Prethrombin-1 as a Drug Substance Promoting Hemostasis with Reduced Risk of Thrombosis

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Abstract

Introduction Prethrombin-1 is a Gla-domain lacking enzymatically inactive split product that results from the cleavage of fragment 1 from prothrombin by thrombin in a feedback reaction.

Methods A prethrombin-1 preparation derived from human plasma was tested for its hemostatic and thrombogenic properties. Animal models of nail clipping (for rabbits) and tail clipping (for mice) were developed to measure blood loss in FVIII-inhibitor or rivaroxaban anticoagulated rabbits and mice, respectively. A modified Wessler test was used in rabbits to assess the thrombogenic potential by Wessler score and clot weight. Studies were performed in groups of three to six for prethrombin-1 dose escalation and comparison with prothrombin, Beriplex®, FEIBA®, and saline as a control. Data were analyzed using t-statistics or the Mann Whitney U test as applicable.

Results Prethrombin-1 has excellent hemostatic properties in anticoagulated mouse and rabbit bleeding models. Wessler tests suggest that in contrast to activated and nonactivated prothrombin complexes, prethrombin-1 has negligible thrombogenic potential.

Conclusion The thrombin zymogen prethrombin-1 promotes hemostasis with reduced risk of thrombosis. Prethrombin-1 may have potential to become a life-saving treatment for patients who bleed or are at risk of bleeding.

Keywords
- coagulation
- vitamin K-dependent proenzyme
- clotting factors
- prothrombin
- prethrombin-1

Introduction

Plasmatic hemostasis is the result of a cascade-like proteolytic activation of inactive zymogens.[1] In its penultimate stage, the concerted action of activated coagulation factors and their respective cofactors leads to the transformation of prothrombin into thrombin.[2] When present in sufficient concentration, thrombin causes the fibrinogen in blood plasma to coagulate in the wound area and, to form, jointly with activated platelets, an insoluble fibrin matrix. Thrombin also plays an important role in the extrinsic pathway, where platelets and the clotting factor cofactors V and VIII are activated.[3]

Thrombin generation from prothrombin is a complex and rigorously controlled enzymatic process.[1] To prevent it from shifting toward thrombogenicity, inhibitors, additional coagulation factors, and cofactors such as factor Xa and cofactor Va as well as platelets and certain endothelial factors are required.[4–6]

Prothrombin is a vitamin K-dependent proenzyme and has a complex structure, characterized by an N-terminal Gla-

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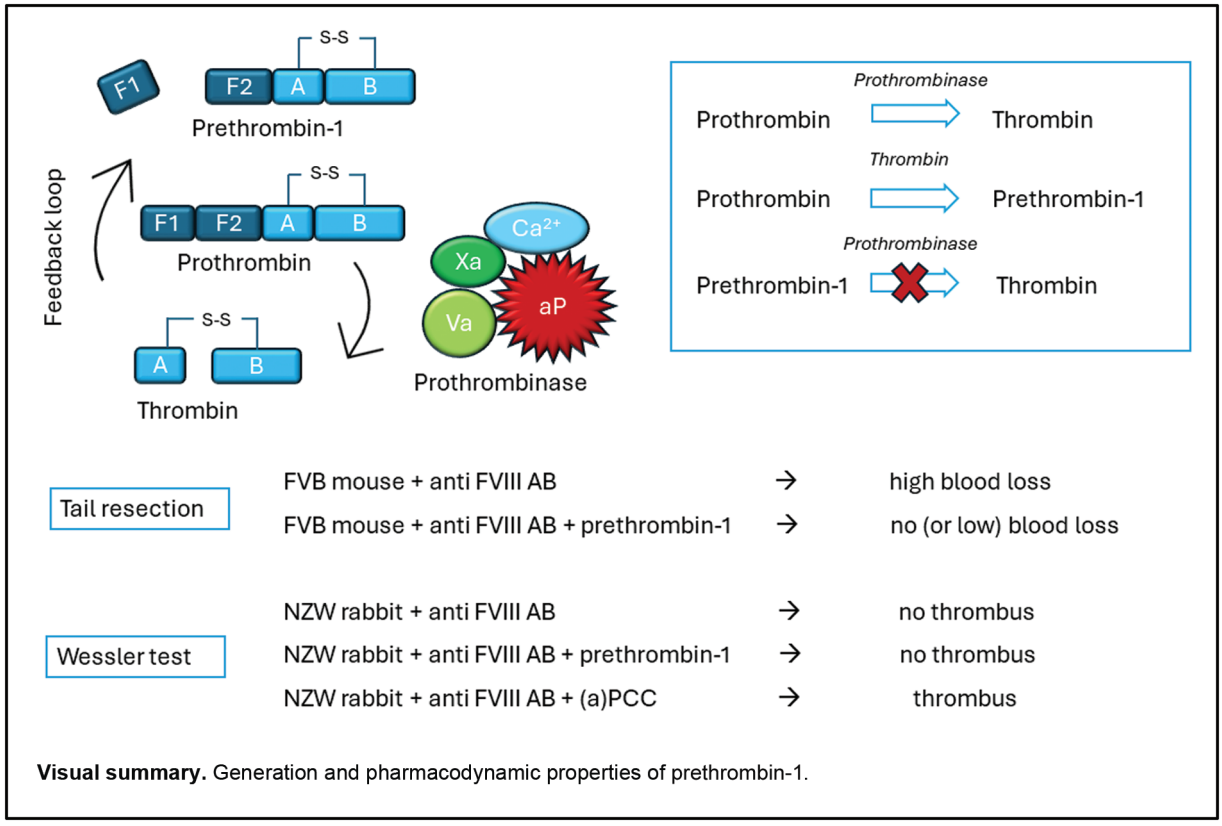

Visual summary. Generation and pharmacodynamic properties of prethrombin-1.

