonly in joints and internal organs, to intracranial bleeds and to excessive bleeding during and following surgery or trauma; frequent bleeding can cause joint swelling, joint damage, severe deformity, frequent infections and reduced mobility in patients with haemophilia.[1] Haemophilia A is caused by defects in or a lack of factor VIII (FVIII) expression, while haemophilia B is caused by defects in or lack of factor IX expression. The prevalence of haemophilia in the United States has been estimated at 1 in 6000 individuals for haemophilia A and 1 in 25 000 individuals for haemophilia B.[2] Patients are primarily treated with exogenous FVIII or FIX and may develop inhibitors (antibodies) against these proteins over time. Activated FVII (FVIIa) enhances the generation of thrombin and can thereby bypass deficiencies or inhibitors that abrogate the intrinsic coagulation pathway.[3,4] Previous experience in humans has established recombinant FVIIa (rFVIIa) as an effective emergency treatment for haemophiliacs with inhibitors through frequent administration of the drug to control haemorrhage.[5] Standard regimens for the treatment of spontaneous bleeding include one infusion of 270 μg/kg of rFVIIa or up to three infusions of 90 μg/kg every 3 hours following a bleeding episode due to the drug's short half-life of 2.9 hours.[6,7] Similar to replacement of FVIII and FIX, the short half-life of rFVIIa decreases the practicality of the drug as a prophylactic agent to reduce the frequency of spontaneous bleeding episodes. Although not an approved indication, the only commercially available rFVIIa product (NovoSeven, Novo Nordisk, Denmark) was shown to be effective as a prophylactic treatment.[8]

MOD-5014 is a long-acting rhFVIIa developed for the treatment of haemophilia A or B using either an intravenous (IV) or subcutaneous (SC) injection. MOD-5014 is being developed for on-demand treatment of spontaneous bleedings (eg, joint bleeding) with the aim of less frequent administrations, as well as for prophylactic use with an anticipated dosing regimen of two to three times a week. The prolonged circulation time of MOD-5014 is the result of three consecutive C-terminal peptide (CTP) repeats at the C-terminus of FVII. The CTP technology is based on the C-terminal peptide of the beta chain of human chorionic gonadotropin.[9,10] This technology has been clinically validated upon the approval of corifollitropin alfa (Elonva, Merck) by the European authorities and is currently utilized to extend the half-life of human growth hormone (MOD-4023),[11] which is in advanced clinical stage in growth hormone-deficient adults and children.[12,13] Understanding the way that the CTP affects the biological function of MOD-5014 in comparison with rhFVIIa is important to gauge any potential differences in the safety profile of MOD-5014 when compared to rhFVIIa. To this end, this study presents a summary of the comprehensive in vitro characterization undertaken to comparatively evaluate the activity of FVIIa-CTP_3 (MOD-5014) vs rhFVIIa following the attachment of CTP, as well as their interaction with cofactors and inhibitors; this work paves the way for in vivo pharmacology and toxicology evaluations of MOD-5014 in animal models.

2 | MATERIALS AND METHODS

2.1 | FVIIa-CTP (MOD-5014) and rhFVIIa

MOD-5014 is a recombinant form of FVIIa with three consecutive C-terminal peptide (CTP) repeats at the C-terminus of the FVII amino acid sequence.[9,10] MOD-5014 is produced in stably transfected DG44 CHO cells and is purified in a series of chromatography steps. During the final step, FVII-CTP_3 is autocatalytically converted to the active form (FVIIa-CTP_3). The calculated molecular weight of FVIIa-CTP_3 (based on the primary amino acid sequence) is approximately 54 kDa. However, due to glycosylation of the CTP domains, the apparent molecular weight is ~75 kDa by SDS-PAGE and 66 kDa by mass spectrometry. The comparator in all experiments was rhFVII (NovoSeven, Novo Nordisk, Denmark). MOD-5014 concentration was calculated based on the mass contribution from the FVII moiety to more directly compare mass quantities to rhFVII. Based on the mass of the amino acid sequence, 84.4% of MOD-5014 corresponds to rhFVIIa and 15.6% corresponds to the CTP moiety. MOD-5014 concentration is calculated on the basis of specific absorbance at 280 nm using an empirically determined extinction coefficient of 1.27 mL/mg/cm.

2.2 | Clotting activity

The in vitro clotting activity of FVIIa was measured using the Staclot VIIa-rTF kit (Diagnostica Stago, Parsippany, NJ, USA). This method includes clotting time measurement of FVII-deficient plasma using the STA Compact MAX or STart 4 instruments. Specific amounts of FVIIa were added to the plasma following the addition of phospholipids, Ca^{2+} and recombinant soluble tissue factor (rsTF). The latter is the extracellular portion of the native TF, which, although it is no longer able to activate FVII to FVIIa by autoactivation, possesses a cofactor function specific for FVIIa. The FVIIa bound to rsTF converts FX to the FXa. The observed clotting time has an inverse relationship with FVIIa level in the plasma, as rsTF does not activate FVII to FVIIa. The obtained clotting time is converted to activity (mU/mL, presented in units of the WHO International Standard for FVIIa) using a FVIIa standard curve, and the specific activity is calculated based on the protein concentration of FVIIa. The Staclot method was also used to compare MOD-5014 and rhFVIIa inhibition by tissue factor pathway inhibitor (TFPI) and antithrombin (AT). Tissue factor pathway inhibitor (TFPI) or AT was added at a range of concentrations (0.625-1250 ng/mL for the former and 1.05-525 000 ng/mL for the latter), following the addition of MOD-5014 or rhFVIIa at a fixed concentration, FVII-deficient plasma, tissue factor and phospholipids, and was incubated for 15 minutes at 37°C. The observed specific activity was converted to % inhibition. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured using a CA-1500 Autoanalyzer (Siemens, Malvern, PA, USA) and validated using routine clinical human plasma diagnostic testing at AML (Herzliya, Israel); MOD-5014 and

