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REVIEW ARTICLE

Turoctocog alfa (NovoEight®) – from design to clinical proof of concept

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Abstract

Turoctocog alfa (NovoEight®) is a recombinant factor VIII (rFVIII) with a truncated B-domain made from the sequence coding for 10 amino acids from the N-terminus and 11 amino acids from the C-terminus of the naturally occurring B-domain. Turoctocog alfa is produced in Chinese hamster ovary (CHO) cells without addition of any human- or animal-derived materials. During secretion, some rFVIII molecules are cleaved at the C-terminal of the heavy chain (HC) at amino acid 720, and a monoclonal antibody binding C-terminal to this position is used in the purification process allowing isolation of the intact rFVIII. Viral inactivation is ensured by a detergent inactivation step as well as a 20-nm nano-filtration step. Characterisation of the purified protein demonstrated that turoctocog alfa was fully sulphated at Tyr346 and Tyr1664, which is required for optimal proteolytic activation by thrombin. Kinetic assessments confirmed that turoctocog alfa was activated by thrombin at a similar rate as seen for other rFVIII products fully sulphated at these positions. Tyr1680 was also fully sulphated in turoctocog alfa resulting in strong affinity (low nM K_d) for binding to von Willebrand factor (VWF). Half-lives of 7.2 ± 0.9 h in *F8*-KO mice and 8.9 ± 1.8 h haemophilia A dogs supported that turoctocog alfa bound to VWF after infusion. Functional studies including thromboelastography analysis of human haemophilia A whole blood with added turoctocog alfa and effect studies in mice bleeding models demonstrated a dose-dependent effect of turoctocog alfa. The non-clinical data thus confirm the haemostatic effect of turoctocog alfa and, together with the comprehensive clinical evaluation, support the use as FVIII replacement therapy in patients with haemophilia A.

Key words haemophilia A; N8; rFVIII; recombinant factor VIII; turoctocog alfa

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The human gene for factor VIII (FVIII) was cloned and expressed in 1984 (1–5), making it possible to produce recombinant FVIII (rFVIII) for prevention and treatment of bleeds in patients with haemophilia A. The FVIII gene encodes a single chain of 2332 amino acid residues with the domain structure A1-A2-B-A3-C1-C2 (Fig. 1) (1). During cellular processing, the molecule undergoes posttranslational modifications, including sulphation of specific tyrosine residues and glycosylation. Furthermore, the protein is processed into a heterodimer consisting of a heavy chain (HC) with the A1-A2-B domains and a light chain (LC) with the A3-C1-C2 domains. The two chains are held together by metal ions (6). While the B-domain plays a role in restricting the expression of endogenous FVIII, it is apparently not needed for the function of FVIII (7–9). Acidic regions C-terminal to the A1 and the A2 domains and N-terminal to the A3 domain (a_1, a_2 and a_3, respectively) are important for interaction with thrombin and von Willebrand factor (VWF). Sulphation of Tyr1680 is essential for high affinity binding of FVIII to VWF (10,11), which is required for protection of FVIII from degradation and rapid clearance. During haemostasis, FVIII is activated by specific thrombin cleavages, thereby producing the A1, A2 and A3-C1-C2 fragments of activated FVIII (FVIIIa). This results in dissociation of

VWF and assembly of the tenase complex (FVIIIa/FIXa) on the surface of activated platelets. FXa is generated, resulting in a thrombin burst, and ultimately leading to the formation of a stable haemostatic plug.

The design of turoctocog alfa

Turoctocog alfa (NovoEight®) is a new rFVIII molecule with a truncated B-domain (Fig. 1). The molecule consists of a heavy chain containing the A1-A2 domains, a truncated B-domain coding for 21 amino acids of the naturally occurring B-domain (amino acid 741–750 fused with 1638–1648) and the light chain (A3-C1-C2 domains). While the B-domain is dispensable for procoagulant activity of FVIII (8), cellular expression and secretion is regulated by the B-domain (12). The truncated B-domain was selected to achieve a well-defined product when produced in Chinese hamster ovary (CHO) cells, thereby providing a solid basis for establishing a robust purification process. In addition, an O-glycosylation site is present in the B-domain allowing for future glyco-engineering (13,14). Upon thrombin activation, the truncated B-domain is removed resulting in rFVIIIa with a structure similar to endogenous FVIIIa (13,15).