domain and the Kringle 1-domain (fragment 1), the Kringle 2-domain (fragment 2), and the serine protease domain. For the generation of thrombin, prothrombin needs to be cleaved at two cleavage sites: R271 and R320.[7,8] Cleavage at R320 results in the formation of the enzymatically active meizothrombin. Subsequent cleavage at R271 results in the release of thrombin. When R271 is cleaved first, the enzymatically inactive prethrombin-2 is generated, which can be transformed into thrombin by further cleavage at R320.[9] In the presence of cofactor Va, cleavage occurs at R320, and meizothrombin is generated. In the absence of factor Va, on the other hand, cleavage occurs at R271, and prethrombin-2 is generated.[10]

Lanchantin et al[11] recognized early on that in a coagulation test using prothrombin deficient plasma, prothrombin activity is lost, due to the action of thrombin on prothrombin. The two split products resulting from the action of thrombin on prothrombin were ultimately designated prethrombin-1 and fragment 1.

Prethrombin-1 is an enzymatically inactive split product which arises after cleavage of fragment 1 from prothrombin by thrombin in a feedback reaction.[12] Prethrombin-1 is not recognized as a component within the activation pathway leading from prothrombin to thrombin. Prethrombin-1 and prethrombin-2 do not contain the Gla-domain necessary for membrane binding capacity and are therefore not efficiently activated by prothrombinase bound to phospholipid vesicles.[13]

Quantitatively, prothrombin is the most prominent procoagulant factor in plasma. From the work of Mann and coworkers[14] we know that high prothrombin concentrations are a risk for thrombosis. Prothrombin has a relatively long half-life in blood, which implies that the repeated administration of nonactivated and activated prothrombin complex concentrates (PCCs) increases the prothrombin concentration in plasma to twice or three times the normal value. In clinical use, prothrombin was identified as a major thrombogenic agent in PCCs.[15]

Highly purified recombinant prothrombin has been shown to be effective in achieving hemostasis.[16] A clinical study to evaluate the safety, toxicity, and pharmacodynamics of recombinant human prothrombin was discontinued prematurely for reasons that were not disclosed.[17]

Bleeding is considered a leading cause of mortality. If untreated, severe or chronic hemorrhaging might lead to organ failure, seizures, coma, joint damage, and eventually death. Even with treatment, severe bleeding is often fatal. The risks of fatal bleeding, major bleeding, and intracranial and gastrointestinal hemorrhage are often underestimated.

Treatment options to arrest bleeding include activated or nonactivated PCCs, and recombinant factor VIIa. PCCs are produced from human plasma.[18] Their composition is complex as they contain clotting factors such as prothrombin, factor VII, factor IX, and factor X as well as the anticoagulant factors protein C and protein S. Activated prothrombin complexes such as FEIBA (Takeda) have an even more

complex composition, as they additionally contain activated factor VII and activated factor X.[19]

In addition to bleeding in congenital clotting disorders like hemophilia, bleeding in acquired disorders often connected to treatment with direct oral anticoagulants (DOACs) continues to be a key complication, affecting 2 to 4% of DOAC-treated patients.[20–22]

In the present preclinical studies, we aimed to identify and explore the potential of prethrombin-1 as a new treatment option for patients who bleed or are at a risk of bleeding.

Materials and Methods

Materials

Prethrombin-1 as well as prothrombin were manufactured in the laboratory of Biomedizinische Forschung & Bio-Produkte AG, Vienna, Austria. FEIBA was purchased from Baxter Healthcare Corporation (Westlake Village, California, United States), and Beriplex from CSL Behring GmbH (Marburg, Germany). Rivaroxaban was purchased from Nschem Shanghai, China, dissolved in DMSO (1000 ug/mL) and diluted with NaCl 0.9%. Sheep polyclonal antibody to human coagulation factor VIII (FVIII) was procured from Haematologic Technologies, Essex Junction, Vermont, United States.

Animals

Animals were obtained from Charles River Laboratories, Sulzfeld, Germany.

All experiments were approved by the animal care committee of the Center for Biomedical Research and Translational Surgery, Medical University of Vienna, Austria.

Study Design

Prethrombin-1 was tested for hemostatic efficacy in rabbits and mice, and for thrombogenicity in rabbits, both species rendered hemophilic by FVIII antibody administration. Studies included dose escalation and comparison with prothrombin, Beriplex, and FEIBA. In one study, the hemostatic efficacy of prethrombin-1 was investigated in mice after rivaroxaban treatment.

Statistics

Descriptive statistics were used in all studies.

Analysis of 2-between group data was performed using *t*-statistics or the Mann–Whitney U-test. For comparison between treatment regimens of more than two groups, the Kruskal–Wallis test was used. Post hoc comparisons were performed with Bonferroni correction. A value of $p < 0.05$ was considered to indicate statistical significance.

Manufacturing of Prethrombin-1

All chemicals and reagents were purchased from Merk Millipore (Burlington, Massachusetts, United States) and Thermo Fisher Scientific (Waltham, Massachusetts, United States). Protein standards were purchased from CoaChrom (Maria Enzersdorf, Austria). Chromatography resins and equipment were purchased from Cytiva (Marlborough, Massachusetts, United States) and BioRad (Hercules, California, United States). Ultra- and dia-filtration (UF/DF) units were purchased from Sartorius (Goettingen, Germany). Coagulation assay reagents and devices were purchased from Werfen (Barcelona, Spain).