rhFVIIa were diluted to 0.5-0.0008 mg/mL with haemophilic (FVIII-deficient) human plasma (BioreclamationIVT, Baltimore, MD, USA) or FVII-deficient plasma (Hyphen BioMed, Neuville-sur-Oise, France).

2.3 | Surface plasmon resonance

The interactions between MOD-5014 and rhFVIIa to sTF were studied using ProteOn 36XPR (Bio-Rad, Hercules, CA, USA) at the ProteOn unit, the National Institute for Biotechnology in the Negev (Ben-Gurion University, Beer Sheva, Israel). Soluble TF (Randox Life Sciences, Kearneysville, WV, USA) was reconstituted in PBS to a final concentration of 100 μg/mL and immobilized on a GLC sensor chip (Bio-Rad) in 10 mmol/L acetate (pH 4.5) using amine coupling according to the manufacturer's protocol. The running buffer contained 20 mmol/L HEPES, 0.1 mol/L NaCl, 5 mmol/L $CaCl_2$ and 0.02% P80 (pH 7.4). The immobilization response was 200 resonance units (RU). All experiments were performed at flow rates of 60 and 40 μL/min at room temperature. Different analyte concentrations (0-20 nmol/L) were injected, followed by surface regeneration using 30 μL of 50 mmol/L EDTA (pH 8.5) after each cycle. Data processing was performed with ProteOn Manager v3.1.0.6 using 1:1 Langmuir model. Immobilized bovine serum albumin (BSA) was used as control and was subtracted from the responses obtained from the reaction surface.

2.4 | Chromogenic substrate assays

The potency of MOD-5014 and rhFVIIa was also assessed using the BIOPHEN FVII chromogenic assay (Hyphen BioMed). All experiments were performed at least 3 times, and error in the fitting parameters was determined by bootstrapping. Factor X is present in the assay in a constant concentration and in excess and is activated upon incubation with FVIIa. The concentration of FXa is measured by its activity on a specific chromogenic substrate (SXa-11), which it cleaves to generate pNA. The amount of pNA is determined by colour development at 405 nm and is directly proportional to FXa activity and to the amount of FVIIa. To evaluate the potency of MOD-5014 and rhFVIIa in the presence of TFPI or AT, the inhibitors were added at a range of concentrations (0.01-20 000 ng/mL for TFPI and 0.16-1680 μg/mL for AT) to constant concentrations of MOD-5014 or rhFVIIa (either 0.6 or 4 ng/mL [EC_{70}] in the TFPI experiments and 0.7 ng/mL in the AT experiments) in the presence of TF (rabbit brain thromboplastin), and FX activation was measured. To monitor FVIIa binding to TF, MOD-5014 or rhFVIIa was incubated with a fixed concentration of TF, followed by the addition of the synthetic substrate Pefachrome FVIIa. Substrate cleavage was monitored at 405 nm by the appearance of yellow colour. As the concentration of TF was well above the expected K_d, at low concentrations, all of FVIIa is bound to TF, and the rate of substrate cleavage is that of the VIIa/TF complex. Once the concentration of FVIIa exceeds that of TF, the rate of substrate cleavage drops to that of free FVIIa. To assess the binding of FVIIa to TF by measuring the rate of FX activation, the synthetic substrate Pefachrome FXa was utilized; cleavage of the substrate is converted to FXa concentration by a standard curve. The rate of FX activation increases as FVIIa binds to TF. Once all of the TF is saturated with FVIIa, the rate of FX activation reaches a maximum value. Pefachrome FXa was also used to measure the rate of FX activation as a function of FX concentration (where FX was incubated with a fixed concentration of FVIIa/TF complex), as well as for measuring FX activation by FVII on TF-free lipid vesicles. In the latter case, the lipid ratio was PC:PE:PS 41:44:14, designed to mimic the composition of highly activated platelets.[14] The lipids were prepared as large unilamellar vesicles (200 nm) that were added in increasing concentrations to FVIIa and FX.