Although the truncated B-domain is derived from the N- and C-terminal sequences of the B-domain from the native full-length FVIII molecule, the junction between these sequences is engineered and might therefore in theory pose an immunological risk. *In silico* methods, based on *in vitro* affinity of peptides towards major histocompatibility complex (MHC) class II molecules, are used to evaluate potential risks of immunogenicity of non-native sequences. Kimchi-Sarfaty *et al.* (16) used this method to compare the predicted immunogenicity of turoctocog alfa with that of a B-domain deleted rFVIII product [ReFacto AF®, Swedish Orphan Biovitrum AB (publ), Stockholm, Sweden]. While the junction between the N- and C-terminal parts of the B-domain is the same for the two proteins, the N-terminal part of the B-domain for the comparator molecule comprises only three amino acids in contrast to 10 in turoctocog alfa, thereby creating a different immunological environment. The *in silico* analysis predicted that the B-domain of turoctocog alfa bound to fewer MHC class II molecules and with lower affinity than the comparator FVIII product (16), suggesting that the B-domain of turoctocog alfa is not associated with an additional risk of inducing an immune response. As the predictive value of *in silico* methods is not established, clinical data are required to assess the immunogenicity of turoctocog alfa. No inhibitors were observed in the pivotal clinical development programme for turoctocog alfa, which included 213 previously treated patients with severe haemophilia A (17,18).

Manufacturing of turoctocog alfa

Turoctocog alfa is produced in CHO cells grown in serum-free conditions and formulated without animal- or human-derived materials. The CHO cell is a well-known production cell line, which has been used to produce therapeutic proteins within many different therapeutic fields (19). The FVIII molecule is intracellularly processed by furin, resulting in cleavage between the B-domain and the LC (Fig. 1). The purification process (Fig. 2) includes the following steps:

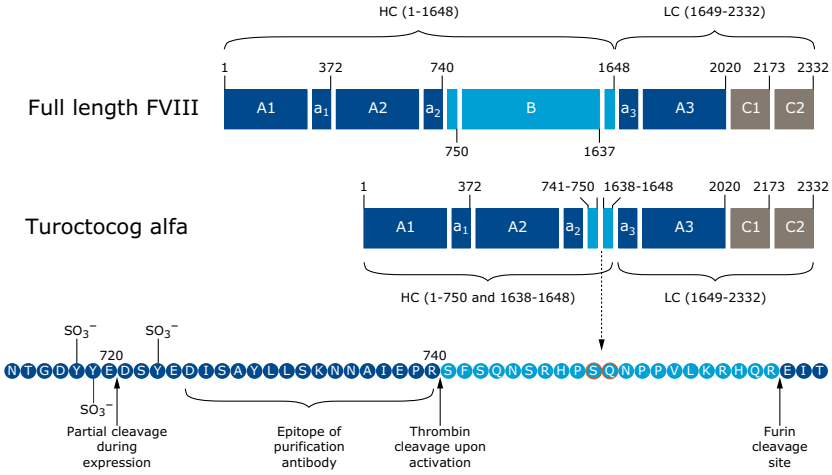

Figure 1 Molecular structure of full-length FVIII and turoctocog alfa. The upper part of the figure illustrates full-length FVIII coding for 2332 amino acid residues. The middle part of the figure shows the structure of turoctocog alfa. The molecule consists of the heavy chain containing the A1-A2 domains, a truncated B-domain coding for 21 amino acids of the naturally occurring B-domain (amino acid 741–750 fused with 1638–1648) and the light chain (A3-C1-C2 domains). The lower part of the figure shows the sequence coding for the 21 amino acid residue truncated B-domain (light blue), which represents 10 amino residues from the N-terminal of the B-domain linked to 11 amino acid residues from the C-terminal of the B-domain. Cleavage sites, tyrosine sulphation sites and the epitope detected by F25 during purification are indicated in this region (13). Reproduced from Thim *et al.* (13) with permission from John Wiley and Sons.