Starting material for the purification of prothrombin (FII) was an intermediate eluate from a human plasma pool containing coagulation factors II, VII, IX, and X (Sanquin, Amsterdam, The Netherlands). The frozen solution was melted to room temperature in a water bath, and after dilution with deionized water was adsorbed batchwise on Sephadex DEAE A50. Desorption of bound protein was performed stepwise with increasing concentration of NaCl in citrate. Buffers used for this scope and all following buffers used during purification of FII before UF/DF contained up to 2 mM benzamidine hydrochloride to preserve the nonactivated form of coagulation factors. Concentrations of FII in samples were monitored in IU with a standard coagulation assay with FII depleted plasma on the ACL TOP Testing System. The fraction containing major amounts of FII from the raw purification step was further processed consecutively on the AEKTA Pure system with a weak anion exchanger (MacroPrepDEAE) and a multi-modal anion exchanger (Capto ADHERE ImpRes). Desorption of bound FII was performed with increasing chloride concentration and adding propylene glycol and arginine to the elution buffer. For polishing, FII was first concentrated in a centrifugal UF/DF device with 30 kDa molecular weight cutoff, and buffer was exchanged on a size exclusion resin (Sephacryl S300 PrepGrade). The FII preparation was stored at −20°C before use for analysis or further processing.

Preparation of prethrombin-1 (Pre-1) started with purified FII to which α-thrombin (FIIa) with a ratio of 0.5 IU FIIa per 1 IU FII. After an incubation period of 24 hours in the mixture obtained, Pre-1 was separated from residual FII and FIIa using chromatography in binding mode with the multimodal resin (Capto ADHERE ImpRes) and in nonbinding mode with an affinity gel (Benzamidine Sepharose FF). In the nonbinding mode, any protein possessing an accessible serine protease active site will bind to the resin, while the zymogen prethrombin-1, lacking such binding capability, undergoes elution from the column, resulting in a higher degree of purification. Purity and conversion rates from FII to Pre-1 were investigated preliminarily by protein electrophoresis on a gradient polyacrylamide gel (SDS-PAGE) and Coomassie blue staining (►**Fig. 1**). Concentration was determined from theoretical extinction coefficient using UV absorption at 280 nm and a standard regression of bovine serum albumin in a Bradford assay.

Analysis of purity, identity, and integrity of the intermediate preparation (FII) and the product (Pre-1) were commissioned to the protein core facility of the Medical University of Innsbruck. Identification of the first six amino acids after N-terminal Edman degradation confirmed the expected sequence known from databases (e.g., UniProt, EBI, Hinxton, United Kingdom). From mass spectrometry analysis coupled with high-pressure liquid chromatography, the main abundant protein (86%) in the FII sample had a measured mass of

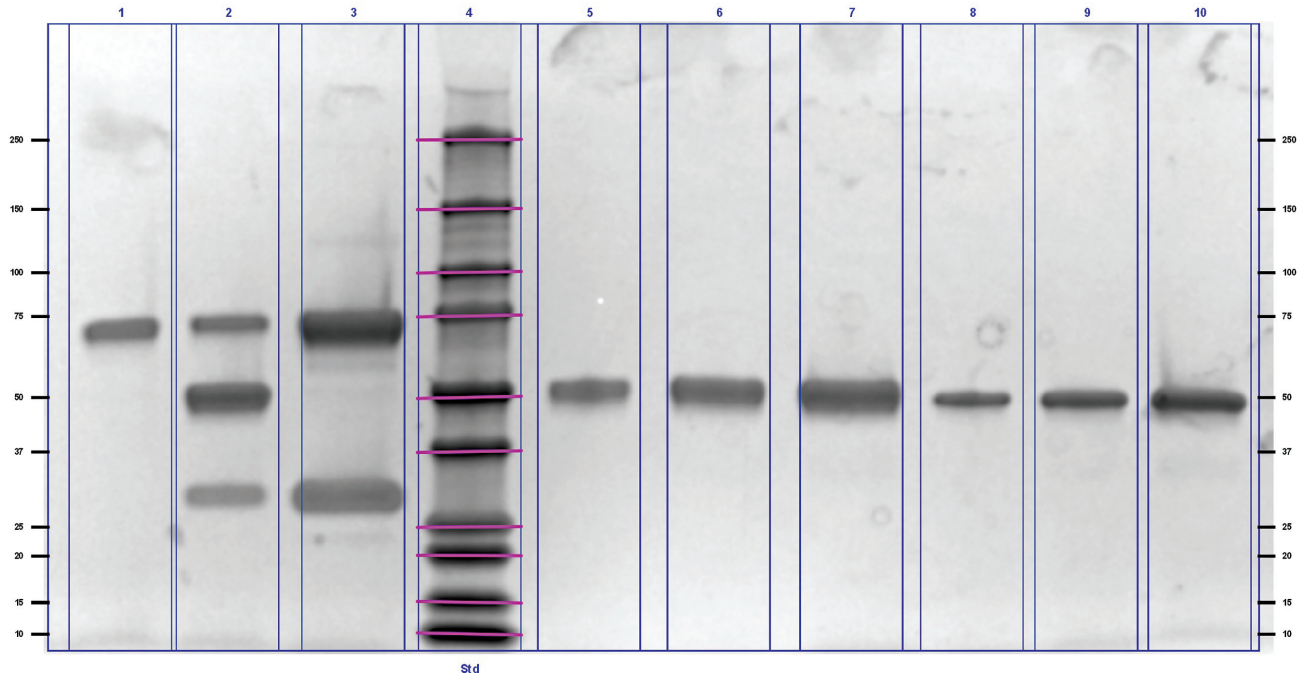

Fig. 1 SDS-PAGE of prethrombin 1 obtained from incubation of prothrombin with thrombin. Lane 1: prothrombin purified with chromatography from human plasma (15 µL, diluted 1:400, nonreduced). Lane 2: prothrombin after incubation with thrombin (15 µL, diluted 1:200, reduced). Lane 3: prothrombin and prethrombin-1 fragment separated with chromatography from incubation mixture (15 µL, diluted 1:32, reduced). Lane 4: molecular weight standard BioRad Precision Plus Protein (5 µL). Lanes 5–7: prethrombin-1 purified with chromatography from incubation mixture (5, 10, 15 µL, diluted 1:300, reduced). Lanes 8–10: prethrombin-1 purified with chromatography from incubation mixture (5, 10, 15 µL, diluted 1:300, nonreduced). Samples were heated with 4 × sample buffer (BioRad, 4x Laemmli Sample Buffer) and reduced with dithiothreitol DTT, loaded on gradient gel (BioRad, 4–20% Mini-PROTEAN TGX Precast) and stained with Coomassie dye (BioRad, Bio-Safe Coomassie Stain).

