A Variant of Recombinant Factor VIIa With Enhanced Procoagulant and Antifibrinolytic Activities in an In Vitro Model of Hemophilia

Geoffrey A. Allen, Egon Persson, Robert A. Campbell, Mirella Ezban, Ulla Hedner, Alisa S. Wolberg

Objective—Recombinant factor VIIa (rFVIIa, NovoSeven) has proven efficacy in treating bleeding in hemophilia patients with inhibitors. A rFVIIa analog with mutations V158D/E296V/M298Q (NN1731) exhibits increased procoagulant activity in vitro and in vivo models. The aim of this work was to define the effects of NN1731 toward factor X activation, platelet activation, thrombin generation, and fibrin clot formation and stability.

Methods and Results—In a cell-based in vitro model of hemophilia, rFVIIa and NN1731 similarly increased factor X activation on tissue factor–bearing cells; however, NN1731 exhibited 30-fold higher factor Xa generation on platelets than similar rFVIIa concentrations. NN1731-mediated thrombin generation depended on platelet activation, but NN1731 did not directly activate platelets. NN1731 produced 4- to 10-fold higher maximal thrombin generation rates than equal rFVIIa concentrations. Both rFVIIa and NN1731 shortened clotting times in the absence of factors IX and VIII; however, NN1731 did so at 50-fold lower concentrations than were required of rFVIIa. In fibrinolytic conditions, both rFVIIa and NN1731 increased fibrin formation and stability; however, NN1731 was effective at 50-fold lower concentrations than were required of rFVIIa.

Conclusions—By increasing factor Xa generation, NN1731 promotes the formation of thrombin and a stable clot to a greater degree than rFVIIa. (Arterioscler Thromb Vasc Biol. 2007;27:683-689.)

Key Words: factor VIIa ■ hemophilia ■ fibrinolysis ■ fibrinogen

Recombinant factor VIIa (rFVIIa, NovoSeven; Novo Nordisk A/S, Copenhagen, Denmark) is effective in treating bleeding in hemophilic patients with inhibitors, and is in trials to expand its application to traumatic bleeding and intracerebral hemorrhage.1 RFVIIa exerts its effects via both tissue factor (TF)-dependent2 and independent3,4 activities. We and others have previously shown that rFVIIa shortens the time to onset and increases the rate of thrombin generation.2,3,5 RFVIIa also shortens the time to onset of fibrin clot formation and normalizes the fibrin structure and porosity of clots formed under hemophilic conditions (absence of factors VIII and/or IX).3,6 Furthermore, rFVIIa improves fibrin clot formation in a fibrinolytic environment, suggesting that rFVIIa may improve hemostasis by improving formation of the primary clot, as well as formation of subsequent clots if the primary clot fails.5

After 10 years of clinical experience with rFVIIa in the treatment of hemophilia, it is increasingly evident that the dosing must be individualized to achieve an optimal treatment outcome. Children especially seem to require higher doses per injection than adults for an optimal effect.7,8 In most cases an optimal dose seems to be achievable if doses are individualized.8–10 However, in some special situations the rapid formation of a strong fibrin plug may be necessary to restore hemostasis.

Several analogs of rFVIIa have been generated that express substantially higher TF-independent activity than rFVIIa in certain in vitro assays,11,12 Several of these analogs also possess increased thrombin-generating activity on activated platelets11 and have been shown to reduce the tail bleeding time and total blood loss in a mouse model of hemophilia A.13

