Preclinical pharmacokinetics and biodistribution of subcutaneously administered glycoPEGylated recombinant factor VIII (N8-GP) and development of a human pharmacokinetic prediction model

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Essentials

- N8-GP is an extended half-life recombinant factor VIII (FVIII) for the treatment of hemophilia A.
- Subcutaneous (SC) FVIII dosing might reduce the treatment burden of prophylaxis.
- SC N8-GP has a favorable PK profile in animal models and disappears from skin injection sites.
- Combined animal (SC) and clinical (IV) data suggest that daily SC dosing may provide prophylaxis.

Summary. Background: N8-GP is an extended half-life recombinant factor VIII (FVIII) for the treatment of hemophilia A. Subcutaneous administration of FVIII may reduce the treatment burden of prophylaxis; however, standard FVIII products have low bioavailability after subcutaneous dosing in animals. Objective: To evaluate the pharmacokinetics, effectiveness and local distribution of subcutaneously administered N8-GP in preclinical models and predict the human pharmacokinetic (PK) profile. Methods: The pharmacokinetics of subcutaneously administered N8-GP were evaluated in FVIII knockout (F8-KO) mice and cynomolgus monkeys; a human PK prediction model in hemophilia A patients was developed. The hemostatic effect was evaluated in a tail vein bleeding model in F8-KO mice. The injection-site distribution and absorption of subcutaneously administered N8-GP were assessed in F8-KO mice by the use of temporal fluorescence imaging and immunohistochemistry. Results: Subcutaneously administered N8-GP had a bioavailability, a first-order absorption rate and a half-life, respectively, of 24%, 0.094 h⁻¹ and 14 h in F8-KO mice, and 26%, 0.33 h⁻¹ and 15 h in cynomolgus monkeys. A dose-dependent effect of subcutaneously administered N8-GP on blood loss was observed in mice. A minimal amount of N8-GP was detected at the injection site 48–72 h after single or multiple dose(s) in F8-KO mice. Subcutaneously administered N8-GP was localized to the skin around the injection site, with time-dependent disappearance from the depot. PK modeling predicted that subcutaneously administered N8-GP at a daily dose of 12.5 IU kg⁻¹ will provide FVIII trough levels of 2.5–10% in 95% of patients with severe hemophilia A. Conclusions: Subcutaneously administered N8-GP may provide effective hemophilia A prophylaxis. A phase I clinical trial is underway to investigate this possibility.

Keywords: factor VIII; hemophilia A; immunohistochemistry; pharmacokinetics; subcutaneous injections.

Introduction

Patients with hemophilia A are deficient in coagulation factor VIII, resulting in a bleeding tendency that varies in severity according to residual FVIII levels [1]. Replacement therapy with FVIII is established as an effective treatment for bleeding in these patients [1]. Regular prophylactic infusion of FVIII is considered to be the optimal treatment for those with severe hemophilia A, providing improved outcomes over on-demand therapy [2]. However, prophylaxis with standard FVIII products requires intravenous administration up to every second day [1,3], and the need for such frequent infusions can
make it challenging for patients to incorporate prophylactic schedules into their daily lives. Although an implanted venous access device can ease the long-term administration of prophylactic infusions, particularly in younger children, concerns including the associated risks of infection and thrombosis complicate the use of these devices [4,5].

A potential approach to substantially reduce the burden of treatment in patients with severe hemophilia A is to administer FVIII subcutaneously, thus avoiding the need for intravenous infusions. However, standard FVIII concentrates have low bioavailability after subcutaneous administration; for example, inadequate exposure of recombinant FVIII (rFVIII) compounds was observed after subcutaneous administration in hemophilia A mice [6] and in cynomolgus monkeys [7]. Thus, low bioavailability makes subcutaneous prophylaxis with standard FVIII products unfeasible.

