Immunogenicity of novel recombinant human activated factor VII analogues on factor VII neonatally-tolerized rats

Christian Sommer¹, Peer Norbert Jørgensen², Zaki Salanti³, Jes Thorn Clausen⁴, Lisbeth Bjerring Jensen²

¹Biopharm Toxicology and Safety Pharmacology, Novo Nordisk A/S, Måløv, Denmark; ²Antibody Analysis, Novo Nordisk A/S, Måløv, Denmark; ³LAB Scantox, Ejby, Denmark; ⁴Antibody and Cell Technology, Novo Nordisk A/S, Måløv, Denmark

Summary
Recombinant activated factor VII (rFVIIa; NovoSeven®) has been widely used to treat bleeding in patients with haemophilia with inhibitors. To increase the intrinsic activity, analogues of rFVIIa (rFVIIaq, rFVIIadqv, and rFVIIadqva) with altered amino acid sequence at or near the active centre have been developed. The immunogenicity of these analogues was tested in a rat immune tolerance model. Neonatal rats received rFVIIa intraperitoneally on post-natal Day 1 and were subsequently challenged with rFVIIa in Freunds Incomplete Adjuvant subcutaneously on Days 10 and 24. Rats were tested for tolerance on Day 32; the tolerant cohort and a parallel cohort of untreated control rats were challenged with rFVIIa, rFVIIaq, rFVIIadqv, or rFVIIadqva on Days 46 and 76. Immune responses determined by enzyme-linked immunosorbent assay (ELISA) on Day 84 showed no statistically significant difference between the responses in the four control cohorts. Immune responses were higher in the control than in the tolerant cohort. Compared with rFVIIa (4/16), there was no difference in the proportion of rats that broke tolerance following challenge with rFVIIadqv (3/16) and rFVIIadqva (7/16), whereas a statistically significant greater proportion broke tolerance after challenge with rFVIIaq (11/16). Therefore, in this model rFVIIadqv or rFVIIadqva were not more immunogenic than rFVIIa.

Keywords
Immunogenicity, Neonatal rat model, rFVIIa, rFVIIa analogues, tolerance

Introduction
Since its introduction in 1996, human recombinant activated factor VII (rFVIIa; NovoSeven®) has demonstrated extensive clinical efficacy and an excellent safety profile in the treatment of bleeding episodes in haemophilia patients with inhibitors to factor VIII and IX (1). The licensed use of rFVIIa covers the treatment of bleeding episodes in patients with congenital haemophilia, acquired haemophilia, factor VII deficiency, and Glanzmann’s thrombasthenia (1). In addition, rFVIIa is currently being investigated for the management of critical bleeding in a number of other clinical settings, including severe trauma and cardiac surgery (2–9).

Variants of rFVIIa with increased intrinsic (tissue factor independent) activity have been developed by rational design in order to improve the treatment of patients with haemophilia by providing increased thrombin generation (10–12). The variants were created in an attempt to mimic the conformational change occurring upon tissue factor binding and contain a limited number of mutations (from 1 to 4). A more rapid thrombin generation has been demonstrated in in-vitro studies (11, 13–15) and improved haemostasis has indeed been obtained in an in-vivo preclinical study (12).

Immune responses to rFVIIa in treated haemophilia patients are rare (16). A point of concern, however, would be if these new rFVIIa analogues were more immunogenic and thereby would break tolerance towards factor VII when administered to humans.

The aim of this exploratory investigation was to evaluate the potential immunogenicity of three rFVIIa analogues, rFVIIaDVQ, rFVIIaDVQA, and rFVIIaQ, using a neonatal rat model of induced tolerance (17, 18). Generally, the induction of antibody formation in animals is not predictive of antibody formation in humans. Humans may develop antibodies against humanized proteins, and frequently the therapeutic response persists in their presence (19). However, as “non-clinical studies may contribute to the interpretation of comparability of the immunogenicity po-
tential" (20), such information is essential if their predictive value for evaluation of immunogenicity is to be estimated.

Materials and methods

Immunogenicity of rFVIIa and analogues in control and tolerance cohorts

Newborn specific pathogen-free (SPF) Wistar rats from time-mated dams were cross-fostered after random allocation amongst the dams. To induce tolerance, on post-natal Day 1, the rats were injected intraperitoneally with rFVIIa 150 µg in 100 µl of vehicle solution (10 mM GlyGly, 150 mM NaCl, 10 mM CaCl$_2$, pH 5.5; filtered through 0.45 µm). On post-natal Days 10 and 24, these rats were given an additional subcutaneous dose of 20 µg rFVIIa in 50 µl of vehicle solution mixed 1:1 with Freund's Incomplete Adjuvant. A parallel group of untreated neonatal rats from the same sets of litters formed the control cohort. All rats were weaned on post-natal Days 23 or 24. On post-natal Day 32, tolerance (i.e. anti-rFVIIa antibody formation) in treated rats was tested in a direct ELISA (0.1 µg antigen per well), using blood sampled from the orbital vein. All sera were tested in four dilutions (1:11, 1:55, 1:275, and 1:1375) on plates coated with either rFVIIa or bovine serum albumin (BSA). Rats with an enzyme-linked immunosorbent assay (ELISA) response (optical density at 450 nm; OD$_{450}$ ≤ 0.5 after serum dilution 1:11 were considered tolerant (the tolerance cohort). For rFVIIa$_Q$, rFVIIa$_{DVQ}$, and rFVIIa$_{DVQA}$, the sera were also tested in an ELISA coated with the identical antigen used for the challenge.

