Nonclinical Safety Assessment of a Long-Acting Recombinant PEGylated Factor Eight (BAY 94-9027) With a 60 kDa PEG

Inge A. Ivens1, David Banczyk2, Katrin Gutberlet2, Shawna Jackman3, Stéphanie Vauléon2, and Anna-Lena Frisk2

Abstract
BAY 94-9027 (Jivi) is a site-specifically PEGylated human B-domain-deleted (BDD) recombinant factor VIII (FVIII), with a 60 kDa branched PEG molecule attached. The nonclinical safety of BAY 94-9027 was evaluated in a toxicology program that included 2 weeks intravenous (IV) toxicity studies in rats and rabbits, a juvenile toxicity study in rats as well as a 26-week chronic study in rats. Doses of 75, 750, or 2250 IU/kg given every other day for 2 weeks did not elicit any findings related to BAY 94-9027. Specifically, no thrombus formation or histological changes such as cellular vacuolation were seen. In the chronic toxicity study, 40, 400, and 1200 IU/kg of BAY 94-9027 given twice weekly did not induce adverse effects related to BAY 94-9027, and no tissue vacuolation was observed. There was no PEG detected in choroid plexus or other areas of the brain, cerebrospinal fluid or in spleen or kidneys. These results were supported by toxicity studies in rats and rabbits treated with PEG 60 kDa attached to the maleimide linker (PEG-60-Mal-Cys). No findings related to PEG-60-Mal-Cys were seen. These results demonstrate the safety of BAY 94-9027 for long-term use.

Keywords
PEGylation, recombinant FVIII, nonclinical studies with polyethylene glycol (PEG), toxicology program, chronic long-term toxicity, safety of polyethylene glycol (PEG)

Introduction
People with the coagulation disorder hemophilia A are treated with factor VIII (FVIII) as replacement for their missing or inactive intrinsic FVIII protein, either on demand (treatment when a bleed occurs) or prophylactically (to prevent bleeding). While prophylactic use can provide continuous protection from bleeding, intravenous (IV) infusions have to be given several times per week due to the short half-life of FVIII. Extending protection with fewer infusions has been a goal in the development of new coagulation factors and can be achieved, for example, by linking the recombinant human FVIII (rFVIII) protein to other, inert large molecules such as the Fc part of an antibody (rFVIIIFc, Elocta/Eloctate) or to polyethylene glycol (PEG) molecules as seen in BAY 94-9027 and Adynovate. These techniques have enabled less frequent IV infusions while maintaining pharmacological activity.1–7

BAY 94-9027 (Jivi) was recently approved for the treatment of hemophilia A. In humans, 2 pharmacokinetic studies have shown that BAY 94-9027 has improved pharmacokinetic parameters compared to recombinant FVIII (rFVIII; Kogenate FS) and extended half-life rFVIIIFc (Elocta/Eloctate) products.2,8 In the PROTECT VIII study and its extension, BAY 94-9027 was efficacious in the prevention of bleeds in previously treated adults and adolescents.9,10 BAY 94-9027 was generally well tolerated, and no patient developed FVIII inhibitory antibodies.11,12

BAY 94-9027 is a site-specifically PEGylated B-domain-deleted (BDD) rFVIII containing a single branched 60 kDa PEG molecule.1 It contains the largest PEG molecule conjugated to a drug protein to date. BAY 94-9027 itself is inactive and requires activation to FVIIIa to be effective in the intrinsic coagulation pathway. The molecule was selected out of several drug candidates with different, smaller PEG sizes and different PEG attachment sites based on its longer half-life in hemophilia A mice and dogs and full coagulation activity. PEGylation was used for BAY 94-9027 since it has been a successful

1 Bayer HealthCare, San Francisco, CA, USA
2 Bayer AG, Berlin, Germany
3 Charles River Laboratories, Horsham, PA, USA

Corresponding Author:
Inge Ivens, Bayer HealthCare, 455 Mission Bay Blvd, South, San Francisco, CA 94158, USA.
Email: ingeivens@aol.com
approach in the modification of biopharmaceuticals to extend their circulation half-life.\textsuperscript{13–16} A single clinical dose of 60 IU/kg body weight of BAY 94-9027 contains a very small amount of PEG, approximately 0.002 mg. Therefore, the overall amount of PEG, also with long-term treatment, is very small in humans.