FVIII products with extended half-lives are being developed to address the limitations of standard FVIII products and reduce the burden of frequent intravenous injections [8]. One such product is N8-GP (turoctocog alfa pegol; Novo Nordisk, Bagsværd, Denmark), which is an extended half-life rFVIII developed for the prophylaxis and treatment of bleeding episodes in patients with hemophilia A [9]. N8-GP is produced by glycoPEGylation of turoctocog alfa, during which the terminal sialic acid on an O-glycan structure in the truncated B-domain is replaced by a conjugated sialic acid containing a branched 40-kDa polyethylene glycol (PEG). The B-domain is cleaved off upon activation of acid containing a branched 40-kDa polyethylene glycol truncated B-domain is replaced by a conjugated sialic acid on an O-glycan structure in the glycoPEGylation of turoctocog alfa, during which the terminal sialic acid on an O-glycan structure in the truncated B-domain is replaced by a conjugated sialic acid containing a branched 40-kDa polyethylene glycol (PEG). The B-domain is cleaved off upon activation of FVIII knockout (F8-KO) mice and cynomolgus monkeys [10]. A first-in-human dose-escalation trial showed that intravenously administered N8-GP has a plasma half-life of ≈19 h in patients with severe hemophilia A, corresponding to a 1.6-fold prolongation versus standard FVIII [10]. Furthermore, in the phase III pathfinder 2 trial, N8-GP administered every 4 days provided effective prophylaxis, as shown by a low median annualized bleeding rate of 1.3 [11]. N8-GP was also effective for the treatment of bleeding episodes, with a single injection resolving 84% of bleeds [11].

The present study describes the pharmacokinetics and the absorption, degradation and biodistribution of subcutaneously administered N8-GP in FVIII knockout (F8-KO) mice and cynomolgus monkeys. Additionally, the hemostatic effect of subcutaneously administered N8-GP was investigated in an F8-KO mouse tail vein transection (TVT) bleeding model. Furthermore, a human pharmacokinetic (PK) prediction model of subcutaneously administered N8-GP in patients with severe hemophilia A was developed to support investigation of the clinical feasibility of prophylaxis with subcutaneously administered N8-GP.

Materials and methods

Descriptions of the F8-KO mice and cynomolgus monkeys, the F8-KO mouse bleeding model and the biodistribution of N8-GP in mouse skin are available in Data S1.

PK studies in F8-KO mice: dosing and plasma sampling

Three groups of nine mice were dosed with 2500 IU kg\(^{-1}\) N8-GP by a single subcutaneous injection in the flank, neck, or lower abdomen. A fourth group of nine mice was dosed intravenously with 280 IU kg\(^{-1}\) N8-GP. Doses were selected to obtain approximately the same maximum plasma concentration for all mice, based on the single-dose study design; this enabled plasma samples with FVIII activity (FVIII:C) above the lower limit of quantification (LLOQ) to be obtained for all groups tested. Blood was sampled from 1 h to 72 h after dosing in a sparse sampling regimen.

Plasma was prepared by diluting the blood 5× in sodium citrate (0.13 M) in FVIII Coatest SP buffer (Coatest SP; Chromogenix, Bedford, MA, USA) and centrifuging at 4000 × g for 5 min at room temperature. The plasma was analyzed for FVIII:C in a chromogenic assay, as described below.

PK studies in cynomolgus monkeys: dosing and plasma sampling

Six cynomolgus monkeys were dosed with 100 IU kg\(^{-1}\) subcutaneously administered N8-GP either in a 500 IU mL\(^{-1}\) formulation (n = 3) or in a 5000 IU mL\(^{-1}\) formulation (n = 3). All monkeys were injected in the lateral region of the upper hind leg on days 1, 2 and 3 to mimic the once-daily dosing regimen intended for the clinical use of subcutaneously administered N8-GP. Five monkeys received a single dose of intravenous N8-GP (either 100 IU kg\(^{-1}\) [n = 2] or 250 IU kg\(^{-1}\) [n = 3]) via the saphenous vein. Doses were selected to obtain approximately the same total area under the plasma concentration–time curve (AUC) for all monkeys, based on the multiple-dose study design; this enabled plasma samples with FVIII:C above the LLOQ to be obtained for all groups tested, while using realistic human intravenous and subcutaneous doses.

Blood samples, drawn from the femoral vein, were obtained over a 96-h period after the first dose; samples were anticoagulated with citrate (0.13 M), and centrifuged for 10 min at 2300 × g at room temperature. Plasma was analyzed for FVIII:C with an N8-GP-specific chromogenic assay that does not measure endogenous FVIII, as described below.