On Days 46 and 77, the rats in the tolerance and control cohorts were injected intravenously with rFVIIa at a nominal dose

![Figure 1: Schedule of treatment procedures in the control and tolerance cohorts, in tolerized rats (A) and sham-tolerized rats (B).](image-url)
of 0.3 mg/kg, one of three rFVIIa analogues (rFVIIaQ, rFVIIaDVQ, or rFVIIaQVA; all nominal 0.3 mg/kg), or ovalbumin nominal 1.0 mg/kg. Blood samples were taken on Days 60 and 84 for determination of antibody titres and tolerance breakage by ELISA, as before. Tolerance was considered broken for OD_{450} readings >0.5. Following collection of the final blood samples, all of the animals were euthanatized without further characterization of the tolerance/antibody response. An overview of the treatment schedule is shown in Figure 1A.

The protein content of the dose formulations was analysed using gel permeation high-performance liquid chromatography (GP-HPLC), and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen) was used to test for aggregates.

**Tolerance test in groups A-D**

In order to test whether the method that was used to induce tolerance was compromising the immune system and increasing mortality, sham tolerance was induced in a parallel experiment in neonatal rats from the same litters and dams as the ones in the tolerance and control cohorts. Group A received one intraperitoneal injection of vehicle only on Day 1; group B received two intraperitoneal injections of vehicle only on Days 1 and 3; and group C received two intraperitoneal doses of 150 µg rFVIIa on Days 1 and 3. In addition, one group (D) received no treatment. Rats in groups A, B, C, and D were all subsequently challenged with subcutaneous doses of 20 µg rFVIIa on Days 10 and 24, and blood samples for antibody determinations were collected on Day 32, as before (Fig. 1B).

**Statistical analysis**

The proportion of tolerant (i.e. antibody-negative) animals in the rFVIIa group was compared with the proportion of tolerant animals in the rFVIIaQ, rFVIIaDVQ, and rFVIIaQVA groups, respectively, using a one-sided Fisher’s exact test. The control groups allowed for possible correction to the proportions, i.e. adjusting for any difference in immunogenicity between groups in non-tolerant animals. The study was planned to have a sufficient power to detect differences in the proportions of tolerant animals at the 0.05 significance level with a probability of 0.8, provided that a minimum proportion of 0.9 in the rFVIIa-treated group was tolerant and a maximum proportion of 0.55 stayed tolerant in the respective rFVIIa analogue groups.

**Results**

**Immunogenicity of rFVIIa and rFVIIa analogues in the control and tolerance cohorts**

A total of 234 neonatal rats were treated with rFVIIa to induce tolerance. Although 94% (219/234) subsequently showed a severely reduced ability to produce anti-rFVIIa antibody (OD_{450} ≤3.5), 34% (79/234) were classified as tolerant on Day 32, i.e. they had OD_{450} ≤0.5 for 10-fold serum dilution. One additional rat, with an OD_{450} marginally higher than 0.5 (0.7), was also included in the tolerance cohort to equalize the numbers in each of the treated groups. The tolerance cohort therefore consisted of 80 rats (16 per treatment group). The control cohort consisted of 120 rats (24 per treatment group).

The titration responses of sera following the challenge experiments were low: hence, no antibody titres could be calculated. It was only possible to use data from the 10-fold dilution at Day 84 for the evaluation. As titration of sera for dilutions lower than 100 may be influenced by an individual-dependent matrix effect, the difference between measurements on sera titrated on rFVIIa and BSA (as an unrelated antigen) were used in the calculations. No differences were observed when sera were tested in an ELISA coated with the identical analogue used for challenge as opposed to coating with the rFVIIa antigen (data not shown).

In the control cohort, the proportion of antibody-negative rats did not differ significantly among the groups challenged with rFVIIa, rFVIIaQ, rFVIIaDVQ, and rFVIIaQVA (p>0.05; Fisher’s exact test). Immune responses were higher in the control than in the tolerant cohort, showing that the tolerance induction altered the response to the subsequent challenge with rFVIIa or

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<th>Table 1: The proportion of tolerant and control rats with non-broken tolerance at Day 84 following two intravenous challenges with rFVIIa, rFVIIaQ, rFVIIaDVQ, or rFVIIaQVA.</th>
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<td><strong>No. of rats</strong></td>
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*One animal killed accidentally on Day 46. **p<0.05 versus the group treated with rFVIIa (one-sided Fisher’s exact test).
the rFVIIa analogues (Table 1, Fig. 2). The tolerance induction did not alter the response to ovalbumin, which was identical for the control and tolerance cohort. The analyses of tolerance breakage in the tolerance cohort were performed without corrections for the proportion of antibody-negative rats in the control cohort groups, as these did not differ significantly.