More than 12 PEGylated biotherapeutics have been approved for IV or subcutaneous (SC) use over the last 2 decades,\textsuperscript{13,16} and no safety signals related to PEG have been identified in humans.\textsuperscript{13–16} Polyethylene glycol molecules $\geq$2 kDa in size are inert, highly water soluble, and have no specific receptor or other target in organs or tissues in the body.\textsuperscript{13} The toxicity of PEGylated drugs usually reflects the toxicity of the parent (unconjugated) drug molecule.\textsuperscript{17} The only effect related to PEG observed in nonclinical toxicity studies was described as cellular vacuolation visible in histological evaluations by light microscopy at higher PEG doses,\textsuperscript{18,19} above the amount of PEG in a clinical dose of BAY 94-9027. In publicly available toxicological information, vacuolation has not been linked with changes in organ function in the absence of changes in cell morphology or changes in the surrounding tissue and therefore cellular vacuolation alone is not considered adverse. Nor have there been any reports of adverse events due to PEG with PEGylated drugs in humans.\textsuperscript{13,16} Cellular vacuolation has been seen histologically with PEG sizes starting already at 5 kDa after repeated high doses in toxicity studies\textsuperscript{14} and has also been reported after a single dose for a different product.\textsuperscript{20} The light microscopically visible cellular vacuoles were round and discrete clear cytoplasmic vacuoles in hematoxylin and eosin (H&E) staining.\textsuperscript{18,19} Polyethylene glycol does not stain with standard histological H&E dyes, and specialized immunohistochemistry (IHC) methods have to be used to detect PEG in cellular vacuoles and recently also in the cytoplasm of cells.\textsuperscript{18,19} These vacuoles likely represent lysosomal/endosomal uptake of PEG or PEGylated drugs. Polyethylene glycol–related vacuolation is seen most frequently in macrophages and other cells of the reticulo-endothelial (RES) system and is likely an adaptive response to remove molecules such as PEG.\textsuperscript{18,21} Vacuoles have also been observed in nonphagocytic cell types including renal tubular epithelium and choroid plexus epithelium. Distribution patterns of PEGylated biotherapeutics may depend on drug receptor/target-mediated uptake and/or ligand-specific transport mechanisms, and therefore organs or tissues with vacuolation and the time and dose when this occurs can vary for different PEGylated biopharmaceuticals. This has been shown by Ivens et al.\textsuperscript{13} in an industry survey of 17 PEGylated biopharmaceuticals under development, which, for example, found that vacuolation of the choroid plexus was seen with 20-, 30-, and 40-kDa site-specific PEGylated proteins. FVIII and BAY 94-9027 have no cellular receptors, it is unlikely that there is specific uptake of BAY 94-9027 in any organ or tissue.

Basophilic storage granules (cytoplasmic of basophilic granules) similar in type to vacuolation seen with PEG have been described for antisense oligonucleotides (ASOs; stained with H&E), and vacuolation has been detected following administration of some sugars and also methylcellulose.\textsuperscript{22}

The nonclinical toxicity program for BAY 94-9027 included systemic, juvenile, and chronic (long-term) toxicity studies in rats and rabbits to address overall safety and specifically the potential for PEG-related cellular vacuolation. The chronic toxicity study with 26 weeks of twice-weekly dosing is the main focus of this publication. It was conducted in immunodeficient (IDF) athymic male rats (Crl; NIH-Foxn1$^{nu}$), which do not mount a humoral immune response against species foreign proteins. The study provides a comprehensive toxicology investigation of BAY 94-9027 utilizing IHC in combination with standard histopathology, and PEG analysis in cerebrospinal fluid (CSF) plus plasma to address recent concerns about PEG accumulation in tissues after administration of PEGylated biopharmaceuticals. The toxicology program BAY 94-9027 was supplemented by studies with the PEG 60 kDa linker moiety alone (unconjugated PEG-60-Mal-Cys) at high doses to assess the potential for formation of cellular vacuolation and the genotoxicity of the maleimide linker.

**Materials and Methods**

**Toxicology Program**

To assess the safety of BAY 94-9027, the nonclinical program was tailored specially for the patient population with hemophilia A. The toxicology program for BAY 94-9027 was conducted in male animals only to reduce the overall animal number since the patient population is male. Factor VIII and BAY 94-9027 are male animals only to reduce the overall animal number since the patient population is male. Factor VIII and BAY 94-9027 are administered IV as highest dose every other day for 2 weeks. This is approximately 30 times the highest human dose of 60 IU/kg. The following endpoints were evaluated: body weight, mortality, clinical signs and symptoms, ophthalmology, hematology and hemostasis (at end of study), clinical chemistry and urinalysis (at end of study and recovery), toxicokinetic (TK), antidrug antibodies (binding and neutralizing), gross pathology and organ weights, histopathology of a set of >40 organs, and tissues per animal according to the Guideline on Repeat Dose Toxicity.\textsuperscript{23} Furthermore, a 2-week study in juvenile rats was conducted with twice-weekly IV dosing of 200 and 1000 IU/kg starting on postnatal day (PND) 17 employing similar endpoints as the systemic studies.

The systemic studies, 2250 IU/kg BAY 94-9027 was given IV as highest dose every other day for 2 weeks. This is approximately 30 times the highest human dose of 60 IU/kg. The following endpoints were evaluated: body weight, mortality, clinical signs and symptoms, ophthalmology, hematology and hemostasis (at end of study), clinical chemistry and urinalysis (at end of study and recovery), toxicokinetic (TK), antidrug antibodies (binding and neutralizing), gross pathology and organ weights, histopathology of a set of >40 organs, and tissues per animal according to the Guideline on Repeat Dose Toxicity.\textsuperscript{23} Furthermore, a 2-week study in juvenile rats was conducted with twice-weekly IV dosing of 200 and 1000 IU/kg starting on postnatal day (PND) 17 employing similar end points as the systemic studies.

Due to recent concerns about PEG safety,\textsuperscript{21} and since the toxicology studies with BAY 94-9027 were limited in duration and doses, the PEG 60 kDa moiety connected to the maleimide
linker (PEG-60-Mal-Cys) was evaluated in further studies up to 4 weeks duration in rats and rabbits. In addition, a set of genotoxicity studies was conducted, since PEG-60-Mal-Cys including the maleimide linker had not been tested before. PEG-60-Mal-Cys was produced by mixing PEG 60 kDa maleimide used in production of BAY 94-9027 with cysteine to quench the reactive part of maleimide by binding it to this amino acid.