FVIII:C assays

In F8-KO mice, FVIII:C was evaluated with a chromogenic assay (Coatest SP; Chromogenix) as described previously [12]. N8-GP was used as a calibrator.
In cynomolgus monkeys, an N8-GP-specific chromogenic activity assay was established. Anti-PEG antibody (mouse anti-PEG clone LHAG-2F8, produced in-house; 1 μg mL⁻¹) was immobilized in MaxiSorp ELISA plates (Nunc A/S, Roskilde, Denmark). After blocking for unspecific binding with FVIII Coatest SP buffer (Chromogenix), plasma samples were diluted in Comate SP assay buffer at least 1:10 and allowed to bind for 1 h. The plates were washed three times in phosphate-buffered saline with 0.05% Tween-20, and the activity of the bound N8-GP was measured with the chromogenic assay as described above. N8-GP (0.27–200 mIU mL⁻¹) was used as a calibrator.

**PK modeling**

For both F8-KO mice and cynomolgus monkeys, subcutaneous and intravenous FVIII activity data were simultaneously modeled with a non-linear mixed-effects model. With an assumption of non-linearity, bioavailability and other PK values were calculated by fitting the desired model parameters to the data to determine the value that gave the least difference between the observed and predicted values.

For F8-KO mice, a one-compartment model with interindividual variability on the volume of distribution was fitted simultaneously to all data. For the cynomolgus monkeys, subcutaneous and intravenous FVIII:C data were simultaneously modeled with a two-compartment distribution model with a first-order absorption rate for estimation of subcutaneous PK parameters. A non-linear mixed-effects model accounted for the effect of the intravenous dose as a covariate on the volume of distribution and clearance, as well as the effect of the subcutaneous injection volume on bioavailability. PHOENIX NLME software (v. 7.0; Certara, Princeton, NJ, USA) was used, with the first-order conditional estimation-extended least squares method, and model evaluation was based on a bootstrap run of n = 900 and 2000 predictions of model estimates. The model fit between different structural models to the data was evaluated on the basis of the -two times log likelihood value (-2LL). A reduction in -2LL of more than 3.84 was considered a significantly better description of the data (P ≤ 0.05 based on the χ² distribution), when adding an extra parameter or covariate.

**Human PK prediction model**

For PK predictions in humans following subcutaneous N8-GP administration, a systemic PK model was developed on the basis of the cynomolgus monkey absorption rate constant and bioavailability parameters, and human elimination parameters. The elimination model structure (one-compartment) and population PK parameters of clearance and volume of distribution were taken from the pathfinder 1 trial, in which patients with hemophilia A received N8-GP intravenously at single doses of 25, 50 or 75 IU kg⁻¹ (n = 7, n = 9, and n = 10, respectively; NCT01205724, www.clinicaltrials.gov [10], and data on file). The human intravenous data were fully described with a one-compartment model, generating 26 PK profiles. The subcutaneous N8-GP absorption rate constant and bioavailability in humans were assumed to be similar to those in cynomolgus monkeys (as determined above), in line with data for recombinant activated FVII (rFVIIa) (NovoSeven; Novo Nordisk), glycoPEGylated rFVIIa (data on file), and recombinant FIX [13]. For calculations of the AUC, which underlies the estimation of bioavailability, the model that best described the data was selected.

**In vivo imaging of the disappearance of N8-GP from the subcutaneous injection site**

The FVIII moiety of N8-GP was randomly labeled on lysine residues with VivoTag680XL Fluorochrome (PerkinElmer, Waltham, MA, USA), according to the manufacturer’s instructions. VT680-N8-GP was purified by size-exclusion chromatography. FVIII:C was maintained > 85%.

One week prior to study initiation, mice were switched to a low-fluorescence rodent chow diet (Altromin C-1039; Cat. no. 10103500, Brogaarden, Lynge, Denmark). On the day before VT680-N8-GP administration, mice were shaved and depilated on the lower body. VT680-N8-GP (1850 IU kg⁻¹ in a concentrated dose volume of 0.2 mL kg⁻¹) was injected subcutaneously into the right flank with Hamilton syringes (Sigma-Aldrich, Copenhagen, Denmark). Nineteen F8-KO mice received a single subcutaneous dose of VT680-N8-GP (1850 IU kg⁻¹), and five mice received multiple subcutaneous doses of VT680-N8-GP (3 × 1850 IU kg⁻¹) at the same injection site, separated by 48-h intervals.