70 kDa with 58% coverage with mature Prothrombin OS = Homo sapiens and the main abundant protein (94%) in the Pre-1 sample had a measured mass of 48 kDa with 79% coverage with Prethrombin-1 OS = Homo sapiens.

In a prothrombin assay with prothrombin-deficient plasma, the prethrombin-1 obtained had only approximately 1% of the activity of prothrombin. With an enzymatic complex of factor Xa and its cofactor Va, one prothrombin molecular equivalent unit of prethrombin-1 generated approximately 200 NIH units of thrombin.

Hemostatic Efficacy of Prethrombin-1 in a Rabbit Nail Clipping Model

A rabbit hemostasis model was developed using 2.5 to 3.5 kg female New Zealand white rabbits. Anesthesia was induced with Ketamine/Xylazine (40 mg/kg and 8 mg/mL; subcutaneous) in the stable area. Then, the transfer to the operating room took place. After orotracheal intubation, the animals were ventilated with 40% oxygen and 2% isoflurane in a volume-controlled manner. As an analgesic, 0.3 mg/kg Buprenorphine was administered. Arterial blood pressure monitoring was performed invasively through the ear artery. The animals received Ringer's solution for fluid substitution (10 mL/kg/h). To simulate a temporary hemophilic state, a FVIII antibody was intravenously infused at a dose of approximately 20,000 Bethesda units (BU) per kilogram of body weight (BW). The administration of the FVIII antibody aimed to replicate the development of a FVIII inhibitor status observed in humans.

Following a minimum 10-minute incubation period, hemophilic status was confirmed by nail clipping. To this end, the cuticle of the nail was excised using a nail clipping device, and blood loss was assessed by immersing the clipped cuticle in a preweighed Falcon tube filled with 37°C 0.9% saline solution. Bleeding was monitored over a 15-minute period. Gravimetric determination of blood loss was performed by differentially weighing the tubes. Then, either the test or the reference substance was administered intravenously. After an additional waiting period of more than 20 minutes, another nail clipping test was performed as described above. Subsequently, the animals were euthanized through an overdose of pentobarbital in anesthesia.

Modified Rabbit Wessler Model

A modification of the Wessler model[23,24] was employed to conduct jugular vein thrombosis experiments on female New Zealand white rabbits weighing 2.5 to 3.5 kg. The animals underwent anesthesia as described above. The contralateral vena jugularis communis was prepared, with all side branches ligated with 8/0 Prolene or coagulated, including smaller outlets. Subsequently, the rabbits were induced into a temporary hemophilic state, following the procedure outlined above.

Ten minutes after the intravenous (i.v.) administration of the test or reference substance, a segment of the vein was ligated both proximally and distally at a distance of 1.5 cm. After a 20-minute interval, the ligated vein segment was excised, placed in a sodium citrate buffer, and dissected

open. Macroscopic examination of the luminal vein surface was conducted. Any observed thrombi were extracted and weighed. The assessment of thrombi utilized a scale (Wessler score) ranging from 0 to 3: 0 denoted an absence of thrombus, 1 represented one thrombus or several small thrombi (<2 mg), 2 indicated one or more non-occluding thrombi, and 3 signified a large, completely occluding thrombus. Finally, the animals were euthanized by an overdose of pentobarbital in anesthesia.

Hemostatic Efficacy of Prethrombin-1 in a FVIII Inhibitor Mouse Model

Hemostasis experiments were performed in 20 to 30 g female FVB mice. To determine the hemostatic efficacy of prethrombin-1 in a FVIII inhibitor mouse model, FVB mice were anesthetized with Ketamine (100 mg/kg)/Xylazine (5 mg/kg) and Dormicum (1 mg/kg) i.p. and VIII antibody at a dose between 25,000 and 30,000 BU/kg BW was injected into the tail vein to ensure increased bleeding after tail resection. In the following 10 minutes, the vena femoralis was exposed for injection of the test substance. Fifteen minutes after administration of the test substance via the vena femoralis and electro-cauterization of the injection site, the tip of the tail was resected using a scalpel at a distance of 3 mm. The tail was suspended in a pre-weighed Eppendorf tube filled with 37°C 0.9% saline solution and bleeding was observed for 30 minutes, at which time the experiment was stopped. Blood loss was measured gravimetrically by differential weighing.

Hemostatic Efficacy of Prethrombin-1 in a Rivaroxaban Mouse Model

The procedure was the same as the one above except for the use of 2 µg Rivaroxaban/mouse instead of FVIII antibody. Rivaroxaban was dissolved in DMSO (1,000 µg/mL) and diluted in 0.9% saline solution.

Results

Rabbit Nail Clipping Study

The rabbit nail clipping model was used to compare the hemostatic efficacy of five doses of prethrombin-1 (4.7, 16, 47, 157, and 469 nmol/kg) against 2 mL of sodium citrate as shown below.

Although the spreads in blood loss observed values among both prethrombin-1 and the control samples appear to be quite high, it must be taken into account that animal models are complex biological systems and variances are inherent.

Blood loss from rabbit nail clipping experiments is shown in ►**Table 1** and ►**Fig. 2**. At a dose of 4.7 nmol prethrombin-1/kg BW, blood loss was above the median blood loss of untreated animals after anticoagulation by FVIII antibody infusion and comparable to sodium chloride. At higher doses, blood loss after i.v. treatment with prethrombin-1 remained below the median blood loss of untreated animals. The difference between sodium chloride and 157 nmol prethrombin-1 was statistically significant at $p < 0.05$.