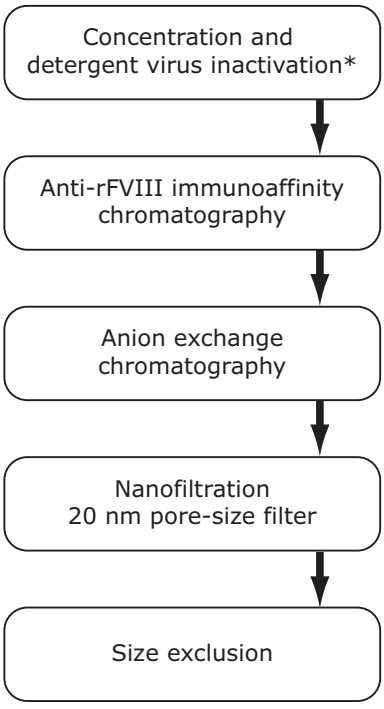

Figure 2 Purification of turoctocog alfa. From Thim *et al.* (13).

concentration (using hydrophobic interaction/cation exchange), anti-rFVIII immunoaffinity chromatography (using a recombinant monoclonal antibody), anion exchange chromatography, and size exclusion (13). Viral inactivation is ensured by detergent treatment (included in the initial purification step) and a 20-nm filtration step prior to the size exclusion step.

During secretion, some rFVIII molecules are cleaved in the A2 domain leaving part of the FVIII molecules with a heavy chain with C-terminus at amino acid 720 or 729 (20,21). As the C-terminal end of the A2 domain (amino acids 720–740) contains residues required for optimal interaction with thrombin (22), the purification process for turoctocog alfa was optimised in order to secure isolation of molecules with intact A2 domain. This was obtained by selecting an antibody (F25) for immunoaffinity chromatography that binds selectively to molecules with intact A2 domain. The antibody binds to the C-terminus of the A2 domain (Fig. 1), enabling removal of FVIII degraded within the A2 domain (13). The gene encoding the antibody was transferred to CHO cells for production and therefore no additional murine antigens or host cell proteins are introduced (23). The removal of degraded heavy chain is clearly seen by SDS–PAGE analysis after thrombin cleavage, where more than one band represents the A2 domain for ReFacto AF® (Fig. 3, lane 8). For turoctocog alfa (Fig. 3, lane 2) or rFVIII derived from the full-length genes (Fig. 3, lanes 4 and 6), no C-terminal degradation in the A2 domain was observed.

The SDS-PAGE analysis also shows that the rFVIII proteins produced by the full-length gene, due to multiple cleavage sites within the B-domain, are a heterogeneous mixture of heavy chains with different lengths of the B-domain included (Fig. 3, lanes 3 and 5). For Advate® (lane 3), one of these bands contains both B-domain sequences and LC sequences, suggesting incomplete processing between the B-domain and the LC (13,24–26). For turoctocog alfa and ReFacto AF®, no such heterogeneity of HC chain fragments was observed and mainly one band at 90 kDa and a minor band representing the single chain protein were detected (lanes 1 and 7). The single chain (representing approximately 3–4% in turoctocog alfa (13)) can be converted into the A1 and A2 domains after thrombin cleavage (lane 2 and 8). The SDS-PAGE analysis also illustrates that the purification process for turoctocog alfa results in a homogeneous rFVIII product, which after thrombin cleavage is identical to native FVIIIa.