In the current study we have used a cell-based reconstituted model system of coagulation to fully characterize the effect of one of these rFVIIa analogs, rFVIIa with mutations V158D/E296V/M298Q (FVIIaIIa, NN1731), on factor X activation, platelet activation, thrombin generation, and fibrin clot formation and stability. This model system has previously been used to examine the effect of elevated prothrombin on fibrin clot formation and structure, as well as the effects of hemophilia and rFVIIa on fibrin clot formation, structure, and stability.5,14 This model system offers several advantages over plasma-based and in vivo murine studies. First, the reconstituted system permits all reactants (pro- and anticoagulants, as well as platelets) to be included at their plasma levels.
level, allowing appropriate biochemical interactions between plasma procoagulants, inhibitors, and cells. Second, the system enables the use of a homogeneous protein mixture, eliminating significant donor-to-donor variability in plasma protein levels, while permitting individual variation in platelet thrombin generating capacity. Third, the system is initiated with cellular TF, permitting the study of cell surface–mediated procoagulant activity. Fourth, the system includes only human proteins (plasma-purified or recombinant) and cells, preventing complications from interspecies variations. For example, the association of human rFVIIa to mouse TF is ~3 orders of magnitude weaker than to human TF, preventing full appreciation of the role of TF-dependent activities in murine-based studies. Additionally, the dose of rFVIIa required to reduce bleeding in mice is substantially (greater than 10-fold) higher than in humans. Finally, the expense of in vivo models can limit the number of concentrations that can be tested in preliminary studies.

Using this in vitro model system, we found that while rFVIIa and NN1731 caused similar rates of factor X activation on TF-bearing cells, NN1731 exhibited significantly greater activity on the platelet surface. NN1731 did not directly activate platelets. Instead, its procoagulant activity was manifest via increased thrombin generation, leading to a further increase in plasma clot stability. Our results suggest that NN1731 was manifest via increased thrombin generation, leading to a direct increase in clot stability. NN1731 did not directly activate platelets. Instead, its procoagulant activity was manifest via increased thrombin generation, leading to a further increase in plasma clot stability. NN1731 did not directly activate platelets. Instead, its procoagulant activity was manifest via increased thrombin generation, leading to a direct increase in clot stability. NN1731 did not directly activate platelets. Instead, its procoagulant activity was manifest via increased thrombin generation, leading to a further increase in plasma clot stability. NN1731 did not directly activate platelets. Instead, its procoagulant activity was manifest via increased thrombin generation, leading to a direct increase in clot stability.

**Methods**

### Proteins and Reagents

Antithrombin was purified from outdated fresh frozen plasma by heparin affinity chromatography. Factor X and plasmogen-free fibrinogen were purchased from Enzyme Research Laboratories, factors V and XI and plasmin from Hematologic Technologies. Factor VIII (as Koate, further purified by gel filtration on Sepharose CL-2B) was generously provided by Dr. Dougald M. Monroe (University of North Carolina, Chapel Hill). Prothrombin and factor IX were purified as described, and prothrombin and factors X and IX were treated with an inhibitor mixture, isolated on Q Sepharose with gelatin-Sepharose to remove fibronectin contamination. RFVIIa and NN1731 were provided by Novo Nordisk. NN1731 (rFVIIa V158D/E296V/M298Q) contains a glutamine substituted for methionine at position 296 as described.11

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### Cell Isolation

Blood was obtained from consenting adults under a protocol approved by the University of North Carolina Institutional Review Board. Monocytes and platelets were prepared as described.8,14,19,20

Platelets were isolated in the presence of 5 μg/mL PG E1 and maintained at 37°C to prevent activation during the isolation protocol.

### Factor X Activation on Monocytes and Platelets

Platelets were activated by incubation with 50 μg/mL of the thrombin receptor agonist peptide SFLLRN for 10 minutes at 37°C. Varied concentrations of rFVIIa or NN1731 were then incubated with TF-expressing monocytes or activated platelets for 15 minutes in the presence of 3 mmol/L CaCl₂. After the addition of factor X (8 μg/mL, final concentration), 10 μL samples were removed at timed intervals (0, 20, and 60 minutes) and assayed for factor Xa activity by adding to 90 μL of chromogenic substrate solution (final concentration: 1 mmol/L S-2765 and 1 mmol/L EDTA in 20 mmol/L HEPES [pH 7.4], 150 mmol/L NaCl, 1 mg/mL BSA [HBS/BSA]). Factor Xa generation over the time course was linear on both monocytes and platelets. Factor Xa concentration, expressed as the slope of the resulting curves in OD/min, was converted to pmol/L by comparison to a standard curve. To account for interindividual platelet variability, 3 separate assays were performed using a different platelet donor for each and the data then combined.