Within the tolerance cohort, the proportions of rats with break in tolerance were not different for the rFVIIaDVQ and rFVIIaDVQA-challenged groups compared with the rFVIIa group (3/16 and 7/16, respectively, vs. 4/16). However, there was a significantly higher proportion of animals in the rFVIIaDVQ-challenged group who broke tolerance compared with the rFVIIa-challenged group (11/16 versus 4/16; p<0.05) (Table 1).

Immunogenicity of rFVIIa in groups A-D
In the sham tolerance-induced rats (groups A and B) and the non-tolerance induced rats (group D), immunization with rFVIIa produced anti-rFVIIa-antibody titres of approximately 20,000. In comparison, the majority (90%) of the rats in the tolerance cohort had titres of <1,000. This indicated that the process of administering intraperitoneal injections in neonatal rats had no apparent effect on their immune response. In addition, mortality was not influenced by the administration of two compared with one intraperitoneal injection (no mortality was observed within four days of administration of vehicle; groups A and B), or by injection with rFVIIa in vehicle compared with vehicle alone. The administration of two intraperitoneal doses of rFVIIa to induce tolerance (group C), as opposed to one dose (tolerance cohort), significantly increased the proportion of tolerant animals (from 34% to 54%; p<0.05, Fisher's exact test).

The measured protein concentrations in the solutions used for immune challenge were lower than the nominal concentrations. However, they were not considered to have altered the outcome of the study. At Day 46 it appeared that rats in the rFVIIaDVQ group may have received a dosage of 0.20 mg/kg, whereas the rFVIIaQ and rFVIIaDVQA groups received 0.09 and 0.12 mg/kg, respectively. At Day 77, the rFVIIaQ, rFVIIaDVQ and rFVIIaDVQA groups received comparable doses of 0.18, 0.21, and 0.18 mg/kg, respectively. As the proportion of animals that broke tolerance in the rFVIIaDVQ group was lower than the corresponding proportions for rFVIIaQ and rFVIIaDVQA, it is unlikely that protein concentrations of the dose formulations have influenced the relative proportions of tolerance breakage between the analogues. On Days 46 and 84, rFVIIa were administered at 0.06 and 0.14 mg/kg, respectively, and ovalbumin at 0.14 and 0.17 mg/kg, respectively. The numbers of antibody-negative rats in the control cohort were unexpectedly high, and this may have resulted from a lower actual dose of rFVIIa being given as a challenge. A clearer bimodal distribution of OD450 values might have been observed in the tolerance cohort if the correct dose had been administered.

Discussion
This preclinical study was designed to compare the immunogenicity of human rFVIIa and three rFVIIa analogues in a neonatal rat model. Since human FVII is a strong antigen in other mammalian species, it was necessary to induce tolerance towards rFVIIa.

The challenge experiment with the FVIIa analogues worked well, despite the fact that the animals were challenged with doses that were lower than planned. In the control groups, 67% to 92% of the rats developed antibodies against FVIIa, and for the tolerated rats only 25% of those that were challenged with FVIIa broke the tolerance. Therefore, a suitable “window” was available for measurement of possible tolerance breakage in the three rFVIIa analogue groups.

A significantly higher proportion of tolerance breakage was observed after administration of rFVIIaQ compared with rFVIIa; however, no difference in tolerance breakage was observed after administration of rFVIIaDVQ or rFVIIaDVQA. Therefore, in this model rFVIIaDVQ or rFVIIaDVQA were not more immunogenic than rFVIIa.

Several studies have investigated the effects of modifying amino acid residues at or around the active site of rFVIIa. In some cases, the activity of rFVIIa has been markedly increased, such as by making single or multiple amino acid substitutions at positions 296, 298, 158, 337, and 305 (11, 12, 21). Other alterations, such as the addition short peptide chains at the active site, eliminated activity creating an inhibitor of thrombosis (22). Few
studies, however, have assessed the immunogenicity of these novel rFVIIa modifications. In the first study to use the rFVIIa immune-tolerant rat model, induced tolerance was seen in 79% of animals by Day 32, and was stable for at least three months in 80% (18). These investigators found that repeated subcutaneous injection with inactivated rFVIIa did not cause breakage of tolerance. This is consistent with the finding for rFVIIaDVQ and rFVIIADVQ of our study. The increased pharmacological activity of these analogues coupled with a lack of immunogenicity indicates that they may have potential for further therapeutic development.

Overall, this study showed that rats with no induced tolerance to rFVIIa showed a markedly higher response to immune challenge with rFVIIa than rats with induced tolerance. In rFVIIa-tolerant rats, a statistically significant higher proportion broke tolerance after challenge with the analogue rFVIIADVQ compared with rFVIIa, but not after challenge with rFVIIA DVQ or rFVIIADVQ. The proportion of tolerant animals was shown to increase with two rather than one intraperitoneal injection of rFVIIa, without increasing the mortality.

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References