Table 1 Blood loss in FVIII-inhibitor rabbits after i.v. treatment with different doses of prethrombin-1 or sodium chloride

Animal #[a]	Dosing of prethrombin-1 or physiological saline solution as a control[b]	Blood loss[c] [mg]
367	2 mL NaCl 0.9%	106.3
368	2 mL NaCl 0.9%	240.5
378	2 mL NaCl 0.9%	43.5
379	2 mL NaCl 0.9%	100.4
383	2 mL NaCl 0.9%	36.7
369	4.7 nmol/kg prethrombin-1	189.2
370	4.7 nmol/kg prethrombin-1	191.6
371	4.7 nmol/kg prethrombin-1	102.3
335	16 nmol/kg prethrombin-1	0
336	16 nmol/kg prethrombin-1	6
332	16 nmol/kg prethrombin-1	23.7
366	16 nmol/kg prethrombin-1	45.5
374	16 nmol/kg prethrombin-1	0
328	47 nmol/kg prethrombin-1	0
330	47 nmol/kg prethrombin-1	8.5
331	47 nmol/kg prethrombin-1	45.7
365	47 nmol/kg prethrombin-1	17.1
375	47 nmol/kg prethrombin-1	0
326	157 nmol/kg prethrombin-1	0
327	157 nmol/kg prethrombin-1	0
334	157 nmol/kg prethrombin-1	0
352	157 nmol/kg prethrombin-1	0
354	157 nmol/kg prethrombin-1	7.3
358	157 nmol/kg prethrombin-1	0
329	469 nmol/kg prethrombin-1	0
345	469 nmol/kg prethrombin-1	4.3
347	469 nmol/kg prethrombin-1	0

Abbreviation: FVIII, factor VIII.
[a]Animal consecutive numbers in the approved animal experimentation protocol.
[b]Dosing of prethrombin-1 or physiological saline solution as control.
[c]Blood loss in [mg] from gravimetric measurements.

Modified Rabbit Wessler Study

Animals receiving the highest dose of prethrombin-1 from the dose escalation study above were compared by Wessler score and clot weight with equimolar doses of prothrombin as well as Beriplex and FEIBA at doses typical for clinical use. The results are shown in ►**Table 2** and ►**Fig. 3** below.

With prethrombin-1, no detectable clots were observed. By comparison, prothrombin showed Wessler scores of 1 to 3 at the same dose. FEIBA gave Wessler scores of 3 and Beriplex a score of 2, both with doses of 70 U/kg BW. Analysis of clot weights gave a significant result for the comparison between prethrombin-1 and FEIBA at $p < 0.05$.

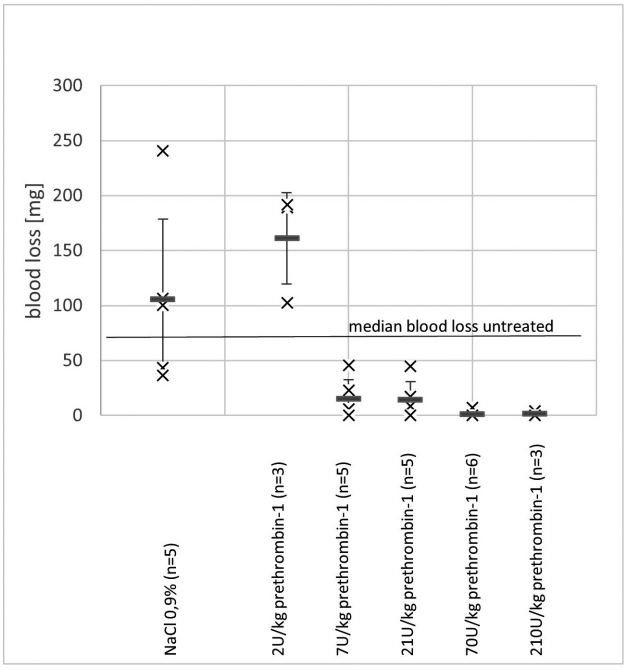

Fig. 2 Blood loss in FVIII inhibitor rabbits after i.v. treatment with different doses of prethrombin-1 or sodium chloride. Individual values are marked with an "x" and means with a horizontal line (-). Median blood loss untreated is the median of all animals before prethrombin-1 treatment. i.v., intravenous.

Hemostatic Efficacy of Prethrombin-1 in a FVIII Inhibitor Mouse Study

The hemostatic efficacy of two doses of prethrombin-1 (224 and 671 nmol/kg) was tested in tail clipping studies in mice treated with FVIII inhibitor. Sodium chloride was used as a control to demonstrate the anticoagulant status induced by the FVIII inhibitor. The results are shown in ►**Tables 3**, **4**, **5**, and ►**Fig. 4** below.

Blood loss in the group receiving sodium chloride amounted to between 424.8 and 823.6 mg.

When prethrombin-1 was administered at a dose of 224 nmol/kg BW, blood loss was greatly reduced in all animals. In three animals, no blood loss was observed and in two more animals blood loss was 3.7 and 10.1 mg (►**Table 4**). When the prethrombin-1 dose was increased to 671 nmol/kg BW, bleeding compared with the control group was also reduced: in five animals no blood loss was seen at all, in one animal it amounted to 51.4 mg (►**Table 5**).

The experiments show that with both, a prethrombin-1 dose of 224 nmol/kg BW and a prethrombin-1 dose of 671 nmol/kg BW, blood loss was reduced compared with the control group. The result is statistically significant at $p < 0.05$.