2.5 | Thrombin generation

Thrombin generation (TG) was measured according to Livnat et al.[15-17] Briefly, pooled plasma from severe FVIII-deficient patients (BioreclamationIVT) was spiked with escalating concentrations of MOD-5014 or rhFVIIa. MP reagent (without TF) or PPP reagent (containing 1 pmol/L TF) (both obtained from Stago, Gennevilliers, France) was used as working buffers for the recalcification and low TF experiments, respectively. Both reagents contained 4 μmol/L phospholipids. Thrombin generation was initiated by adding 20 μL of fluorogenic substrate/$CaCl_2$ buffer (FluCa-kit, Stago). Fluorescence was measured using a fluorometer (Fluoroskan Ascent, Lab System, Helsinki, Finland) using an excitation filter at 390 nm and an emission filter at 460 nm. Results were displayed as plots and derived parameters, that is, lag time, endogenous thrombin potential (ETP) and peak height, and were calculated using specialized computer software (v3.0.0.29, Thrombinoscope-BV, Maastricht, The Netherlands). Each sample was tested independently in duplicates. To evaluate platelet binding by MOD-5014 and rhFVIIa, platelets were isolated from healthy human donor blood and activated with thrombin and convulxin (a collagen receptor agonist). The platelets were incubated with escalating concentrations of MOD-5014 or rhFVIIa and subsequently added to plasma concentrations of FX (135 nmol/L) and prothrombin (1.2 μmol/L) in the presence of the chromogenic substrate Gly-Pro-Arg-paranitroanalide (500 μmol/L). Thrombin generation was detected by colour development at 405 nm.

2.6 | Rotation thromboelastometry

Pooled plasma from severe FVIII-deficient patients was spiked with either MOD-5014 or rhFVIIa at concentrations of 0, 2.5, 10 or 15 μg/mL. Rotation thromboelastometry (ROTEM) measurements were conducted with a ROTEM device (Pentapharm, Munich, Germany) using 300 μL of FVIII-deficient plasma, with a subsequent addition of ellagic acid (contact activation, INTEM) or low concentration of TF (EXTEM reagent diluted 1:1700). ROTEM tests were performed according to the manufacturer's instructions at 37°C and were run for a minimum of 45 minutes. The following variables were measured: clotting time, α-angle (reflecting clot propagation) and maximum clot firmness (MCF).

TABLE 1 Prothrombin time (PT) and activated partial thromboplastin time (aPTT) measurements of MOD-5014 and rhFVIIa

Test article	Tested concentration (mg/mL)	PT (sec)		aPTT (sec)	
		Haemophilic plasma	FVII-deficient plasma	Haemophilic plasma	FVII-deficient plasma
MOD-5014 2.5 mg/mL	0.5	10.0	10.2	21.0	21.0
	0.1	8.7	8.9	22.8	21.0
	0.02	8.6	8.8	30.9	<21.0
	0.004	8.5	8.8	45.0	24.0
	0.0008	8.8	9.3	62.5	25.9
rhFVIIa 0.943 mg/mL	0.5	N/A[a]		N/A[a]	
	0.1	8.5	8.7	21.0	21.0
	0.02	8.3	8.6	26.6	21.0
	0.004	8.3	8.6	38.9	22.7
	0.0008	8.5	8.7	55.0	25.4
Control (Plasma only)	0	11.9	No coagulation	87.1	27.5

[a]Outside the detection range of the instrument.

3 | RESULTS

3.1 | In vitro activity

The specific activities of MOD-5014 and rhFVIIa were evaluated using a Staclot VIIa-rsTF assay. MOD-5014 has 35% of the specific activity of rhFVIIa (16 720 and 47 489 U/mg, respectively). However, when evaluated on a molar basis, MOD-5014 and rhFVIIa showed an identical cleavage rate of the synthetic substrate Pefachrome FVIIa (27.5 mol substrate/min/mol VIIa). The EC_{50} values of MOD-5014 and rhFVIIa were assessed using a chromogenic assay intended for testing FVII activity by measuring FX activation and were found to be 0.41 and 0.38 ng/mL, respectively. To compare the PT and aPTT profiles of MOD-5014 and rhFVIIa, the two compounds were spiked in human haemophilia and FVII-deficient plasma. When MOD-5014 and rhFVIIa were spiked at a similar range of concentrations, PT measurements were comparable for both compounds, and aPTT was slightly longer for MOD-5014 (Table 1). To compare the effect of the two compounds on TG, severe haemophilia A pooled plasma was spiked with MOD-5014 and rhFVIIa at a wide range of concentrations. A dose-dependent response was observed following the addition of the two compounds (Figure 1A-D). When TG was initiated by recalcification only, no TG was detected in the absence of MOD-5014 or rhFVIIa as reflected by the flat curve. As shown in Figure 1A,B, at low concentrations (1.25-2.5 μg/mL, equivalent approximately to 45 and 90 μg/kg, respectively), lower TG was observed with MOD-5014 in comparison with rhFVIIa, as reflected by increased lag time and a lower thrombin peak (estimated as 30%-40% lower than in rhFVIIa). When TG was initiated by low TF, baseline TG was observed. A similar pattern of dose-dependent response was observed in the presence of TF for both compounds, albeit with larger amplitude (Figure 1C,D). Spiking with increasing concentrations of TF with fixed doses of either MOD-5014 or rhFVIIa provided a TF-dependent increase in TG performance. For both compounds at all doses and TF concentrations below 5 pmol/L, poor to moderate TG response was observed; the presence of 5 pmol/L TF provided a pronounced improvement in the TG profile. Comparison of the TG profiles of MOD-5014 and rhFVIIa suggested a slight reduction in MOD-5014 response (estimated as 20%-30% lower) without TF or at very low TF concentration (0.5 pmol/L). As the level of TF increases, a similar response is observed at all MOD-5014 and rhFVIIa concentrations (see Figure S1 and Figure S2). Although the higher concentration provided a pronounced improvement in the TG profile, none of the tested compounds were able to provide a complete restoration of the TG as compared to normal TG obtained with spiking of the FVIII-deficient plasma with 100% FVIII (Figure 1E). Finally, the ability of MOD-5014 binding of platelets to support platelet surface thrombin generation was assessed. As shown in Figure 1F, MOD-5014 supported rates of thrombin generation that were slightly lower than rhFVIIa. The lag prior to the onset of thrombin generation was ~40% longer with MOD-5014 than with rhFVIIa.