### Thrombin Generation on Activated Platelets

To determine whether NN1731 is more efficient than rFVIIa at detecting low levels of platelet TF, platelets were activated by incubating with thrombin (10 nmol/L) in the presence of factor V and CaCl₂ (20 mmol/L, and 3 mmol/L, final concentrations, respectively) for 10 minutes at 37°C. Activated platelets were then incubated with an inhibitory anti-TF antibody (HTF-1) or nonspecific IgG control (MOPC) at 5 μg/mL for 10 minutes at room temperature. Factor X and prothrombin (135 mmol/L and 1.4 μmol/L, final concentrations, respectively), and rFVIIa (25 nmol/L, final) or NN1731 (5 mmol/L, final) were then added to the activated platelet solutions and thrombin generation was measured at timed intervals by sub-sampling reaction supernatants into chromogenic substrate.

### Cell-Based Model System

Procoagulant proteins and inhibitors were co-incubated at 4°C overnight, before the assay, to ensure that any trace of contaminating proteases in the zymogen preparations were inhibited.5,14,15 To start the reactions, freshly-isolated unactivated platelets were combined with the protein/inhibitor concentrate and cofactors, and immediately added to TF-bearing monocytes. Final concentrations of the model system assay components were as follows: TF-bearing monocytes (500 monocytes/well), unactivated platelets (100 000 platelets/μL), plasma levels of factors II (100 μg/mL), V (7 μg/mL), VIII (0.1 μg/mL), IX (4 μg/mL), X (8 μg/mL), XI (5 μg/mL), antithrombin (150 μg/mL), TFPI (0.1 μg/mL), factor XIII (5 μg/mL), and either 10 nmol/L factor VII or a catalytic amount of rFVIIa (0.01 μg/mL, 0.2 nmol/L). Hemophilia was simulated by omitting both factors IX and VIII. Because the normal donor platelets used in these experiments contain trace amounts of factors VIII and IX, this dual omission prevented the formation of even low levels of tenase activity. Reactions were performed at room temperature and simultaneously in parallel with (100 μL final volume) and without (200 μL final volume) fibrinogen (2 mg/mL). Plasmin challenge assays were performed by adding plasmin (60 nmol/L) to some fibrinogen-containing wells, as indicated.

### Platelet Activation

Samples (10 μL) were removed from model system wells lacking fibrinogen, added to 50 μL paraformaldehyde, incubated for at least 30 minutes, and diluted with Tyrodes/BSA. Samples were then stained with the phycoerythrin-conjugated anti-CD62 for at least 60 minutes at room temperature. Platelet activation was determined by measuring the percentage of platelets expressing the activation-specific alpha granule marker CD62 (P-selectin, GMP140) using a FACScan flow cytometer (BD Biosciences).
Thrombin Generation
Timed aliquots (10 μL) were removed from model system wells lacking fibrinogen and assayed for thrombin activity by adding to 90 μL of chromogenic substrate solution (final concentration: 0.5 mmol/L Chromozym Th, and 1 mmol/L EDTA in HBS/BSA) at room temperature. After sufficient color development, reactions were quenched with the addition of 100 μL 50% acetic acid, and the absorbance at 405 nm was compared with a standard curve of thrombin.

Characterization of Fibrin Clot Formation and Lysis by Turbidity
Clot formation and lysis were detected by turbidity changes at 405 nm in a SpectraMax 340PC plate reader (Molecular Devices). The time to onset of clot formation is a function involving the time to initiation of thrombin generation plus the time of prototibril formation and was defined as the time to the inflection point before the turbidity increase during clot formation. The maximum slope was defined as the slope of a line fitted to the maximum rate of turbidity increase during clot formation (“%max” in the Molecular Devices software) using 5 to 10 points to determine the line. The final optical density of the clot reflects the structure of the fibrin fibers comprising the clot. For plasmin challenge assays, area under the curve (AUC) values were calculated as the sum of the trapezoids formed by the data points, less a baseline established by the lowest measurement taken (Kaleidagraph 3.6.4, Synergy Software, http://www.synergy.com).