Doses of PEG-60-Mal-Cys were selected as very high multiples of the weekly PEG dose present in BAY 94-9027 assuming that a high dose given for 4 weeks will reveal the potential for formation of cellular vacuolation. In the 4-week rat study, animals were dosed with 11 mg/kg given every second day, which is more than 5000-fold higher than the amount of PEG 60 kDa in a single human dose of 60 IU/kg. The study design of the chronic toxicity study in IDF rats is described in more detail below, and Table 1 summarizes the toxicology studies conducted with BAY 94-9027, while Table 2 lists supplemental toxicology studies with PEG-60-Mal-Cys including group size and doses.

The chronic toxicity study was conducted in IDF athymic male rats (Crl: NIH-Foxn1nu immuno-deficient nude rat) since immune-competent animals mount an antibody response to human FVIII within 2 weeks due to the differences in the protein structure between humans and animals. Binding antibodies (ADA) and drug-neutralizing antibodies (nADA) are generated, which abolish exposure to active FVIII.

Good laboratory practice status and regulations applied. The chronic study in IDF rats complied with the US Animal Welfare Act. The models in immune-competent rats and rabbits for the initial characterization of BAY 94-9027 were chosen in line with the international guidelines for assessment of the toxicological profile on drugs, especially biologics. In line with German regulations, animal studies requested by regulations for risk assessment of human use were authorized by the local authority responsible for animal protection by granting a “license for the use of animals for scientific purposes” (Anzeige der Verwendung von Tieren für wissenschaftliche Zwecke) for the specific type of experiment. This includes the animal species used, the procedures performed, and the general size of the study based on the German Animal Welfare Law. The approval process included a thorough check whether the principle design of the animal studies was adequate for the intended purpose and the potential suffering of the animals ethically justified. All animal studies performed in Germany were performed with valid licenses for such experiments.

All in vivo studies were good laboratory practice (GLP) compliant. The peer reviews and all assays and assessments

### Table 1. List of Repeated Administration Toxicity Studies With BAY 94-9027.

| Type of Study                        | Species (Animal Number per Group), Gender, Strain | Dosing                  | BAY 94-9027 Doses, IU/kg |
|-------------------------------------|-------------------------------------------------|-------------------------|--------------------------|
| Systemic toxicity: 2-week dosing (4 week recovery) | Rat (10), male, Sprague-Dawley                   | IV bolus (every other day) | 0, 75, 750, 2250          |
| Systemic toxicity: 2-week dosing (4 week recovery) | Rabbit (6), male, New Zealand White              | IV bolus (every other day) | 0, 75, 750, 2250          |
| 2-week juvenile study in rats (PND 17-32) | Rat (22), male, Wistar                           | IV bolus (twice weekly)  | 0, 200, 1000              |
| 13- and 26-week chronic toxicity study (26-week recovery) | Crl: NIH-Foxn1nu immuno-deficient nude rat (13 weeks:10, 26 weeks 20), male | IV bolus (twice weekly)  | 0, 40, 400, 1200          |

### Table 2. List of Repeated Administration Toxicity Studies With PEG 60 kDa Bound to Maleimide (PEG-60-Mal-Cys).

| Type of Study                        | Species (Animal Number per Group), Gender, and Strain | Dosing                  | PEG-60-Mal-Cys, mg/kg |
|-------------------------------------|-------------------------------------------------|-------------------------|----------------------|
| 4-week dosing (4-week recovery)     | Rat (12), male, Sprague-Dawley                   | IV bolus (every second day) | 0, 0.045, 0.7, 11.0  |
| 4-week dosing (13-week recovery)    | Rabbit (6), male, New Zealand White              | IV bolus (twice weekly)  | 0, 0.02, 0.2, 2.00   |
| 4-week juvenile study in rats       | Rat (19), male, Wistar                           | IV bolus (twice weekly)  | 0, 0.4, 2.0          |
| Genotoxicity: ames assay            | Salmonella strains                                | In vitro                | 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mg/plate |
| Genotoxicity: mouse lymphoma assay  | Mouse lymphoma cells                              | In vitro                | 0.15, 0.5, 1.5, and 5 mg/mL |
| Genotoxicity: micronucleus assay in rats | Rat, male, Sprague-Dawley                        | IV bolus (every second day) | 0.045, 0.7, 11        |

Abbreviations: IV, intravenous; PND, postnatal day.
except the assay for PEG concentration in rat plasma in the chronic rat study were conducted under GLP.

### Chronic Toxicity Study With BAY 94-9027 in IDF Rats (Crl: NIH-Foxn1

**Animals and study design.** Male Crl: NIH-Foxn1

**IDF rats were approximately 8 weeks old at the start of treatment, assigned to study groups using computer-generated randomization procedures, housed two per box when possible in isolator equivalent animal rooms, and handled according to procedures for immunocompromised rodents.** Rats were housed in filter-top, autoclaved, polycarbonate nesting boxes containing environmental enrichment with autoclaved bedding and nesting material. Rats were provided with Irradiated Rodent Diet available ad libitum, and sterilized water in sipper tube bottles was available ad libitum. Veterinary care was available throughout the course of the study. All cage sizes and housing conditions are in compliance with the US Guide for the Care and Use of Laboratory Animals.

The highest dose of 1200 IU/kg twice weekly was selected to be 30-fold above the highest weekly dose in humans. BAY 94-9027 and the control item were administered to the animals via IV injection into the tail vein (alternating between the left and right caudal veins, when possible) by straight needle bolus administration. The lots used were tested for potency, stability, and other attributes to assure the accuracy of dosing in the study.