The ROTEM performance of both MOD-5014 and rhFVIIa was evaluated in severe haemophilia A plasma at a range of concentrations. The tests were conducted with the addition of either ellagic acid (recalcification with contact activation of plasma, INTEM) or low concentration of TF (recalcification with activation of extrinsic pathway, EXTEM). In the INTEM test, clotting time was shorter and the α-angle increased gradually in plasma treated with either MOD-5014 or rhFVIIa at 2.5 and 10 μg/mL in comparison with non-treated plasma. By increasing MOD-5014/rhFVIIa concentration to 15 μg/mL, the changes in clot formation did not differ from that of plasma treated with 10 μg/mL FVIIa. No difference was found between the activities of MOD-5014 and rhFVIIa. In the EXTEM test, there was a decrease in clotting time and increase in MCF in both MOD-5014 and rhFVIIa-treated plasma compared to non-treated plasma. An increase in clot propagation (α-angle) was observed to a similar extent in plasma treated with both agents at 2.5 and 10 μg/mL, with no further change at 15 μg/mL (Table 2).

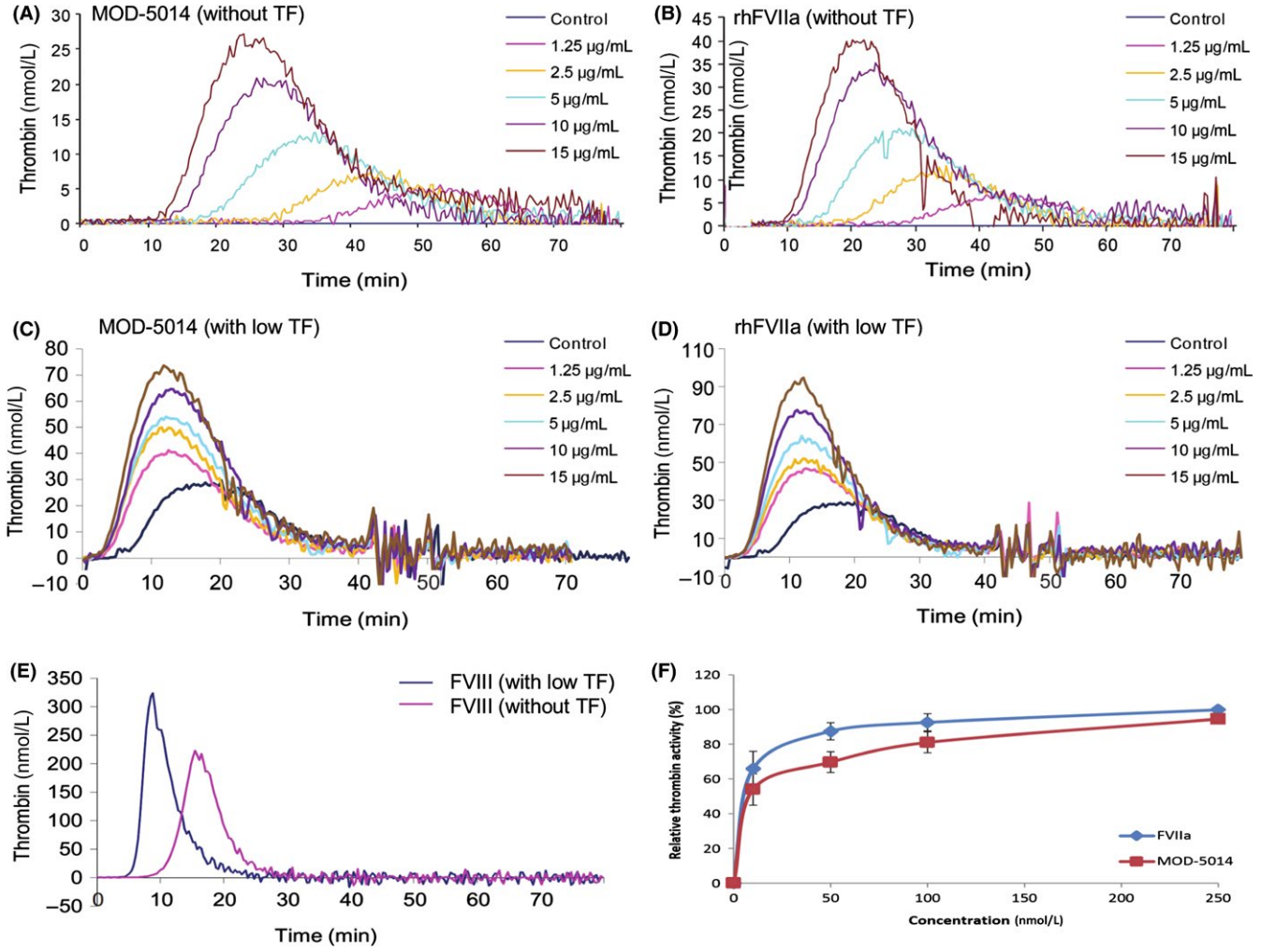

FIGURE 1 Thrombin generation by MOD-5014 vs rhFVIIa. Thrombin generation (TG) was evaluated in severe haemophilia A pooled plasma following spiking of MOD-5014 (A,C) or rhFVIIa (B,D). Thrombin generation (TG) was initiated following either by recalcification (A,B) or by addition of low levels of tissue factor (TF) (C,D). The control samples contained the reaction mix without added FVIIa. Panel (E) shows thrombin generation following spiking with 100% FVIII. Panel (F) shows TG by different concentrations of activated platelet-bound MOD-5014 or rhFVIIa. Note the different *y*-axis scale in each panel