Hemostatic Efficacy of Prethrombin-1 in a Rivaroxaban Mouse Study

Female FVB mice were treated with 2 µg rivaroxaban to induce an anticoagulant state. After rivaroxaban administration, the tip of the tail was resected, and blood loss was recorded. ►**Table 6** shows the data obtained from five mice.

Prethrombin-1 at a dose of 224 nmol/kg was used to reverse the anticoagulant effect. Again, tail-clipping was used to record blood loss after reversal of anticoagulation. The results are shown in ►**Tables 6** and **7**. See also ►**Fig. 5**.

The results demonstrate that the anticoagulant effect of rivaroxaban is reversed by prethrombin-1. The result is significant at $p < 0.05$.

Table 2 Wessler score and clot weight in FVIII-inhibitor rabbits after i.v. treatment with prethrombin-1, prothrombin, Beriplex, and FEIBA

Rabbit #[a]	Substance[b]	Dose[c]	Wessler score[d]	Clot weight[e]
329	Prethrombin-1	469 nmol/kg	0	No clot
345	Prethrombin-1	469 nmol/kg	0	No clot
347	Prethrombin-1	469 nmol/kg	0	No clot
342	Prothrombin	469 nmol/kg	3	13.9 mg
344	Prothrombin	469 nmol/kg	3	7.5 mg
351	Prothrombin	469 nmol/kg	1	No clot
164	Beriplex	70 U/kg	2	2.4 mg
191	Beriplex	70 U/kg	2	2.8 mg
222	Beriplex	70 U/kg	3	10.2 mg
165	FEIBA	70 U/kg	3	5.0 mg
227	FEIBA	70 U/kg	3	12.3 mg
298	FEIBA	70 U/kg	3	14.3 mg

Abbreviations: FVIII, factor VIII; i.v., intravenous.
[a]Animal consecutive numbers in the approved animal experimentation protocol.
[b]Substance applied by i.v. administration.
[c]Dose in nmol/kg or FIX U/kg.
[d]Wessler score as described in the Materials and Methods section.
[e]Wet clot weight in [mg] from gravimetric measurements.

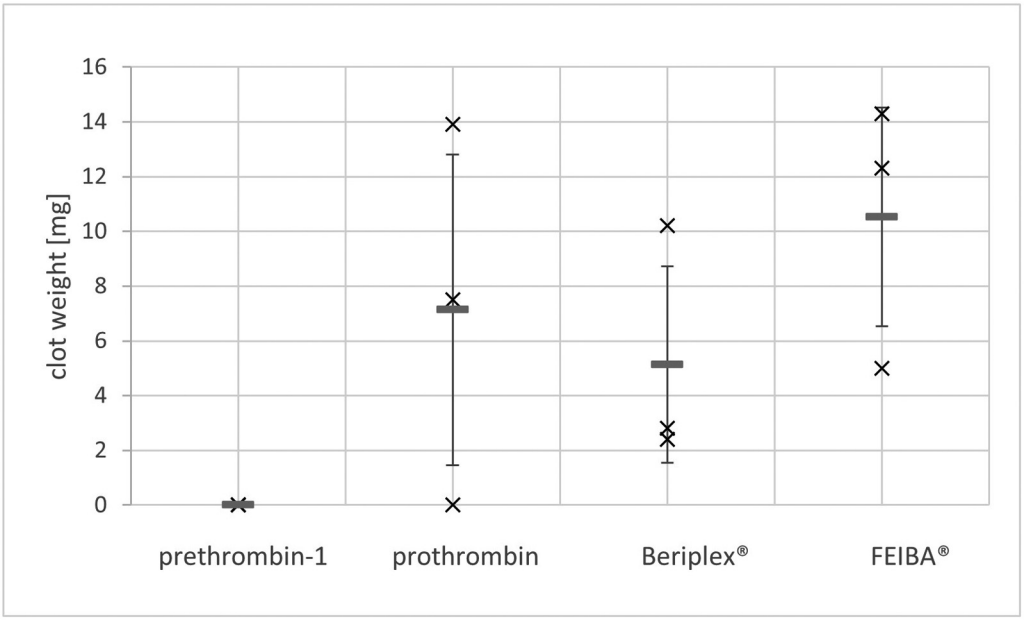

Fig. 3 Clot weight (Wessler test) in FVIII-inhibitor rabbits after i.v. treatment with prethrombin-1, prothrombin, Beriplex, and FEIBA. Individual values are marked with an "x" and means with a horizontal line (-). Standard deviations are presented in bars. i.v., intravenous.

Table 3 Blood loss in FVIII-inhibitor mice treated with sodium chloride

NaCl 0.9%		Ab[c] [µL]	BU[d]/kg	NaCl[e] [µL]	Blood loss[f] [mg]
Mouse[a]	BW[b] [g]				
MH300	23.7	40	31,472	144	504.4
MH301	23.8	40	31,339	144	603.0
MH302	24.3	40	30,695	144	424.8
MH303	24.7	40	30,198	144	823.6
MH304	24.6	40	30,320	144	740.3

Abbreviation: FVIII, factor VIII.
[a]Animal consecutive numbers in the approved animal experimentation protocol.
[b]Body weight.
[c]The antibody solution had a concentration of 13.8 mg/mL with an activity of 3,820 BU/mg. For the mouse studies, the solution was diluted 1:3 prior to application.
[d]Bethesda units per kg body weight.
[e]Physiological saline administered by i.v. administration.
[f]Blood loss in [mg] from gravimetric measurements.

Discussion

It has been known for quite some time that thrombin cleaves fragment 1 from prothrombin, resulting in the formation of prethrombin-1.[25] However, the potential physiological significance of this phenomenon has remained unexplored.

One may be tempted to assume that a missing fragment 1 largely inactivates prothrombin. The fact that prethrombin-1 is indeed able to arrest bleeding in several conditions such as hemophilia or anticoagulation caused by direct oral anticoagulants is remarkable and unexpected.