Animals received volumes ranging between 0.1 and 3.0 mL/kg body weight. Groups of rats received 0, 40, 400, and 1200 IU/kg of BAY 94-9027 twice weekly IV for 13 or 26 weeks at volumes between 0.1 and 3.0 mL/kg. Additional animals received the test item for 26 weeks twice weekly followed by 26 treatment-free weeks (see Table 3 for the study design). The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight gains, food consumption, ophthalmology, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), TK parameters for BAY 94-9027 to show exposure, PEG concentrations in plasma, PEG in CSF, ADAs in serum, gross necropsy findings, organ weights, IHC evaluation for PEG in tissues, and histopathologic examinations.

**Laboratory evaluations for clinical chemistry, hematology, coagulation and urinalysis.** Blood was collected from jugular vein (in-life) or via the vena cava while under isoflurane/oxygen anesthesia (terminal) from individual animals. After collection, samples were transferred to the appropriate laboratories for processing. Table 4 shows the sample schedule for clinical pathology. Clinical pathology parameters were assessed after 13 and 26 weeks and after recovery.

Hematology parameters included red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, red blood cell distribution width, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, reticulocyte count (absolute), platelet count, white blood cell count, neutrophil count (absolute), lymphocyte count (absolute), monocyte count (absolute), eosinophil count (absolute), basophil count (absolute), large unstained cells, and other cells (as appropriate).

Clinical chemistry parameters included alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, creatine kinase, total bilirubin, urea nitrogen, creatinine, calcium, phosphorus, total protein, albumine, globulin (calculated), albumine/globulin ratio, glucose, cholesterol, triglycerides, sodium, potassium, and chloride.

Coagulation parameters included activated partial thromboplastin time by assay that did not react to PEG, fibrinogen, and prothrombin time.

Urinary parameters included color, appearance/clarity, specific gravity, pH, protein, glucose, bilirubin, ketones, blood. Urine were collected from individually housed animals overnight. Animals were fasted overnight prior to collection.

**Toxicokinetics and antidrug antibody analysis.** Plasma concentrations of BAY 94-9027 were determined by a validated method from individual rats. Starting on dosing day 78 (week 11) and dosing day 169 (week 24), blood samples were collected at 0, 0.25, 8, 24, 48, and 72 hours after dosing. Due to the spacing of the time points, each animal was sampled at least twice, allowing assessment if exposure was impacted/low due to antidrug antibodies. BAY 94-9027 activity was determined in diluted plasma by an enzymatic assay with absorbance readout using an antihuman FVIII monoclonal antibody as capture and a commercially available kit (Biophen FVIII: C; Hyphen BioMed, Neuville-sur-Oise, France) for measuring activity. The ADAs were evaluated in serum samples for all animals at termination using a validated enzyme-linked immunosorbent assay (ELISA). Samples were heat treated at 56°C for 30 minutes before analysis at a minimum required dilution of 1:20. BAY 94-9027 was coated onto a microtiter plate and used to capture antidrug antibodies present in samples. Captured antibodies were detected using a goat anti-rat IgG antibody conjugated to horseradish peroxidase (HRP) and tetramethylbenzidine as a substrate. Samples were analyzed initially in a screening assay followed for samples screened positive by a confirmatory assay.

**Polyethylene glycol analysis in plasma.** Plasma samples for PEG analysis were collected at necropsy from each animal. Samples

### Table 3. Study Design of 26 Weeks Chronic Toxicity Rat Study Followed by 26 Weeks of Recovery With BAY 94-9027 Dosed Twice Weekly.

| Group | Test Material | Dose Level, IU/kg* | Number of Animals |
|-------|---------------|-------------------|-------------------|
| 1 | Saline Control | 0 | 10 20 15 |
| 2 | BAY 94-9027 | 40 | 10 20 15 |
| 3 | BAY 94-9027 | 400 | 10 20 15 |
| 4 | BAY 94-9027 | 1200 | 10 20 15 |

*Polyethylene glycol (PEG) 60 kDa amounts for 40, 400, and 1200 IU/kg of BAY 94-9027 were approximately 1.3, 13, and 40 μg/kg per dose.
were analyzed under non-GLP conditions for PEG by liquid chromatography–mass spectrometry after protein precipitation with methanol. PEG-60-Mal-Cys was used as reference standard and a stable isotopically labeled 60 kDa PEG as internal standard. The method was validated for a lower limit of quantitation (LLOQ) of 20 ng/mL.

**Cerebrospinal fluid collection and analysis.** Cerebrospinal fluid was collected from all animals (when possible) at termination under anesthesia before necropsy for analysis of PEG. The determination of PEG in CSF was conducted using a validated direct competitive ELISA with an LLOQ of 20 ng/mL: Diluted samples were added on a 96-well plate coated with bovine serum albumin conjugated to methoxy-PEG. An anti-PEG antibody specific for the polyoxyethylene backbone and conjugated to HRP was used as detection reagent. PEG-60-Mal-Cys was used as the reference standard. Some samples were excluded from the analysis since they had a blood contamination level higher than 0.5%, which was shown to generate false-positive results during validation.

**Necropsy, histology, and immunohistochemistry.** A gross necropsy was performed for all animals, and organ weights were taken. Tissues were collected for histology and IHC in 10% neutral-buffered formalin. The number of organs and tissues weighed and the list of 46 organs and tissues per animal fixed and evaluated histologically closely followed the Guideline on Repeated Dose Toxicity. Histological evaluation was performed by a pathologist and peer-reviewed according to internal SOPs (SOPs).