3.2 | Interaction with TF and lipids

A binding study of MOD-5014 and rhFVIIa to immobilized sTF using surface plasmon resonance (SPR) yielded K_d values of 2.32-2.4 nmol/L for MOD-5014 and 1.72-1.91 nmol/L for rhFVIIa (Table 3). When factor VIIa binds TF, a conformational change occurs in factor VIIa that results in an increase in the rate of substrate cleavage, which can be used to monitor factor VIIa binding to TF. To compare the TF binding capability of MOD-5014 and rhFVIIa, varying concentrations of the two substances were incubated with TF and the cleavage rate of Pefachrome FVIIa was measured. As the concentration of TF was well above the expected K_d, at low concentrations, all of the FVIIa should be bound to TF, and the rate of substrate cleavage is that of the VIIa/TF complex. Once the concentration of FVIIa exceeded that of TF, the rate of substrate cleavage dropped to that of free FVIIa. As shown in Figure 2A, TF binding by MOD-5014 accelerated substrate cleavage by MOD-5014 to a similar extent as rhFVIIa. Binding of FVIIa to TF can also be assessed by measuring the rate of FX activation, as cleavage of FX by FVIIa is slow relative to cleavage by the FVIIa/TF complex. Factor X activation was assessed by cleavage of the synthetic substrate Pefachrome FXa; there was a very slight negative cooperativity (Hill value < 1) in the binding of FVIIa to TF (either MOD-5014 or rhFVIIa). When bound to TF, MOD-5014 showed a slightly reduced rate of FX activation (93%) relative to rhFVIIa, and the affinity of MOD-5014 for TF was equivalent to that of rhFVIIa (Figure 2B). To establish the kinetic profile of the MOD-5014/TF complex, the rate of FX activation as a function of FX concentration was measured. When bound to TF, MOD-5014 had a slightly reduced turnover of FX (92%) relative to rhFVIIa; the binding of FX to the MOD-5014/TF complex was similar to its binding to the rhFVIIa/TF complex (Figure 2C). To evaluate the activity of MOD-5014 in the absence of TF (such as may occur on the surface of platelets), FX activation by FVIIa was studied on lipid vesicles using a lipid ratio that mimics the composition of activated platelets. As shown in Figure 2D, FXa generation increased with

TABLE 2 The effect of MOD-5014 and rhVIIa on clot formation in FVIII-deficient plasma

Test	Treatment	Conc. (μg/mL)	Clot time (sec)	α-angle (°)	MCF (mm)
INTEM	Control	-	1123	9	11
	rhVIIa	2.5	810	12.5	10
		10	531	25	13
		15	472	26	12
	MOD-5014	2.5	797	14.5	12
		10	611	22.5	14.5
		15	660	27	13
EXTEM	Control	-	404	19.5	10.5
	rhVIIa	2.5	371	20.5	12.5
		10	340	27	13
		15	377	30	14
	MOD-5014	2.5	355	22.5	12.5
		10	324	28.5	14
		15	323	30	13.5

TABLE 3 Binding affinity of MOD-5014 and rhFVIIa to tissue factor (TF)

Test article	Immobilized sTF (μg)	k_{on} (1/Ms)	k_{off} (1/s)	K_d (M)	R_{max} (RU)	χ^2 (RU)	χ^2/R_{max} (%)
MOD-5014	0.65	6.08×10^5	1.46×10^{-3}	2.4×10^{-9}	132.77	7.02	5.28
	1.25	5.88×10^5	1.36×10^{-3}	2.32×10^{-9}	403.73	41.03	10.1
rhFVIIa	0.65	11.5×10^5	1.98×10^{-3}	1.72×10^{-9}	118.98	6.86	5.7
	1.25	9.54×10^5	1.82×10^{-3}	1.91×10^{-9}	391.28	35.94	9.1

the increasing concentration of lipids as more surface area was available for the reaction; at a sufficiently high concentration of lipids, the rate of the reaction decreased as FVIIa and FX were segregated onto different lipid vesicles. The rate of FX activation in the absence of TF was lower for MOD-5014 (~65%) in comparison with rhFVIIa for all lipid concentrations.