Structural characterisation of turoctocog alfa

The purity and homogeneity of turoctocog alfa allowed crystallisation of the protein. The resulting X-ray crystallographic structure of turoctocog alfa (Fig. 4) confirms that the protein has a structure similar to those previously reported for other rFVIII molecules (27,28). The X-ray fluorescence wavelength scan of the turoctocog alfa crystals identified two significant peaks, indicating copper (Cu^{+}) and zinc (Zn^{2+}), respectively (29). The presence of both copper and zinc was supported by colorimetric data, indicating that the ion-binding site in the A1 domain is predominantly populated by zinc, while that in the A3 domain is predominantly populated by copper (29).

Posttranslational modifications

Known posttranslational modifications of plasma-derived FVIII include N- and O-linked glycosylations (30). As for the plasma-derived FVIII, turoctocog alfa contains four N-linked glycosylations (Asn41 and Asn239 in the A1 domain, Asn1810 in the A3 domain and Asn2118 in the C1 domain) and one O-linked glycosylation (Ser750) in the B-domain (13). Structure analyses have shown that the N-linked glycosylation sites are fully glycosylated, while the degree of occupancy of the O-linked glycan at Ser750 is approximately 65%. The majority of the complex N-linked glycans at Asn41 in the A1 domain and Asn1810 in A3 are di-sialylated core fucosylated bi-antennary structures, while high mannose glycans are present at Asn239 in A1 and Asn2118 in C1 (13).

Recombinant proteins derived from non-human cell lines can contain detectable amount of non-human glycoforms (31). As observed for Advate® (31), N-glycolyl neuraminic

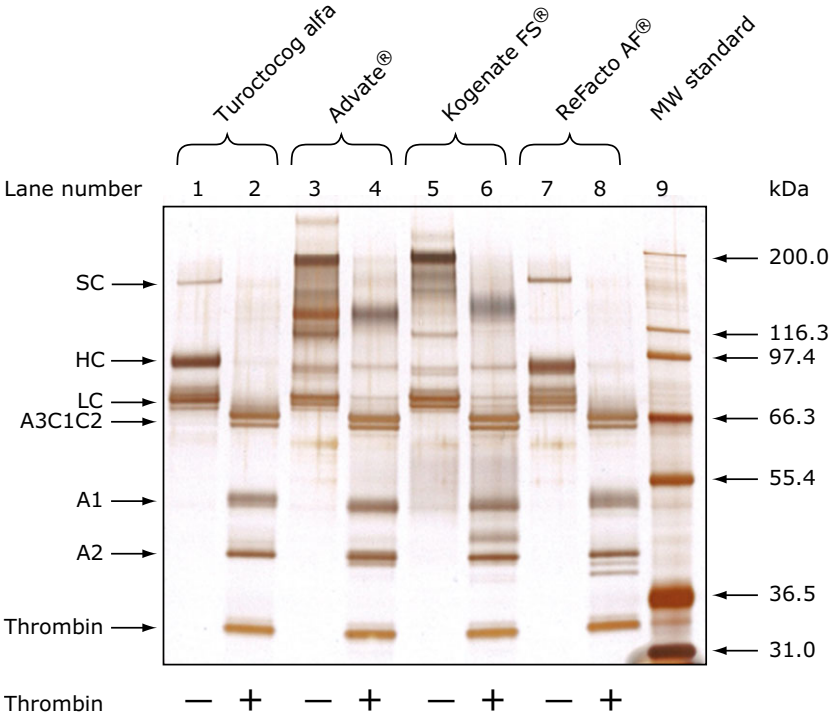

Figure 3 SDS-PAGE of rFVIII molecules before and after thrombin cleavage. From Kristensen *et al.* (26).

acid is also detected in low amounts in turoctocog alfa on di-sialylated core fucosylated bi-antennary structures (13). Furthermore, the O-linked glycosylation at Ser750 contains a doubly sialylated GalNAc-Gal structure, which has also been described to be present in other rFVIII molecules (32). The clinical relevance of these observations is unknown. In agreement with the lack of capacity of producing the antigenic epitope in Galα(1,3)Gal structures in CHO cell lines (33), these structures have not been detected in turoctocog alfa (13).