Sections for IHC of brain with choroid plexus, kidneys, and spleen (weeks 13, 26, and 52) were prepared after fixation in 10% neutral-buffered formalin (2-7 days; approximately 5 μm thick), dehydration, and embedding in paraffin wax. From each animal, 4 unstained sections from each of the 3 organs were mounted on Super frost slides and air dried for at least 24 hours at room temperature.

Positive controls for IHC were sectioned from formalin-fixed, paraffin-embedded rat skeletal muscle injected postmortem with cell culture suspension containing untreated THP-1 human macrophages. The design of the positive controls was similar to the one described by Rasmussen et al. Tissue sections were deparaffinized and rehydrated, and for each IHC procedure antigen-blocking was performed to eliminate nonspecific binding by endogenous avidin/biotin and 10% normal goat serum for proteins. The primary antibody (Ab) used was a rabbit monoclonal anti-PEG Ab (PEG-B-47; Epitomic, CA, USA). The company indicates binding to the terminal methoxy group of conjugated PEG molecules, and Rudmann et al. have shown that this detection antibody also binds to unconjugated PEG. The optimal antibody concentration was determined to be 5 μg/mL. In order to confirm negative staining results at 5 μg/mL, the 2-fold concentration of 10 μg/mL antibody was applied on brain, spleen, and kidney sections from 5 of 20 controls and 5 of 17 high-dose animals from the 26-week test period. The secondary antibody was a link anti-rabbit antibody (HK326-UR; Biogenex, 49026 Milmont Drive, Fremont, CA 94538, USA) used per manufacturer recommendation, and labeling was performed with a streptavidin-alkaline phosphatase followed by development with the chromogen permanent AP-red solution and counterstaining with hematoxylin. Isotype-matched immunoglobulin from nonimmunized rabbit serum (rabbit IgG, IS600, Dako, 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA) matched by concentration was used in place of the primary antibody for negative controls. All steps were performed at room temperature, and incubation was performed in a humid chamber at Bayer AG, Germany. Peer reviews of the immunohistochemical data were conducted according to internal SOPs.

**Statistical Analysis**

Proportions and/or incidences were calculated to summarize categorical data as appropriate. Means and standard deviations were calculated, where appropriate. For clinical pathology data, analysis of variance was employed, comparing the groups treated with BAY 94-9027 to saline control (group 1).

**Results**

**Two-Week Toxicity Studies With BAY 94-9027**

The following briefly summarizes the results of the 2-week studies. The analysis of anti-FVIII antibodies confirmed that approximately 50% of rats and rabbits had developed nADAs.

---

**Table 4. Scheduled Clinical Pathology Time Points for Chronic Rat Study.**

| Numbers | Time Point | Hematology | Hemostasis | Clinical Chemistry | Urinalysis |
|---------|------------|-------------|------------|--------------------|------------|
| 10 rats/group, 13 week subset | At termination | X | X | X | X* |
| 20 rats/group, 26 week subset | Day 23 and 86 (24 ± 1 hours after dose) | / | X | / | / |
| 20 rats/group, 26 week subset | At termination | X | X | X | X* |
| 15 rats/group, recovery subset | At termination | X | X | X | X* |

Abbreviations: X, sample collected; /, no samples taken. * Samples collected from cages within the week prior to euthanasia.
after 2 weeks, while animals treated with full-length FVIII in concurrent dose groups had a higher frequency of close to 100%. This confirmed that a longer duration of the studies would not be meaningful since the nADAs abolish exposure to active drug.

Rats and rabbits dosed IV with BAY 94-9027 every other day at 75, 750, and 2250 IU/kg for 2 weeks tolerated BAY 94-9027 without any toxicity. No treatment-related mortality, effects on general behavior, or relevant changes in organ weights or histopathologic changes were observed. Specifically, no PEG-related cellular vacuolation in any of the >40 organs and tissues evaluated per animal was seen. In rabbits, a prolongation of the activated partial thromboplastin time (aPTT) was recorded after 2 weeks of dosing in the 750 (153% of controls) and 2250 IU/kg (166%) dose groups. This was also seen at the end of the recovery period. Most likely, the prolongation is due to the development of inhibitory antibodies (nADAs) that cross-reacted with rabbit FVIII, causing the prolongation of aPTT that persisted throughout the recovery period. No anti-PEG antibodies were detected in rats or rabbits. The no-adverse effect level (NOAEL) is the highest dose of 2250 IU/kg given every other day. No test article-related adverse effects were seen in juvenile rats (first dose at PND 17) up to the highest dose of 1000 IU/kg given twice weekly for 2 weeks.

Four-Week Toxicity Studies With PEG 60 kDa

PEG-60-Mal-Cys was well tolerated after single and repeat administration up to very high doses (Table 2). In none of the studies conducted, treatment-related mortality or effects on general behavior or organ weights were observed. Up to the highest doses tested, no evidence of any organ-specific or other toxicities were seen. Specifically, no tissue vacuolation in histopathologic evaluations was seen after 4 weeks of high doses of PEG-60-Mal-Cys in rats and rabbits.

A juvenile animal study was conducted with PEG-60-Mal-Cys with the first IV dose on PND 17, dosed twice weekly for 4 weeks. No toxicologically relevant effects were observed for PEG-60-Mal-Cys up to 2 mg/kg, the highest doses tested in these young animals.