The disappearance of VT680-N8-GP after subcutaneous injection was followed by temporal in vivo fluorescence imaging (FMT 2000; PerkinElmer) at 665-nm excitation/688-nm emission up to 72 h after dosing; mice were under continuous isoflurane anesthesia. Scans were analyzed and quantified with TRUEQUANT 3.5 (PerkinElmer); fluorescence was quantified in fixed two-dimensional regions of interest around the injection site, and no threshold was applied to the fluorescence datasets. Intrinsic fluorescence was measured on the opposite flank. After the final scan, mice were killed by cervical dislocation, and skin samples were collected from injection sites for further characterization by SDS-PAGE and western blot analysis, as described in Data S1.

**Results**

**Subcutaneous pharmacokinetics of N8-GP in F8-KO mice**

To evaluate pharmacokinetics, N8-GP was administered to F8-KO mice as single subcutaneous or intravenous injections, and the observed FVIII:C–time profiles were
used to estimate the PK parameters (Table S1). The data obtained and visual predictive checks of the model fit for the subcutaneous and intravenous profiles are shown in Fig. 1A,B; the shaded areas demonstrate that 95% of the predicted values capture most of the observed data points. Owing to the sparse sampling regimen and very fast initial distribution phase observed in these animals, a one-compartment model was selected, and adequately described the FVIII:C–time profiles after subcutaneous and intravenous administration of N8-GP to the mice (Fig. 1A,B). When N8-GP was administered subcutaneously, maximal FVIII:C in plasma was reached at 14 h after dosing, the estimated bioavailability of N8-GP was 24%, and the terminal half-life was 14 h.

Subcutaneous pharmacokinetics of N8-GP in cynomolgus monkeys

N8-GP was then administered to cynomolgus monkeys as three consecutive subcutaneous doses at 0, 24, and 48 h, or as a single intravenous dose. The observed FVIII:C–time profiles were used to estimate the PK parameters (Table S2) and predict the subcutaneous and intravenous profiles (Fig. 2A,B). For subcutaneous administration, the shaded areas show that 95% of the predicted values capture most of the observed data after the first and third doses; however, the model appears to underpredict FVIII:C after the second dose (from 24 h to 48 h) (Fig. 2A). For intravenous administration, 95% of the predicted values capture most of the observed data points (Fig. 2B). On the basis of the more frequent sampling regimen and slower distribution, a two-compartment model was selected, and adequately described the FVIII:C–time profiles after subcutaneous and intravenous administration of N8-GP to cynomolgus monkeys (Fig. 2A,B). When N8-GP was administered subcutaneously, maximal FVIII:C in plasma was reached at 6.8 h after dosing; the estimated bioavailability of N8-GP was 26%, with a terminal half-life of 14 h. Two different formulations were compared for subcutaneous dosing: the 5000 IU mL⁻¹ formulation (in a volume of 0.02 mL kg⁻¹) showed 32% lower bioavailability than the 500 IU mL⁻¹ formulation (in a volume of 0.2 mL kg⁻¹). This was confirmed by non-compartmental analysis, which resulted in a 36% difference in the AUC.

Simulated human pharmacokinetics: prediction of high daily trough levels

The observed PK parameters in cynomolgus monkeys were used to estimate human PK parameters for a 70-kg individual (Table S3) and simulate PK profiles after multiple subcutaneous or intravenous dosing in humans (Fig. 3). On the basis of this modeling, a daily dose of 12.5 IU kg⁻¹ subcutaneous N8-GP is predicted to provide FVIII:C trough levels of 2.5–10%, and corresponding peak levels of 5–15%, in 95% of patients with hemophilia A (Fig. 3).