Statistics
For the comparison of thrombin generation rates and plasmin challenge assay AUCs, two-sample t-tests were performed for all conditions with ≥4 measurements per group versus hemophilia as the control, and a step down method (beginning with testing for the highest dose first and subsequently proceeding to the lowest dose) was used to control the type I error rate. Significance at the 0.05 level was required at all preceding steps to proceed to the next successive step. Wilcoxon-Mann-Whitney rank sum tests were used to compare the onsets and rates of clot formation of equivalent doses of rFVIIa and NN1731 in both clot formation and plasmin challenge assays; probability values <0.05 are reported as significant.

Results
Factor X Activation
NN1731 activation of factor X on the surface of TF-expressing monocytes was similar (only 1.2-fold higher) to rFVIIa (Figure 1, inset) suggesting that the TF-dependent properties of NN1731 were similar to those of rFVIIa. In contrast, compared with equivalent concentrations of rFVIIa, NN1731 exhibited dramatically (30-fold) increased factor Xa generating activity in the presence of activated platelets (Figure 1).

To examine whether NN1731 procoagulant activity on platelets depended on the presence of platelet-derived TF, thrombin-activated platelets were preincubated with a monoclonal inhibitory anti-TF antibody and rFVIIa- and NN1731-dependent thrombin generation were measured. No difference in thrombin generation was observed in the presence or absence of anti-TF antibody for either rFVIIa or NN1731 (results not shown). Furthermore, thrombin generation was not observed in the absence of factor X, indicating that thrombin generation on platelets depended on rFVIIa or NN1731-mediated activation of factor X. Together, these findings suggest that the mechanism of action of NN1731 results primarily from platelet-dependent (TF-independent) activities.

Platelet Activation
Platelet activation in a model of “normal” hemostasis has a predictable pattern: an initial quiescent period preceding platelet activation, followed by a rapid rise to a maximum level of fully activated platelets. In the model of hemophilia, the duration of the quiescent period was similar, but the rate of platelet activation, as evidenced by the slope of the curve, was decreased (Figure 2). When added to the model of hemophilia, 25 nmol/L rFVIIa increased, but did not normalize, the rate of platelet activation. In contrast, 25 nmol/L NN1731 increased the rate of platelet activation in the system to near normal. Moreover, the effects of NN1731 were concentration-dependent across all concentrations tested (1 to 25 nmol/L NN1731). Importantly, when NN1731 (alone) was incubated with unactivated platelets, no platelet activation above normal baseline was observed, suggesting that NN1731, itself, does not activate platelets.

Figure 1. NN1731 increases the rate of factor Xa generation on platelets more potently than rFVIIa. SFLRN-activated platelets were incubated with rFVIIa and NN1731 followed by the addition of factor X. Factor Xa present one hour after the addition of factor X to LPS-stimulated monocytes or SFLLRN-activated platelets (outset) is shown. Twenty-five and 50 nmol/L rFVIIa correspond to the recommended doses of rFVIIa, 90 and 180 μg/kg, respectively. Bars show pmol/L generated (±SD). Note the difference in y axis scaling between the inset and outset figures.