Chronic Toxicity Study With BAY 94-9027

The IDF rat model has been used previously to evaluate other coagulation factors such as FVIII and FIX. This rat strain has severely impaired thymus-related immunoreactions and T-cell development, preventing ADA and nADA formation as shown for FVIII or FIX. The number of granulocytes, monocytes/macrophages, erythrocytes, B cells, and natural killer (NK) cells in the blood of IDF rats were usually within the normal range; the innate immune system in IDF rats is well developed. There is indication that the IDF rat strain has a reduced survival time likely due to pathogen-free housing requirements with a reported life span of 1.5 years. Also, detailed mortality data and histological background lesion information are not available since this model is usually not used for toxicology studies.

Results of In-life Assessment. The following summarizes the study results. No BAY 94-9027-related observations were recorded during in-life or recovery phase up to the highest dose of 1200 IU/kg twice weekly. There were no treatment-related changes in body weights, body weight gains, or food consumption (data not shown). In the highest dose group, 4 of 45 animals per group died or were killed moribund within 52 weeks (end of recovery phase), while in the other groups 1 or 2 deaths were recorded (Table 5). For 2 of the 8 animals that died, no clear cause of death could be determined (animal number 78 and 136). Findings such as paraphimosis (1 rat in group 4), kidney pathology (1 rat in group 4), and lymphoma (2 rats in group 4) are known to also occur in other rat strains as background changes, especially in older rats. It is likely that the IDF rat strain can develop these pathologies and that the mortality seen at the high dose is likely unrelated to treatment with BAY 94-9027.

Clinical Chemistry, Hematology, Coagulation, and Urinalysis. No changes were seen in any of the hematology, clinical chemistry, or urinary parameters. The only effect measured after administration of BAY 94-9027 was limited to a transient minimal decrease in the aPTT, mainly at the highest dose on days 23 and 86. This was not noted at subsequent time points. A reduction in aPTT is an expected pharmacological effect of BAY 94-9027. There were no BAY 94-9027-related alterations in
clinical chemistry parameters. There were no BAY 94-9027-related alterations in urinalysis parameters.

Toxicokinetics and Antidrug Antibody Evaluation. The goal of the TK assessment of BAY 94-9027 was to demonstrate exposure and identify animals that may have had low/no exposure due to antibodies to rFVIII. The data were supported by the ADA analysis. The nADAs were not measured since ADAs together with TK already indicates neutralization of exposure. After 13 and 26 weeks, the lowest dose of 40 IU/kg was mostly below limit of quantitation of the assay. At the higher dose groups, all animals showed exposure except one rat in the mid-dose group. This animal also was the only animal with ADAs. Group means are listed in Table 6.

Analysis of PEG in Plasma. The analysis of PEG in plasma showed that only 1 sample from each of the week 13 and week 26 time points had measurable PEG levels in the group 2 (40 IU/kg) animals (21.1 and 24.5 ng/mL), and all other group 2 samples were below the LLOQ (20 ng/mL). Mean concentrations of PEG in plasma increased with dose in the group 3 (400 IU/kg) and group 4 (1200 IU/kg) animals as presented in Table 7. No PEG was measurable in plasma after 26 treatment-free weeks.

Polyethylene Glycol in CSF. All CSF samples analyzed for PEG had values below the method LLOQ of 20 ng/mL.

Macroscopic and Microscopic Pathology. No BAY 94-9027-related macroscopic or microscopic findings were noted after 13 and 26 weeks or at the end of the recovery phase. The macroscopic findings observed were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence in control and treated animals and, therefore, were considered unrelated to administration of BAY 94-9027. No test item-related organ weight changes were seen in any of the organs evaluated at the 3 necropsy time points.

No BAY 94-9027-related microscopic findings were noted (Figure 1). Only high-dose and control animals were evaluated by histology since no BAY 94-9027-related findings were seen that would have triggered an evaluation of the other dose groups.

Cellular vacuolation seen in some animals of all groups including those in the control group in cortical epithelial cells in the adrenal glands, tubules of the kidneys, and hepatocytes was considered normal morphologic variability for these tissues and not attributed to PEG.

In the brains of some animals including controls, free erythrocytes, pigmented macrophages (golden-brown/brown), and/or erythrophagocytosis were seen primarily in the third ventricular space and meninges and were considered related to the terminal cerebral spinal fluid collection at necropsy. At the end of the recovery period, no BAY 94-9027-related microscopic findings were noted.

Immunohistochemistry for PEG Detection. No positive immunoreaction for PEG was present in the following tissues examined: brain (choroid plexus [lateral, third, and fourth ventricle], cerebral cortex, corpus callosum, globus pallidus, hippocampus, thalamus, hypothalamus, pyramids, cerebellum, trigeminal nuclei and tracts, lateral [dentate] cerebellar nuclei, medulla oblongata, circumventricular organs and pineal gland), spleen, and kidney (Figure 2). In positive controls, a strong immunopositive reaction for PEG was observed in the expected structures (Figure 3). Negative controls were negative (data not shown).