The exact mode of action by which prethrombin-1 promotes hemostasis will require further exploration. It appears that prethrombin-1 intervenes when thrombin is generated by activated factor X independently of activated platelet surfaces. This is the case in the extrinsic phase when such surfaces are generated.

Prothrombin and prethrombin-1 are processed to thrombin with a low but similar kinetic rate when no activated platelet surfaces are available. One might speculate that in the extrinsic phase, when only minute amounts of thrombin are generated, its precursor, the thrombin-zymogen concentration plays an important role in the final concentration of generated thrombin. At this stage, it does not matter whether the zymogen form is prothrombin or prethrombin-1.[26]

The thrombin generated at the extrinsic stage is used to activate factors V and VIII as well as to activate platelets. It would be plausible that increased factor V activation in the extrinsic phase is the reason for the macroscopic effect to arrest bleeding. Other modes of action are also conceivable,

Table 4 Blood loss in FVIII-inhibitor mice after i.v. treatment with 224 nmol/kg prethrombin-1

Prethrombin-1 (25.6 nmol/mL)					Dose Pre-1[e] [nmol/kg]	Blood loss[f] [mg]
Mouse[a]	BW[b] [g]	Ab[c] [µl]	BU[d]/kg	Pre-1[e] [µl]		
MH626	28.4	40	24,749	48.9	224	0
MH630	26.1	40	26,930	45.1	224	3.7
MH631	29.2	40	24,071	50.6	224	0
MH646	27.7	40	25,375	47.4	224	0
MH648	25.7	40	27,349	45.4	224	10.1

Abbreviations: FVIII, factor VIII; i.v., intravenous.
[a]Animal consecutive numbers in the approved animal experimentation protocol.
[b]Body weight.
[c]The antibody solution had a concentration of 13.8 mg/mL with an activity of 3,820 BU/mg. For the mouse studies, the solution was diluted 1:3 prior to application.
[d]Bethesda units per kg body weight.
[e]Prethrombin-1 administered by i.v. administration in [µL] and nmol/kg body weight.
[f]Blood loss in [mg] from gravimetric measurements.

Table 5 Blood loss in FVIII-inhibitor mice after i.v. treatment with 671 nmol/kg *prethrombin-1*

Prethrombin-1 (25.6 nmol/mL)					Dose Pre-1[e] [nmol/kg]	Blood loss[f] [mg]
Mouse[a]	BW[b] [g]	Ab[c] [µL]	BU[d]/kg	Pre-1[e] [µL]		
MH633	28.1	40	25,014	146.6	671	51.4
MH634	26.2	40	26,827	136.1	671	0
MH643	27.6	40	25,467	146.6	671	0
MH644	26	40	27,034	136.1	671	0
MH647	22.3	35	27,579	115.2	671	0
MH664	26	40	27,034	136.1	671	0

Abbreviations: FVIII, factor VIII; i.v., intravenous.
[a]Animal consecutive numbers in the approved animal experimentation protocol.
[b]Body weight.
[c]The antibody solution had a concentration of 13.8 mg/mL with an activity of 3,820 BU/mg. For the mouse studies, the solution was diluted 1:3 prior to application.
[d]Bethesda units per kg body weight.
[e]Prethrombin-1 administered by i.v. administration in [µL] and nmol/kg body weight.
[f]Blood loss in [mg] from gravimetric measurements.

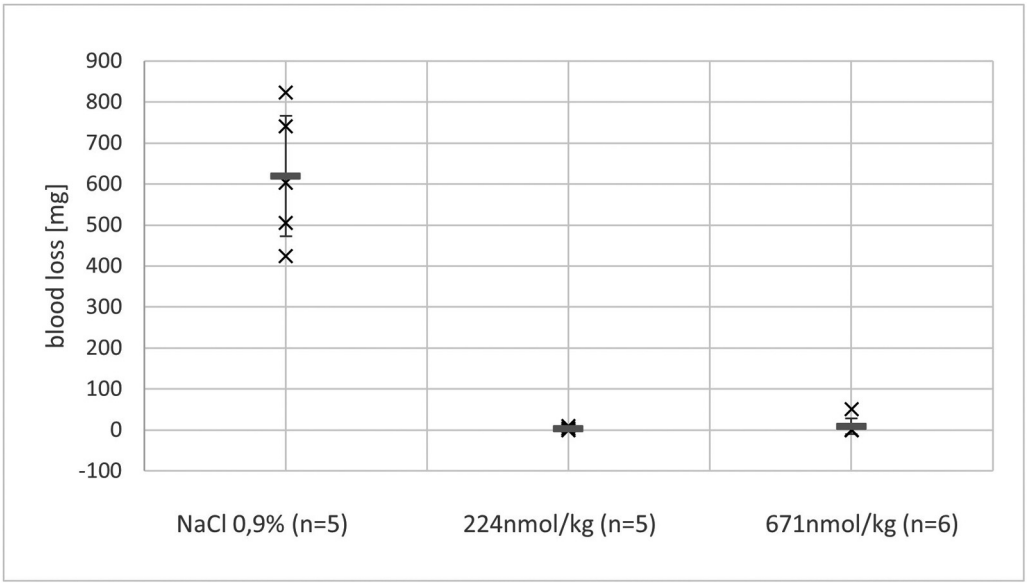

Fig. 4 Blood loss in FVIII-inhibitor mice after i.v. treatment with prethrombin-1 compared with untreated animals. Individual values are marked with an "x" and means with a horizontal line (-). Standard deviations are presented in bars. NaCl means physiological saline 0.9%. Two concentrations of prethrombin-1 are displayed. The number of animals per group (*n*) is shown. i.v., intravenous.