Hemostatic effect in F8-KO mice

The in vivo effect of subcutaneously administered N8-GP was evaluated in F8-KO mice. TVT was performed 24 h after subcutaneous administration of N8-GP (Fig. 4A). Mice in each of the four treatment groups receiving 52, 105, 210 and 419 IU kg⁻¹ subcutaneous N8-GP had statistically significantly reduced blood loss as compared with the vehicle-injected group. Furthermore, blood loss decreased with increasing doses of subcutaneous N8-GP (P < 0.0001 for dose dependency). Next, N8-GP (352 IU kg⁻¹) was administered subcutaneously at 24, 28,
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72 or 96 h prior to TVT. The effect of subcutaneous N8-GP decreased over time (P < 0.0001 for time dependency) (Fig. 4B). Subcutaneous N8-GP had a significant effect as compared with the vehicle group when administered 24 h (P < 0.0001) and 48 h (P < 0.001) before injury.

Disappearance of fluorescently labeled N8-GP from the subcutaneous injection site

To characterize the fate of subcutaneously administered N8-GP at the injection site, temporal fluorescence imaging combined with ex vivo analysis of injection-site homogenates was used to describe the time-dependent disappearance and intactness of N8-GP after subcutaneous administration. A single subcutaneous dose of VT680-N8-GP (1850 IU kg\(^{-1}\)) was administered in the flanks of F8-KO mice. Imaging of the depot was carried out up to 72 h after dosing (Fig. 5A), and the fluorescence from VT680-N8-GP was quantified in a defined area around the injection site (Fig. 5B). Time-dependent disappearance of subcutaneously administered VT680-N8-GP was observed over a period of 72 h. Fluorescent quenching was detected immediately after administration, owing to the high amount of fluorescence in the small administered volume.

The stability of subcutaneously administered VT680-N8-GP was determined in skin samples obtained from F8-KO mice that had been fluorescently imaged. Homogenates were lysed and separated by SDS-PAGE, and this was followed by fluorescence gel scanning and western
blot analysis (Fig. 5C,D). Owing to the random labeling of N8-GP and the use of a polyclonal anti-FVIII antibody for western blotting, bands representative of N8-GP single, heavy and light chains were observed on gels (Fig. 5C) and on western blots (Fig. 5D). VT680-N8-GP remained intact at the injection site, and gradually disappeared, with no visible fragmentation. Very little protein was detectable at 72 h (Fig. 5C,D). On the western blot, an approximately 30-kDa band was present in all samples (including the predose samples), and therefore corresponds to unspecific staining (Fig. 5D). On the fluorescence-scanned gels, a faint band of approximately 40 kDa was observed in the 5-min sample (Fig. 5C). The apparent mass corresponds to the mass of the A2 domain after activation of the heavy chain. This band was also faintly observed in the VT680-N8-GP stock on the western blot (Fig. 5D), and this band is therefore considered unlikely to represent any major degree of FVIII activation/fragmentation after subcutaneous injection. FVIII chromogenic activity in plasma supports the idea that VT680-N8-GP peaked at 6–24 h and that the signal was below the detection limit by 72 h (data not shown).

Subsequently, the disappearance and sequential stability of multiple subcutaneous N8-GP doses was examined in F8-KO mice. Three injections of VT680-N8-GP (1850 IU kg\(^{-1}\)) at the same site were administered at 48-h intervals. Quantitative fluorescence imaging showed similar absorption kinetics of subcutaneously administered VT680-N8-GP as observed in the single-dose study (Fig. 6A). Furthermore, very little VT680-N8-GP remained in the depots in skin homogenates collected 48 h after the last administered dose. This was observed both with fluorescence gel scanning and with western blot analysis (Fig. 6B,C). Thus, multiple injections of VT680-N8-GP show a disappearance pattern similar to that of single-dose VT680-N8-GP.

**Biodistribution of N8-GP in skin and lymph nodes of F8-KO mice**

Immunohistochemical (IHC) analysis of skin samples from F8-KO mice subcutaneously injected with N8-GP and killed at 5 min and 6, 24 and 72 h after injection (\(n = 3–4\) at each time point) readily identified FVIII and PEG (Fig. 7A,B). There was time-dependent disappearance from the skin injection sites, and minimal levels of FVIII and PEG were detected at 72 h after dosing. The IHC stain for PEG was much more sensitive than that for FVIII, but the two antigens of N8-GP disappeared synchronously from the injection sites. Another set of antibodies against FVIII and PEG were applied in parallel, and provided similar results (data not shown). Semi-quantitative assessment of the disappearance of N8-GP from the skin injection sites is shown in Fig. 7C,D.