3.3 | Interaction with TFPI, AT and heparin

A comparison of MOD-5014 and rhFVIIa clotting activity inhibition profiles in the presence of tissue factor pathway inhibitor (TFPI), a natural inhibitor of FVIIa, showed that TFPI inhibited rhFVIIa and MOD-5014 and in a similar dose-dependent manner (Figure 3A). The addition of heparin to either MOD-5014 or rhFVIIa resulted in >96% inhibition for both compounds, even at the extremely low concentration of 0.1 U/μL (results not shown). The clotting activities of MOD-5014 and rhFVIIa were also evaluated in the presence of antithrombin, which resulted in a similar rate of inhibition (Figure 3B). In the presence of TFPI, MOD-5014 and rhFVIIa demonstrated a very similar FXa activity inhibition curve (Figure 3C). The addition of heparin had no significant influence on the inhibition profile when added with TFPI (results not shown). MOD-5014 and rhFVIIa exhibited similar activation of FX at the presence of AT (Figure 3D). When heparin was added to MOD-5014 or rhFVIIa in the presence of a constant AT concentration, MOD-5014 and rhFVIIa exhibited similar and moderate inhibition; the same was true when heparin was present in a constant concentration, and AT was added at different concentrations (not shown).

4 | DISCUSSION

In vitro, based on the data collected as of today, MOD-5014 behaves similarly to rhVIIa in its mechanism of action by forming a complex with TF. This complex, in the presence of calcium and phospholipids, activates FX with the subsequent conversion of prothrombin to thrombin. A reduced specific activity was obtained for MOD-5014 compared to rhFVIIa as observed by functional activity assays such as Staclot and TG. FX activation test by a chromogenic assay in the presence of TF, phospholipids and calcium also demonstrated a slightly lower activity of MOD-5014 in comparison with rhFVIIa as reflected by EC_{50}. The lower activity of MOD-5014 might be due to the fact that 84.4% of MOD-5014 corresponds to FVIIa and 15.6% corresponds to CTP. This might also be due to the attachment of the latter to the proteolytic domain (C-terminus) of FVIIa, which can presumably reduce the enzymatic activity of the protein without interfering with its basic interactions with other biological components. The question of whether this has a direct effect on the capability of MOD-5014 to