An additional posttranslational modification of FVIII is sulphation of specific tyrosines. A total of six tyrosine sulphation sites (Tyr346 in a_1, Tyr718, 719, 723 in a_2 and Tyr1664 and Tyr1680 in a_3) have been described for plasma-derived FVIII (30). The tyrosine sulphation in turoctocog alfa was analysed, and Tyr346, Tyr1664 and Tyr1680 were found to be fully sulphated (Table 1) (34). The high negative charge of the peptide containing Tyr718, Tyr719 and Tyr723 resulted in weak response in the mass spectrometry analysis thereby preventing reliable quantification. However, only fully sulphated peptides containing Tyr718, Tyr719 and Tyr723 were detected. Similar data were found for a plasma-derived FVIII concentrate Octanate®, whereas Tyr1680 was not fully sulphated in Advate® (34). The existence of a small amount (2.6-16.7%) of non-sulphated Tyr1680 in Advate® was also observed in other studies (31,35). In these studies, other rFVIII products (Kogenate FS® and ReFacto AF®) also contained similar amounts of non-sulphated Tyr1680, while FVIII produced in human embryonic kidney (HEK) cells (31) or plasma-derived FVIII (35) did not have detectable amounts of non-sulphated Tyr1680 (Table 1). The full sulphation of Tyr1680 in turoctocog alfa demonstrates that CHO cells are capable of providing full tyrosine sulphation.

In agreement with the existence of a small amount of non-sulphated Tyr1680 in Advate®, the affinity of turoctocog alfa to immobilised VWF was slightly higher than observed for Advate®, both when measured by ELISA and by surface plasmon resonance (SPR) (Table 2) (15). However, for both FVIII proteins, the K_d values are in the sub-nanomolar range (15) and the excess VWF in the circulation (30–50 nM VWF monomer) means that essentially all FVIII will be bound to VWF after infusion. In agreement with this, comparable pharmacokinetics of turoctocog alfa and Advate® were observed in *F8*-knockout (*F8*-KO) mice (36), in haemophilia A dogs (37) and in human clinical trials (38) (see below).

Functional characteristics – *in vitro* and *in vivo*

Sulphation of Tyr346 and Tyr1664 are required for optimal interaction with thrombin (11). In line with the observed full tyrosine sulphation at Tyr346 and Tyr1664 for both turoctocog alfa and Advate®, similar rates of activation by thrombin were observed for turoctocog alfa ($13.5 \pm 6.7 \times 10^{-3}$ per minute) and Advate® ($10.6 \pm 3.5 \times 10^{-3}$ per minute) (15). The kinetic assessment furthermore revealed similar affinity towards FIXa and similar Michaelis constants (K_m) of FX activation for turoctocog alfa and Advate®,