Representative high-magnification images of the anti-FVIII IHC staining for each treatment group receiving N8-GP are shown in Fig. 8A. In samples taken 5 min after dosing, the IHC reactivity for FVIII (identifying N8-GP) was mostly interstitial and, to a lesser degree, cell-associated (Fig. 8A). This distribution pattern was also observed in samples removed 6 h after dosing, but with more evidence of cell-associated staining (Fig. 8A). Double immunofluorescence identified a large proportion of the cells that stained for FVIII at 6 h as being CD11b-
positive myeloid cells (Fig. 8B). There was no significant staining at the 72-h time point (Fig. 8A).

The lymph nodes showed very little IHC staining for FVIII at all time points assessed. The more sensitive IHC stain for PEG identified the transient presence of N8-GP in the subcapsular space of all four draining lymph nodes recovered 6 h after the skin injection, but not at any of the earlier or later time points (5 min, 24 h, and 72 h) (Fig. 8C). No IHC staining for PEG was identified in the lymph nodes from any of the animals that received vehicle only ($n = 4$).

Discussion

Intravenously administered N8-GP has a 1.6-fold longer half-life than standard rFVIII products in patients with

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severe hemophilia A [10,11]. This allows prophylaxis with intravenous administration of 50 IU kg⁻¹ N8-GP every fourth day [11]. Intravenous administration can, however, be troublesome, particularly in young children, and limit compliance. The relatively low bioavailability of FVIII after subcutaneous administration in hemophilia A mice and cynomolgus monkeys [6,7] has so far suggested that subcutaneous prophylaxis in humans with standard FVIII would be unfeasible. In the present study, we evaluated the feasibility of subcutaneous administration of N8-GP in F8-KO mice and cynomolgus monkeys, and developed a human PK prediction model.

When N8-GP was administered subcutaneously to F8-KO mice and cynomolgus monkeys, N8-GP maximal activity was observed at 14 h and 6.8 h, respectively. Plasma half-lives was 14 h and 15 h, respectively. In mice, this observed half-life is similar to that reported in an earlier study following intravenous administration of N8-GP (14 h), whereas, in cynomolgus monkeys, the half-life observed in the present study is similar to or slightly shorter than the reported half-life following intravenous administration (18 h) [9].

N8-GP was hemostatically active after subcutaneous administration. A dose-dependent effect on blood loss was observed in F8-KO mice when N8-GP was administered 24 h before TVT, with significant reductions in blood loss being seen at 24 h and 48 h after dosing.

The estimated levels of subcutaneous bioavailability of N8-GP were 24% and 26% in F8-KO mice and cynomolgus monkeys, respectively. This represents a marked increase over the levels shown in earlier preclinical studies investigating the subcutaneous administration of standard FVIII. In mice, bioavailability of < 1% for rFVIII was observed [6]. In cynomolgus monkeys, the bioavailability of standard FVIII reached a maximum of 3–11% as determined with an assay that was not specific for human
FVIII (making the actual bioavailability uncertain) [7]. The bioavailability of subcutaneously administered N8-GP is also greater than what was recently reported for the extended-half-life rFVIII Fc fusion protein (rFVIIIFc) and the rFVIIIFc–von Willebrand factor (VWF)–XTEN fusion protein; rFVIIIFc had a bioavailability of approximately 1% in cynomolgus monkeys, and the rFVIIIFc–VWF–XTEN fusion protein had bioavailability levels of 20% in hemophilia A mice and 8% in cynomolgus monkeys, following subcutaneous administration [14].