Figure 2. NN1731 accelerates the rate of platelet activation in hemophilia, but NN1731 does not directly activate platelets. Thrombin generation was initiated in the cell-based model of hemophilia (absence of factors IX and VIII) as described in Methods. Normal conditions were simulated by the inclusion of factors IX and VIII. Aliquots of the reaction supernatants were removed, fixed in paraformaldehyde, and the percentage of activated platelets present was determined by assaying for expression of CD62 by flow cytometry, as described in Methods.
Both rFVIIa and NN1731 shortened the time to onset and increased the rate of thrombin generation and peak thrombin concentration in hemophilic conditions (Figure 3). In some experiments with high concentrations of rFVIIa or NN1731, the time to onset of thrombin generation was shorter than that seen under normal conditions. Because the only variable between assays was the platelet donor, this finding likely reflects differences in the ability of the donor platelets to support factor X activation and/or thrombin generation.22 Thrombin generation rates for NN1731 were 4- to 10-fold higher than equivalent concentrations of rFVIIa. All concentrations of NN1731 tested (1 to 500 nmol/L) produced thrombin generation rates that were significantly higher than hemophilia (P<0.05). Whereas 250 to 500 nmol/L rFVIIa failed to normalize the rate of thrombin generation, 25 nmol/L and higher concentrations of NN1731 produced rates of thrombin generation that were statistically indistinguishable from normal (Figure 3a and 3b). In some experiments, extremely high levels of NN1731 (50 nmol/L) produced thrombin generation rates slightly higher than that observed in normal conditions (presence of factors IX and VIII). Because we omitted both factors VIII and IX, our data indicate that rFVIIa and NN1731 increased thrombin generation by direct factor X activation, and not via a factor IX-activation pathway.4

In all experiments, platelet activation preceded and was a prerequisite for measurable thrombin production (lower limit of detection 1 nmol/L), suggesting that the presence of NN1731, alone, does not support thrombin generation in the absence of activated platelets (Figure 4).

**Fibrin Clot Formation**

Previously, we have shown that rFVIIa shortens the time to onset of fibrin clot formation in hemophilia B.5 In the present study, NN1731 also shortened the time to onset of clot formation in hemophilic conditions (Figure 5a and 5b), with a more pronounced effect than equivalent rFVIIa concentrations (P<0.01). These findings suggest that NN1731 would produce rapid formation of a hemostatic plug, compared with other hemostatic agents. Similar to findings with thrombin generation, the time to onset of clot formation in the presence of either rFVIIa or NN1731 was, in some experiments, shorter than that seen under normal conditions (presence of factors IX and VIII; Figure 5b).

Previous work has shown that clot turbidity is related to the composition of fibrin fibers present in a clot. Increased turbidity reflects fibers with increased mass/length ratios (thicker fibers), whereas decreased turbidity reflects fibers with decreased mass/length ratios (thinner fibers). We have previously shown that hemophilic clots are composed of
thicker fibrin fibers than normal clots. The current findings suggest that NN1731, like rFVIIa, produces thinner fibers in hemophilic clots.

**Plasmin Challenge Assay**

The plasmin challenge assay provides a functional measure of the procoagulant activity of the system by measuring the ability of a fibrin clot to form in a fibrinolytic environment. In this assay clot formation must compete with clot lysis, and so clot formation is exquisitely sensitive to the rate of thrombin generation and, therefore, fibrin polymer formation. As seen in the absence of plasmin, both rFVIIa and NN1731 shortened the time to onset and increased the rate of clot formation versus hemophilic conditions (P<0.05). The onset of clot formation in the presence of NN1731 was shorter than observed with equivalent concentrations of rFVIIa (P<0.01), indicating more rapid formation of the fibrin clot. Both rFVIIa and NN1731 increased the AUC in hemophilic conditions; doses of NN1731 above 5 nmol/L significantly increased the AUC versus hemophilic conditions (P<0.05). The effects of NN1731 were observed at lower concentrations than rFVIIa; NN1731 (25 nmol/L) induced a significantly higher AUC than 25 nmol/L rFVIIa (Figure 6a and 6b, P<0.0001), indicating that NN1731 leads to the production of a more stable and longer lasting fibrin clot. Because TAFI was not included in these experiments, the effects of both rFVIIa and NN1731 were independent of TAFI activity. Rather, these results were entirely dependent on the rFVIIa or NN1731-mediated increase in fibrin generation rate in the system, resulting from increased thrombin generation.