### Table 6. Toxicokinetics: Summary of Mean Plasma Concentrations (Geometric Mean and SD) of BAY 94-9027 After 11 and 24 Weeks of Dosing (Each Value is Comprised of 3 [Week 11] or 5 [Week 24] Rats Per Time Point).

| Time Points After Dosing | 40 IU/kg (Week 11) | 400 IU/kg (Week 11) | 1200 IU/kg (Week 11) | 40 IU/kg (Week 24) | 400 IU/kg (Week 24) | 1200 IU/kg (Week 24) |
|--------------------------|-------------------|-------------------|---------------------|------------------|------------------|---------------------|
| IU/L                     | IU/L              | IU/L              | IU/L                | IU/L             | IU/L             | IU/L                |
| 0                        | /                 | /                 | /                   | /                | /                | /                   |
| 0.25                     | 692 (1.27)        | 8090 (1.12)       | 27 200 (1.08)       | /                | 3090 (6.23)      | 27 000 (1.07)       |
| 4                        | ND                | ND                | ND                  | /                | 8420 (1.14)      | 12 500 (4.53)       |
| 8                        | /                 | 4940 (1.11)       | 5740 (7.91)         | /                | 4020 (1.99)      | 10 900 (4.50)       |
| 24                       | /                 | 2530 (1.36)       | 7960 (1.14)         | /                | 3030 (5.55)      | 14 900 (2.01)       |
| 48                       | ND                | ND                | ND                  | /                | 1060 (1.21)      | /                   |
| 72                       | /                 | /                 | /                   | /                | /                | /                   |

Abbreviations: ND, not determined; /, below LLOQ; LLOQ, lower limit of quantitation; SD, standard deviation.

### Table 7. Plasma Levels of Free PEG After 13 and 26 Weeks.

| Group | Time Point | Mean ± SD, ng/mL | Number of Samples |
|-------|------------|------------------|------------------|
| 2     | Week 13    | Below LLOQ       | 10               |
|       | Week 26    | Below LLOQ       | 19               |
|       | End of recovery | Below LLOQ     | 15               |
| 3     | Week 13    | 122 ± 15         | 10               |
|       | Week 26    | 127 ± 33         | 20               |
|       | End of recovery | Below LLOQ   | 12               |
| 4     | Week 13    | 399 ± 52         | 10               |
|       | Week 26    | 473 ± 57         | 17               |
|       | End of recovery | Below LLOQ | 14               |

Abbreviations: LLOQ, lower limit of quantitation; SD, standard deviation.
Figure 1. Histopathological evaluation after 13 and 26 weeks of treatment or at the end of the 26 weeks recovery period noted no BAY 94-9027-related microscopic findings up to the highest dose of 1200 IU/kg in any of the more than 40 organs or tissues evaluated per animal. Brain with the choroid plexus epithelia (A and B), spleen (C and D), and kidneys (E and F) from rats administered BAY 94-9027 at 1200 IU/kg twice weekly (B, D, and F) showed no indication of test item-related cellular vacuolation when compared to the controls given the vehicle (A, C, and E). Note the golden brown pigment in macrophages unrelated to BAY 94-9027 present in both controls and animals given BAY 94-9027 (C and D). Hematoxylin and eosin (H&E), original scan ×20.
Biotherapeutics is difficult. Although comparing data between different PEGylated biopharmaceuticals, vacuoles were related to cellular vacuolation seen in toxicology studies with some PEGylated molecules.13,21 Recently published nonclinical studies and reviews with molecules of different PEG sizes either unbound18 or conjugated to drug protein13,14,19 have shown that as long as vacuolation is not associated with pathologic changes like tissue degeneration, inflammation, necrosis, and cellular distortion related to vacuolation, or changes in study end points including hematology, clinical chemistry, urinalysis, or organ weight vacuolation, cellular vacuolation is not considered to be adverse. It was shown that cellular vacuolation was dose and time dependent when testing either unconjugated or conjugated PEG molecule13,18 and seen at higher doses than present in BAY 94-9027, which is in the microgram/kg range.14 Although comparing data between different PEGylated biotherapeutics is difficult.

Rudmann et al18 evaluated very high IV doses of linear unconjugated PEG 10, 20, or 40 kDa. Normalized to a weekly dose the PEG doses were 10 kDa—700 mg/kg/week; 20 kDa—350 mg/kg/week; and 40 kDa—200 mg/kg/week. The 3-month study in rats showed that the molecular weight of PEG influenced both the tissue distribution and the vacuolation. For 10 and 20 kDa PEG, PEG immunoreactivity was most prominent in the renal tubule epithelium, in alveolar macrophages, and hepatic Kupffer cells, while cellular vacuolation was absent. Rats given 40 kDa PEG had strong PEG immunoreactivity in splenic subcapsular red pulp macrophages, renal interstitial macrophages, and choroid plexus epithelial cells frequently associated with cellular vacuolation. No PEG IHC staining or vacuolation was detected in the brain parenchyma, suggesting that even at very high PEG doses the choroid plexus acts as a barrier between the blood and the ventricular CSF for PEGs. It is not known how far data from unconjugated PEG can be accurately extrapolated to PEGylated biopharmaceutics since vacuolation, distribution and excretion patterns of PEGylated biotherapeutics may depend more on drug receptor/target-mediated cellular uptake or ligand-specific transport mechanisms, and therefore organs or tissues with vacuolation and the time and dose when this occurs can vary.