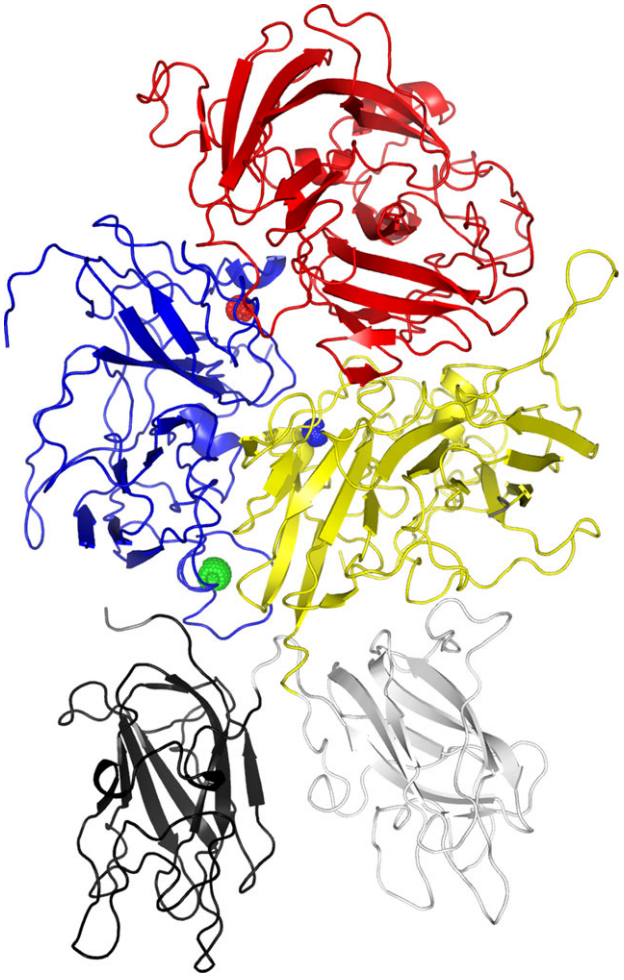

Figure 4 Crystallographic structure of turoctocog alfa. The heavy chain consists of the A1 (blue) and A2 (red) domain while the light chain consists of the A3 (yellow), C1 (grey) and C1 (black) domains. The three ions, calcium (green), copper (blue) and zink (red), observed in the structure are shown as spheres. The structure is available at protein database with the entry code 4bdv (http://www.rcsb.org/pdb/explore/explore.do?structureId=4BDV) (29).

Table 1 Tyrosine sulphation in turoctocog alfa and other FVIII products

Product	Origin	Non-sulphated Tyr1680 (%)
Turoctocog alfa	CHO	Below detection limit (0.5%)
Octanate®	Plasma-derived	Below detection limit (0.5%)
Advate®	CHO	2.6–16.7
ReFacto AF®	CHO	4.5–13.9
Kogenate FS®	BHK	1.0–6.5
Human-cl rhFVIII	HEK	Below detection limit

From Nielsen *et al.* 2012 (34), Kannicht *et al.* 2013 (31) and Grancha *et al.* 2011.(35).
CHO, Chinese hamster ovary; BHK, baby hamster kidney; HEK, human embryonic kidney.

Table 2 VWF binding in turoctocog alfa and Advate®

VWF binding	Turoctocog alfa	Advate®
VWF binding (ELISA) K_d (nM) ($n = 4$)†	0.24 ± 0.04*	0.48 ± 0.13
VWF binding (SPR) (turoctocog alfa $n = 9$, Advate® $n = 2$)[1]		
K_d (nM)	0.23 ± 0.13*	0.45 ± 0.07
k_{on} ($\times 10^6$/M/s)	4.5 ± 2.1	1.2 ± 0.6
k_{off} ($\times 10^{-4}$/s)	9.3 ± 4.7	5.5 ± 1.9

From Christiansen *et al.* 2010 (15).
VWF, von Willebrand factor; SPR, surface plasmon resonance.
*Significantly different ($P < 0.05$) from the value obtained for Advate® using one-tailed Student's t-test.
†Values are mean and SD of the numbers of experiments indicated.

suggesting the same cofactor activity of the two FVIII compounds. Inactivation of activated turoctocog alfa and Advate® by activated protein C (APC) was also similar for the two products (15). In standard FVIII:C assays, such as one-stage clotting assay and chromogenic assay, similar activities were obtained showing in essence no assay discrepancies (15). The specific activity was 9300 ± 400 U/mg (mean and SD of seven batches) (15). In addition, a field study using spiked haemophilia A plasma samples mimicking post-infusion samples showed that turoctocog alfa can be reliably measured using routine FVIII:C assays in clinical laboratories without use of a product-specific standard. The field study also showed that the variability between Advate® and turoctocog was similar across all the laboratories irrespectively of the assay conditions used (39).