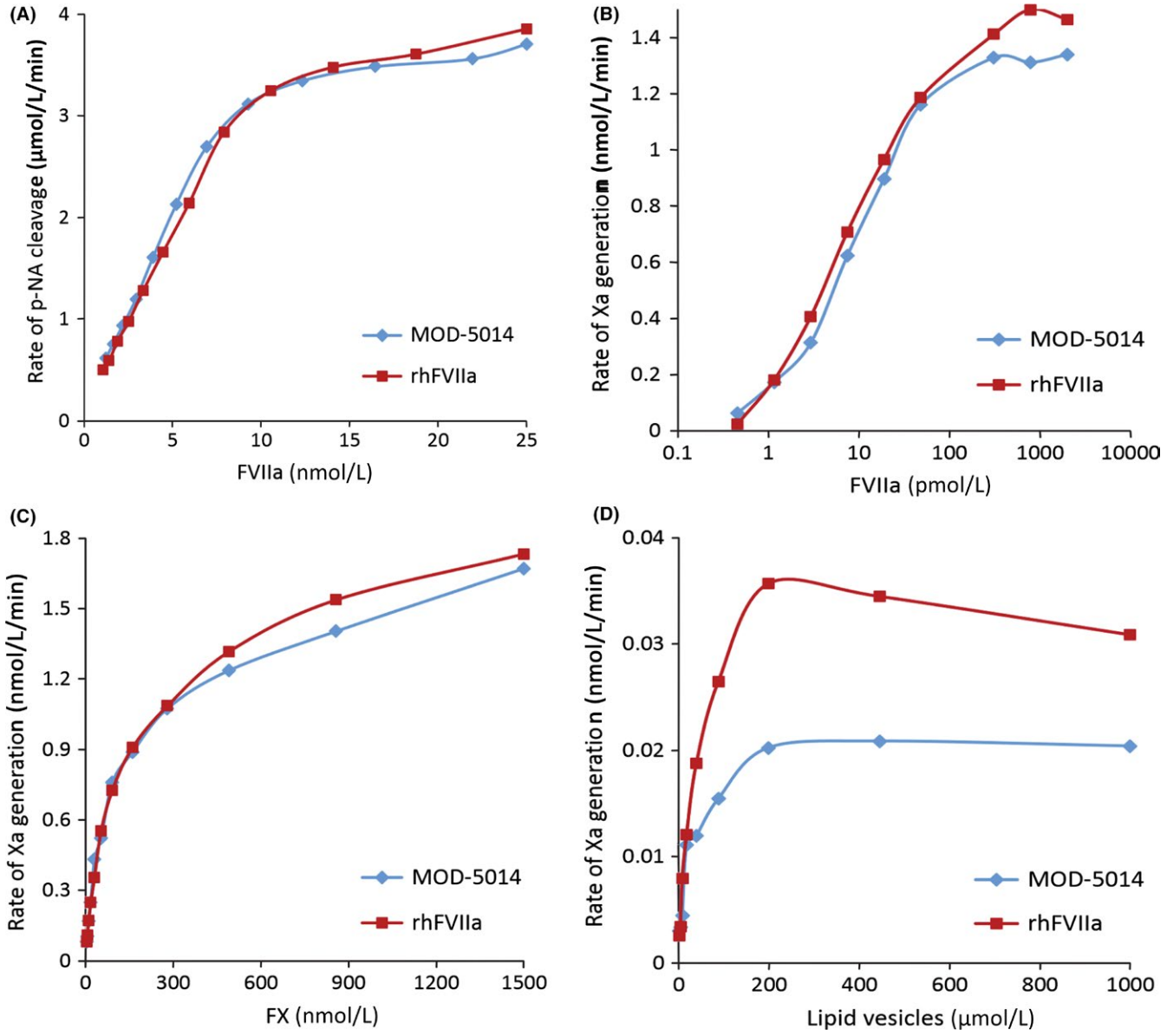

FIGURE 2 Interaction of MOD-5014 and rhFVIIa with cofactors. A, Tissue factor (TF) (8.7 nmol/L) was incubated with rhFVIIa or MOD-5014, and binding was assessed by cleavage of synthetic substrate. The inflex point occurs when TF is saturated with rhFVIIa or MOD-5014. B, TF (10 pmol/L) was incubated with rhFVIIa or MOD-5014. 135 nmol/L FX and substrate were added, and factor VIIa binding to TF was measured by activation of FX. V_{max} calculation is ±5%. C, Kinetic profile of the FVIIa/TF complex (1 nmol/L/10 pmol/L) assessed using the rate of FX activation as a function of FX concentration. D, The rate of FXa generation with 20 nmol/L rhFVIIa or MOD-5014 and 500 nmol/L FX is plotted against the concentration of lipid vesicles in the absence of TF. The representative data sets shown in panels (A-D) were run on the same day using a single batch of protein solutions that were titrated to have the same molar concentrations

maintain proper haemostasis and control bleeding will be evaluated in a clinical setting.

The binding affinity of MOD-5014 to TF was found to be similar to that of rhFVIIa and comparable to previously reported K_d values.[18-23] The interaction of FVIIa and TF mainly takes place at the light-chain region of the coagulation factor,[24] which is distant from the FVII-CTP fusion area (located in the heavy-chain region of FVII). Therefore, the fused CTP is unlikely to interfere with FVII/TF binding. Both MOD-5014 and rhFVIIa demonstrated a dose-dependent TG response when spiked to severe haemophilia A pooled plasma, with an initial poor response at concentrations mimicking clinical doses of 40-80 µg/kg. These results suggest that doses lower than 40-80 µg/kg will not provide an adequate in vivo response. These results are in line with published data demonstrating a successful use of FVIIa at doses of 200 µg/kg FVIIa and above to obtain an improved TG profile.[25] MOD-5014 demonstrated reduced TG performance when spiked at a similar concentration as rhFVIIa, suggesting that an increased concentration (30%-40%) might be needed in a clinical setting to provide proper initial haemostatic effect comparable to that of rhFVIIa. Spiking both compounds at fixed