Table 6 Blood loss in mice after rivaroxaban administration

Mouse #[a]	Blood loss[b] after rivaroxaban administration [mg]
736	35.8
737	44.6
738	28.6
739	45.7
740	38.9

[a]Animal consecutive numbers in the approved animal experimentation protocol.
[b]Blood loss in [mg] from gravimetric measurements.

Table 7 Blood loss in mice after rivaroxaban reversal with 224 nmol/kg prethrombin-1

Mouse #[a]	Blood loss[b] after rivaroxaban reversal [mg]
741	0
742	0
743	0
744	0
745	0

[a]Animal consecutive numbers in the approved animal experimentation protocol.
[b]Blood loss in [mg] from gravimetric measurements.

and it is necessary to study the kinetic effects in greater detail and with well thought-through approaches.

Elevated concentrations of prothrombin may escalate the risk of blood clots, including those from deep vein thrombosis and pulmonary embolism. In our current preclinical studies, we investigated the potential benefit of prethrombin-1 as an alternative to administering prothrombin or prothrombin-containing PCCs in cases of acute bleeding. Our findings suggest that prethrombin-1 effectively controls bleeding and exhibits a lower thrombogenic potential compared with prothrombin and prothrombin-containing PCCs. This unexpected discrepancy may be attributed to prethrombin-1's inability to participate in the prothrombinase process, which converts the entire prothrombin in the proximity of the enzyme complex into thrombin.

We postulate that prethrombin-1 facilitates the separation of processes reliant on prothrombin as a zymogen in the natural system—specifically, the formation of minute amounts of thrombin through the extrinsic pathway and the primary massive generation of thrombin by prothrombinase in the common pathway, where thrombin cleaves fibrinogen into fibrin. The detachment of fragment 1 and subsequent loss of substrate suitability for prothrombinase may preserve zymogen for the extrinsic process.

Interestingly, prethrombin-2 does not promote hemostasis. The major difference between prethrombin-1 and prethrombin-2 is the missing factor Va-binding site.[27] This implies that the factor Va-binding site has significant importance for the hemostatic efficacy of prethrombin-1.

Our results from preclinical research have shown the potential of prethrombin-1 for human therapeutic use and suggest that its thrombogenic potential is low, even at high doses. However, only testing in humans will be able to reveal a therapeutic window for prethrombin-1 in different bleeding situations. Although prethrombin-1 for our preclinical studies was sourced from human plasma, it can alternatively

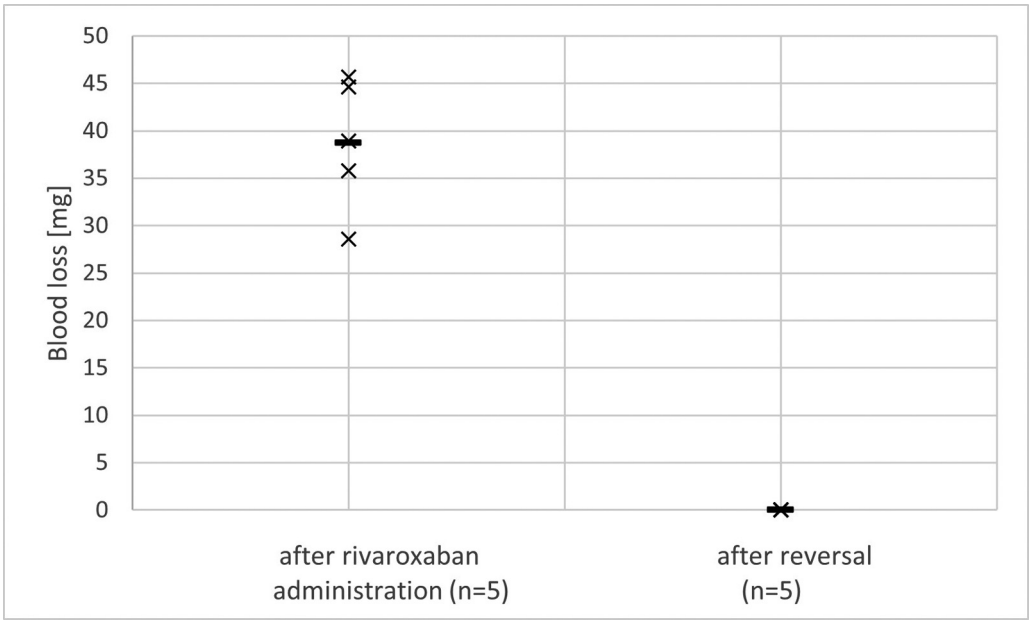

Fig. 5 Blood loss in mice after rivaroxaban administration and after reversal with prethrombin-1. Individual values are marked with an "x" and means with a horizontal line (-). Standard deviations are presented in bars.

be produced relatively easily by recombinant DNA technology. This might be another major advantage over activated or nonactivated PCCs.

What is known about this topic?

- It has been known for quite some time that thrombin cleaves fragment 1 from prothrombin, resulting in the formation of prethrombin-1.
- The potential physiological significance of this phenomenon has remained unexplored.

What does this paper add?

- The thrombin zymogen prethrombin-1 is promoting hemostasis with reduced risk of thrombosis.
- In the present preclinical studies, we aimed to identify and to explore the potential of prethrombin-1 as a new treatment option for patients who bleed or are at a risk of bleeding.

Authors' Contribution

J.G.G. wrote the first draft, conceived the project, and supervised experiments. J.G.G., M.P., H.B., C.M., A.T., and R.G. designed and performed experiments, analyzed, and interpreted data. J.G.G., M.P., H.B., C.M., A.T., R.G., M.K., and W.S. contributed to the concept, literature search, and conclusions. All authors edited the final version.

Conflict of Interest

None declared.

Acknowledgment

We dedicate this work to Dr. Johann Eibl. His relentless spirit of inquiry and academic curiosity made the present work possible. Dr. Eibl passed away on 29 January 2023, in his 97th year of life. Sit tibi terra levis.

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