**Discussion**

The bypassing agent, rFVIIa, induces hemostasis in most hemophilic patients with inhibitors. Additionally, rFVIIa use is being expanded to a number of clinical situations, including the treatment of traumatic bleeding and intracerebral hemorrhage. Some individuals appear to have a poor clinical
response to the dose of administered rFVIIa. For this reason, rFVIIa variants with improved activity are being considered because they may improve efficacy per dose of the drug and induce more rapid formation of a firm hemostatic plug. We tested the activity of the highly active analog of rFVIIa, NN1731, in a cell-based in vitro model of hemophilia. In this model, NN1731 shortened the time to onset and increased thrombin generation rates and peaks compared with rFVIIa. Furthermore, NN1731 increased the formation and stability of fibrin clots similar to rFVIIa, but at significantly lower concentrations than were required of rFVIIa. In total, these results are consistent with previous findings that NN1731 has increased activity on platelets, in thrombelastography studies of hemophilic blood, and in hemophilic mice. The present study extends these findings with analysis of platelet activation, dependence on platelet activity, and effects on fibrin clot formation and stability. Importantly, the current study delineates a biochemical mechanism for the earlier findings. It is believed that rFVIIa activity in hemophilia results primarily from rFVIIa-mediated increased tenase and, subsequently, prothrombinase activity on the platelet surface. Our data indicate that rFVIIa- and NN1731-dependent generation of factor Xa on the TF-bearing cell were similar; however, platelet-dependent factor Xa generation was significantly increased by NN1731 relative to rFVIIa. Thus, the NN1731-dependent increase in the thrombin generation rate likely results from increased platelet-dependent factor Xa formation in a mechanism similar to rFVIIa. This activity is likely attributable to a greater enzymatic activity of NN1731, but increased binding of the analog to the platelet surface may also play a role.

Safety is an important consideration in the development of any new therapeutic. It is believed that the safety of rFVIIa is attributable to its dependence on the presence of an activated platelet surface for significant factor X activation and thrombin generation. Thus, it would be important to demonstrate that any potential analog possesses similar characteristics. We observed that NN1731 did not directly activate platelets (Figure 2). Additionally, thrombin generation was not detectable by chromogenic substrate (lower limit of detection 1 nmol/L) before platelet activation, suggesting that only very low levels of extrinsic TF-mediated thrombin generation on the TF-bearing cell occurred before platelet activation (Figure 4). These important and novel findings suggest that NN1731 would not lead to thrombin generation in the absence of injury and active TF exposure. Ghosh et al (2006) recently showed that NN1731 (“FVIIaDVQ”) is more rapidly inhibited by TFPI and antithrombin/heparin than wild type rFVIIa. Because the activity of NN1731 is restricted to the activated platelet surface, these results suggest that the effects of NN1731 would be localized and limited to the wound site and thus not expected to result in disseminated activity. However, because rFVIIa may contribute to thrombosis under certain circumstances, caution should be used in extrapolating from these in vitro experiments to human studies. Clinical trials are warranted to further evaluate the safety of this molecule.

A major finding in this work is that, unlike rFVIIa, NN1731 can normalize the thrombin generation rate in hemophilic conditions. Normalization of the thrombin generation rate has not previously been observed at any concentration of rFVIIa tested (up to 500 nmol/L) (Figure 4b). Additionally, while even very high concentrations of rFVIIa failed to normalize the clot formation rate, NN1731 at 25 nmol/L or higher appeared to do so (Figure 5a). In the plasmin challenge assay, NN1731 shortened the time to clot formation, increased the clot formation rate, and also increased the area under the curve (“lifespan” of the fibrin clot, Figure 6). These findings suggest that NN1731 improved the conversion of fibrinogen to fibrin and that NN1731 would be more likely to support clot formation in tissues with high endogenous fibrinolytic activity or in situations in which a primary clot has failed and residual plasmin remains at the wound site.

Given these findings, our data suggest that NN1731 may be especially efficacious in situations in which rapid formation of a firm fibrin clot with increased resistance to fibrinolysis is necessary to achieve hemostasis. We anticipate that these results from our in vitro model system will help in the design and optimization of dose in future in vivo studies.

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