In additional functional studies, the thrombin generation assay and thromboelastography (TEG®) were used to characterise the activity of turoctocog alfa in human blood or plasma (15). In the thrombin generation assay, haemophilia A was mimicked either by using severe haemophilia A plasma and adding normal human platelets and lipidated tissue factor (40), or by a reconstituted cell-based model (41) where coagulation factors V, VIII, VIIa, IX, X XI, prothrombin, tissue factor pathway inhibitor (TFPI) and antithrombin were added to normal human platelets and monocytes stimulated to express tissue factor. In both systems, a delayed and lowered thrombin generation was seen when no FVIII was present, while addition of turoctocog alfa improved the thrombin generation in a concentration-dependent manner. When turoctocog alfa was added at 1 IU/mL, the rate of thrombin generation in the plasma-based model system was 3.7 ± 0.8 nM/min similar to the value obtained for Advate® (3.7 ± 0.7 nM/min) (15). Thromboelastography using whole blood from patients with haemophilia A showed as expected that the clot formation time was delayed and the rate of clot formation decreased in this population. Addition of turoctocog alfa resulted in a dose-dependent improvement and normalisation of these parameters.

The *in vivo* effect of turoctocog alfa was evaluated in *F8*-KO mice. These mice do not have spontaneous bleeds, but bleed significantly after injury. In a tail bleeding model using a 4 mm tail cut, turoctocog alfa induced a dose-dependent shortening of the bleeding time and a reduction of the blood loss (36). The turoctocog alfa dose causing 50% reduction (ED_{50}) of these parameters were 29 and 27 IU/kg, respectively (Fig. 5). These ED_{50} values were not significantly different from the values obtained for Advate® (39 and 28 IU/kg, respectively). At a sufficiently high dose, both FVIII compounds normalised the bleeding time and blood loss. Lower FVIII doses were required for normalisation when more subtle injuries were made in the mice. In a saphenous vein bleeding model, the ED_{50} of turoctocog alfa was 5.7 ± 1.3 IU/kg (42), while an ED_{50} of 1.1 IU/kg [95% confidence intervals 0.09–2.0 IU/kg] was observed in a tail vein transection model (Peter Johansen and Tom Knudsen, Novo Nordisk, manuscript submitted). A single high dose of turoctocog alfa (280 IU/kg) was evaluated in a needle-induced joint bleeding model in *F8*-KO mice (36). While untreated mice showed bleeds in the joint and concomitant swelling 1 and 3 d after injury, turoctocog alfa-treated animals showed no signs of or only minor bleeds in the joints. In conclusion, all mice studies demonstrated haemostatic efficacy of turoctocog alfa, and turoctocog alfa was capable of normalising the bleeding phenotype.

Pharmacokinetics

The pharmacokinetics of turoctocog alfa were assessed in *F8*-KO mice and in haemophilia A dogs before initiating the human clinical trials (36,37). In the human phase 1 clinical trial, where the pharmacokinetic profile of a single i.v. dose of turoctocog alfa was comparable to the profile of a single i.v. dose of Advate® (Table 3) (38).

Table 3 Pharmacokinetic parameters for turoctocog alfa in patients with haemophilia A

	Turoctocog alfa Mean (SD)	Advate® Mean (SD)
F8-KO mice (280 IU/kg), *N* = 3–4		
$t_{1/2}$ (h)	7.2 (0.9)	7.7 (1.4)
Clearance (mL/h/kg)	11 (1)	10 (2)
Mean residence time (h)	10 (1.3)	11 (2.1)
Haemophilia A dogs (100 IU/kg), *N* = 3		
$t_{1/2}$ (h)	8.9 (1.8)	8.2 (0.2)
Clearance (mL/h/kg)	3.6 (0.6)	5.2 (0.7)
Mean residence time (h)	12 (2)	11 (0)
Humans (50 IU/kg), *N* = 20		
Incremental recovery (IU/mL)/(IU/kg)	0.019 (0.002)	0.019 (0.003)
AUC (h*IU/mL)	12.97 (3.48)	13.03 (4.25)
$t_{1/2}$ (h)	10.83 (4.95)	11.19 (3.51)
Clearance (mL/h/kg)	4.11 (1.06)	4.17 (1.20)

From Martinowitz *et al.*, 2011 (38), Elm *et al.*, 2012 (36) and Karpf *et al.*, 2009 (44) and 2011 (37).
Values were based on FVIII:C measurements. Incremental recovery: FVIII activity 30 min after end of infusion relative to the administered dose. AUC, area under the plasma concentration curve; $t_{1/2}$, terminal half-life.