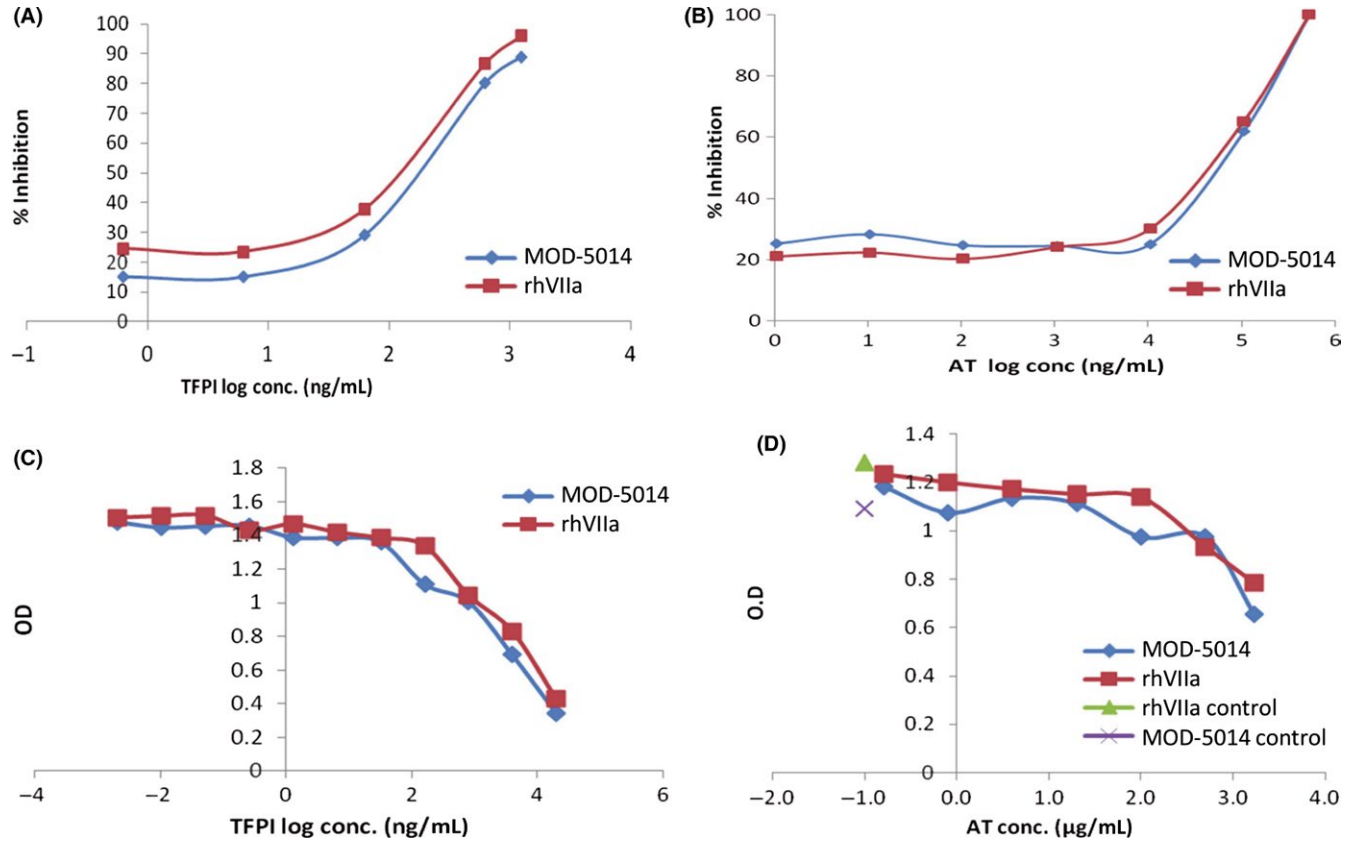

FIGURE 3 Clotting activity inhibition of MOD-5014 and rhFVIIa. A, Clotting inhibition by FVIIa in the presence of tissue factor pathway inhibitor (TFPI). B, Clotting inhibition in the presence of antithrombin (AT). C, FX activation by FVIIa in the presence of TFPI. D, FX activation by FVIIa in the presence of AT. For all panels, representative data are shown

concentrations in the presence of escalating concentrations of TF demonstrated that the amount of TF in the sample was predominantly responsible for the increased TG response, further confirming a biological similarity between MOD-5014 and rhFVIIa. A reduced response of MOD-5014 in comparison with rhFVIIa in the absence of TF was observed; this may imply that the attachment of CTP can slightly interfere with non-TF-dependent FVIIa activity, as this attachment is located close to the proteolytic domain of the protein. The finding that MOD-5014 had a slightly lower affinity for binding to activated platelets and slightly lower proteolytic activity on platelet surfaces is not surprising, as the modification added to the native FVIIa molecule has the potential to sterically hinder binding to both the platelet membrane and to protein substrates. When ROTEM was utilized to compare the activity of MOD-5014 and rhFVIIa, a dose of 2.5 µg/mL, which mimics an in vivo dose of 80 µg/kg,[16] resulted in a low response for both compounds, further confirming that concentrations below 2.5 µg/mL might be considered as ineffective and will provide a very limited or undetectable in vivo response. Increasing concentrations of MOD-5014 and rhFVIIa to 10 µg/mL were followed by dose-dependent stimulatory effect on clot formation, with no essential difference in the effects of either agent.

The catalytic activity of MOD-5014 in terms of TF binding was shown to be slightly reduced in comparison with rhFVIIa. The lower rate of MOD-5014 activation when bound to TF could be a consequence of reduced affinity for FXa or reduced turnover of FX once it is bound to the complex. Measuring the rate of FX activation as a function of FX concentration established the kinetic parameters of the complex and showed that when bound to TF, MOD-5014 had a slightly reduced turnover of FX (92%) relative to rhFVIIa, while the binding of FX to the MOD-5014/TF complex was the same as its binding to the rhFVIIa/TF complex. In a low TF environment, or in the total absence of TF, FX activation on platelets is thought to contribute to the haemostatic effect of FVIIa. An evaluation of FX activation on TF-free lipid vesicles by either MOD-5014 or rhFVIIa revealed a lower rate of FXa generation by the former. This difference was not due to differences in affinity to the lipids, as the rate of FXa generation relative to the maximum for each was similar when plotted against lipid concentration. This also suggests that the segregation of rFVIIa and FX is the same as the segregation of MOD-5014 and FX when phospholipids are in excess. In addition, the binding of FX to MOD-5014 on the lipid surface was the same as its binding to rhFVIIa; this suggests that the difference is due to the lower catalytic activity for MOD-5014 in comparison with rhFVIIa.

The inactivation of FVIIa plays a crucial role in maintaining proper haemostasis. Understanding the inhibition process of MOD-5014 following the attachment of CTP is critical to its safety assessment prior to the first-in-human study. Incubating MOD-5014 or rhFVIIa with TFPI, a major FXa-dependent inhibitor of the

extrinsic coagulation pathway, resulted in a very similar deactivation pattern for both compounds, reflected by % clotting inhibition. Antithrombin was previously reported to inhibit FVIIa at a slow rate and also demonstrated augmented inhibition at the presence of heparin. Antithrombin demonstrated a similar inhibition pattern of both MOD-5014 and rhFVIIa; this pattern was maintained following the addition of heparin. The comparability of MOD-5014 and rhFVIIa in terms of inhibition suggests that MOD-5014 can be properly deactivated in vivo. The slightly reduced rate of AT-induced inhibition may suggest a slight extension of MOD-5014 half-life in vivo with a proper inhibition response.

5 | CONCLUSION

The present work has established the comparable in vitro activity of MOD-5014 and rhFVIIa, thus paving the way for subsequent in vivo pharmacology evaluations of this novel compound in preparation for clinical studies.

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ABI, TL, RG, YF, LM, MH, LB and MZ designed the experiments and analysed the results. BS provided technical assistance. DM, OH, GK and GH supervised the research. GH wrote the manuscript. Prof. Maureane R. Hoffman (Duke University) performed the platelet binding experiments. Dr. Doron Calo (OPKO Biologics) provided assistance in technical editing of the manuscript.

DISCLOSURES

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SUPPORTING INFORMATION

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