The increased subcutaneous bioavailability of N8-GP may be a result of reduced cellular clearance caused by the presence of the PEG moiety. Indeed, we previously demonstrated that N8-GP is taken up more slowly than standard rFVIII in cells expressing lipoprotein receptor-related protein-1, human monocyte-derived dendritic cells [9], and primary rat hepatocytes [15]. Likewise, PEGylation may protect FVIII against uptake by cells in the subcutis. Furthermore, PEGylation may protect against proteolytic degradation and increase solubility [16]. Finally, higher than physiological sodium chloride concentrations are required to keep FVIII in solution [17], which could result in precipitation of standard FVIII under the isotonic conditions in the subcutis. N8-GP, however, has increased solubility under isotonic conditions (data not shown).

The 24–26% bioavailability of subcutaneously administered N8-GP demonstrates that some of the administered N8-GP did not reach the circulation, and prompted us to evaluate the distribution of N8-GP after subcutaneous administration. Temporal imaging of F8-KO mice dosed with fluorescently labeled VT680-N8-GP showed that minimal N8-GP was detected at the injection site 48–72 h after a single dose or after repeated dosing at 48-h intervals, suggesting that there is no accumulation of protein at the site of injection. The stability of N8-GP in the subcutis was confirmed on fluorescence gel scans and western blots of skin homogenates collected 5 min to 72 h after dosing.

The subcutaneous PK data obtained in cynomolgus monkeys, together with data on N8-GP administered intravenously in cynomolgus monkeys and in patients
with hemophilia A ([10] and data on file), were used to develop a human PK prediction model for subcutaneously administered N8-GP. With this model, it was predicted that a daily dose of 12.5 IU kg\(^{-1}\) subcutaneous N8-GP could provide FVIII trough levels of 2.5–10% in 95% of patients with hemophilia A. The results obtained with this model suggest that subcutaneously administered N8-GP may allow for a daily prophylaxis dosing regimen in patients with hemophilia A that results in higher minimum plasma FVIII:C, with significantly less difference between trough and peak levels than with current intravenous FVIII replacement therapy. Generally, the goal of FVIII prophylactic therapy is to maintain plasma FVIII levels above a target threshold; studies have shown patients with FVIII trough levels of >1% to have reduced numbers of breakthrough and joint bleeds [18]. Comparisons of baseline FVIII levels and bleeding frequencies across patients with severe and moderate hemophilia suggest that further reductions in bleeding frequency can be obtained with even higher trough levels [19]. For many patients, the inconvenience of frequent intravenous infusions is a challenge, and can lead to poor adherence [20], which negatively impacts on maintenance of trough levels. Venous access devices are used by some patients, but are associated with risks of infection and thrombosis [4,5]. It is anticipated that the relative ease of subcutaneous administration, together with the altered PK profile of subcutaneously administered N8-GP, may enable convenient daily treatment providing more stable FVIII:C levels, including the desired higher trough levels. This concept is currently under evaluation in a phase I trial investigating the safety, tolerability, pharmacokinetics and preliminary efficacy of once-daily subcutaneous administration of N8-GP in patients with hemophilia A (NCT02994407).

**Addendum**

F. Rode designed the animal PK studies and analyzed the results, and contributed to the design of the distribution studies and the human subcutaneous PK modeling. K. Almholt designed and performed the IHC analyses. M.
Petersen designed and performed the in vivo imaging studies and ex vivo quantification in skin homogenates. M. Kreiggaard contributed to the PK modeling. M. Kjalke contributed to the initial conception of subcutaneous PK studies in mice. D. Karpf designed the studies in cynomolgus monkeys and analyzed the results, and performed initial subcutaneous PK studies in mice. A. V. Groth performed the intravenous PK modeling. P. B. Johansen designed and performed the mouse blood loss experiments. L. F. Larsen performed plasma sample analyses. M. Loftager developed the N8-GP-specific chromogenic activity assay and performed plasma sample analyses. J. Haaning contributed to the conception and overall design of the studies. All authors contributed to critical writing or revision of the intellectual content, and gave final approval of the manuscript.

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Disclosure of Conflict of Interests

All authors are or were full-time employees of Novo Nordisk during the conduct of this study. All authors are minor shareholders of Novo Nordisk A/S.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Data S1. Supplementary methods.
Table S1. Mouse PK parameters.
Table S2. Cynomolgus PK parameters.
Table S3. Human PK parameters for a 70-kg individual.

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