Clinical safety and efficacy

The clinical safety and efficacy of turoctocog alfa was demonstrated in 213 previously treated patients with severe haemophilia A (FVIII activity ≤ 1%) and no history of inhibitors. None of these patients developed FVIII inhibitors, no safety concerns were identified and a prophylactic effect of turoctocog alfa was demonstrated (17,18). Overall, the median annualised bleeding rate was 3.0 bleeds per patient per year (estimated mean annualised bleeding rate 5.3 [95% CI: 3.9–7.3] bleeds per patient per year) in children and the median annualised bleeding rate was 3.7 bleeds per patient

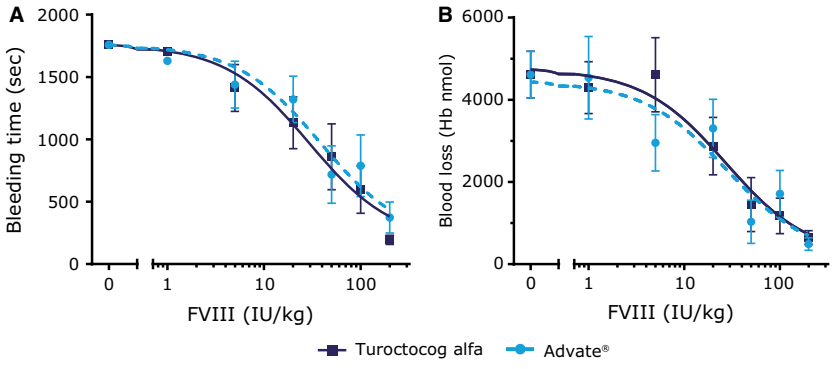

Figure 5 Effect of turoctocog alfa on bleeding in *F8*-KO mice. Turoctocog alfa or Advate® was administered intravenously at the doses noted to *F8*-KO mice 5 min prior to a 4-mm tail cut. Bleeding time (A) and blood loss (B) were measured for 30 min. Data are means and SEM of *n* = 8. The left data point (0 IU/kg) reflects the bleeding observed in *F8*-KO mice receiving vehicle only. Reproduced from Elm *et al.* (36) with permission from John Wiley and Sons.

per year (estimated mean annualised bleeding rate 6.5 [95% CI: 5.3–8.0] bleeds per patient per year) in adults and adolescents. The majority of the bleeding episodes (95% in children and 89% in adults and adolescents) were controlled with 1–2 infusions of turoctocog alfa. Furthermore, the haemostatic responses during and after surgery were rated as excellent or good in all surgeries (43). These data confirm the clinical efficacy of turoctocog alfa.

Conclusions

Turoctocog alfa, a new rFVIII, has been carefully designed to achieve a safe, well-defined and homogeneous product and the molecular characterisation confirms the quality of the FVIII protein. The molecular properties of turoctocog alfa were supported by functional assays, which showed that turoctocog alfa is fully functional in haemophilia A plasma and blood. Furthermore, studies in *F8*-KO mice using bleeding and injury models of different severity all showed that turoctocog alfa was capable of normalising the bleeding phenotype. The data are in line with clinical results, confirming the haemostatic effect of turoctocog alfa and support the use of turoctocog alfa as FVIII replacement therapy in patients with haemophilia A.

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Author contributions

All authors gave input, reviewed and approved the manuscript.

Conflict of interest disclosure

All authors are employees of Novo Nordisk A/S.

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