In an industry survey summarizing data from 17 PEGylated biopharmaceutics under development in 2013 found that vacuolation of the choroid plexus was seen also with 20, 30, and 40 kDa site-specific-PEGylated proteins.13 Again, no PEG was detected in the brain parenchyma except in one case where the PEGylated molecule was designed to cross the blood–brain barrier (development of this molecule was stopped).13

**Discussion**

**Safety of Large PEG Molecules Used in PEGylated Biopharmaceuticals**

Concerns have been raised about the detection of PEG-related cellular vacuolation seen in toxicology studies with some PEGylated biopharmaceuticals.13,21 Vacuoles were observed by histopathology in cells of the RES but also in some studies in pivotal organs such as kidneys and the choroid plexus.13–16,18,21 Recently published nonclinical studies and reviews with molecules of different PEG sizes either unbound18 or conjugated to drug protein13,14,19 have shown that as long as vacuolation is not associated with pathologic changes like tissue degeneration, inflammation, necrosis, and cellular distortion related to vacuolation, or changes in study end points including hematology, clinical chemistry, urinalysis, or organ weight vacuolation, cellular vacuolation is not considered to be adverse. It was shown that cellular vacuolation was dose and time dependent when testing either unconjugated or conjugated PEG molecule13,18 and seen at higher doses than present in BAY 94-9027, which is in the microgram/kg range.14 Although comparing data between different PEGylated biotherapeutics is difficult.

**Long-term Safety of PEGylated Coagulation Factors**

PEGylated rFVIII (turoctocog alfa pegol, N8-GP) with a 40 kDa branched PEG and PEGylated FIX (Refixia, nonacog beta pegol, N9-GP) using the same 40 kDa PEG were tested in the IDF chronic rat model.29,30 With turoctocog alfa pegol, no cellular vacuolation was detected after 26 or 52 weeks of every fourth day dosing up to 1200 IU/kg (containing up to 0.024 mg PEG/kg/dose). In the chronic study with Refixia with 6 and 26 weeks of treatment, no light microscopic vacuolation was reported by the authors, but PEG was detectable in the choroid plexus and spleen macrophages by IHC. The Refixia doses given every fifth day were 40 IU/kg (clinical dose; 0.2 mg PEG/kg/dose), 150 IU/kg (0.8 mg PEG/kg/dose), 600 IU/kg (2.7 mg PEG/kg/dose), and 1200 IU/kg (6.4 mg PEG/kg/dose). At the low dose of 40 IU/kg, IHC was positive for PEG in choroid plexus of about 50% of examined animals after 26 weeks, while the high dose of 1200 IU/kg showed positive IHC after 6 weeks of treatment.

N9-GP, turoctocog alfa pegol (N8-GP), and BAY 94-9027’s distribution to the blood stream as blood coagulation proteins may differ to many of the other PEGylated biopharmaceautics, which usually have a drug target receptor or bind in organs/issues. Factor VIII and also BAY 94-9027 are usually bound to van Willebrand Factor (vWF) in circulation. The only receptors known for FVIII are clearance receptors in the liver although the pathways that regulate the clearance of vWF-bound and vWF-free FVIII are not well characterized.34,35 It is likely that BAY 94-9027 including its PEG-60 will be removed by the liver, the protein part is degraded intracellularly, while PEG 60 kDa remains and is excreted or recirculated.24 It was shown that unconjugated PEG-60-Mal-Cys is excreted mainly via the kidney.24

It can be assumed that a small amount of PEG 60 kDa from BAY 94-9027 may be taken up also in the liver as was shown with PEG 40 kDa16 for N8-GP and may be excreted in bile although this has not been confirmed. The small difference in organ distribution seen between conjugated and unconjugated PEG 40 kDa did not have any impact on the liver function or morphology as seen from the nonclinical studies since no vacuolation in any of the liver cell types including Kupffer cells occurred with BAY 94-9027, turoctocog alfa pegol, and Refixia in the chronic IDF rat model.

The PEG doses in Refixia in the chronic rat study were distinctly higher than those tested here for BAY 94-9027. They were a multiple of 100 to 3200 over a clinical dose of 0.2 mg PEG/kg/dose. IHC was positive for PEG in choroid plexus of about 10% of evaluated animals after 26 weeks, while the high dose of 3200 IU/kg (12.8 mg PEG/kg/dose) showed positive IHC after 52 weeks of treatment.

Safety of BAY 94-9027

The toxicology program for BAY 94-9027 evaluated the systemic, juvenile, long-term, and genotoxic properties of
BAY 94-9027 or its PEG 60 kDa moiety. There was no thrombus formation in rats or rabbits up to the highest dose of 2250 IU/kg (>30 times the maximal human dose) in the 2-week studies. Thrombus formation or other reactions in the coagulation system could be a potential effect at high doses when FVIII is activated to FVIIIa. In the systemic and juvenile toxicity

Figure 2. In the immunohistochemical evaluation, no PEG immunoreactivity could be detected after 13 weeks, 26 weeks, or at the end of the 26 weeks treatment-free period in the brain and in the choroid plexus epithelial cells (A and B), spleen (C and D), and kidneys (E and F) from rats administered vehicle (A, C and E) or BAY 94-9027 (B, D, and F). Note the brown background staining in macrophages unrelated to BAY 94-9027 present in both controls and animals given BAY 94-9027 (C and D). Immunohistochemistry (IHC), original scan 20×.
studies with BAY 94-9027 or PEG-60-Mal-Cys, there was no cellular vacuolation and no toxicity seen.

The chronic toxicity of BAY 94-9027 was assessed in the IDF rat. These animals mount a no humoral immune response to species-foreign proteins such as FVIII. They can therefore be treated longer than immune-competent animals although due to the special housing requirements (pathogen free) and the sensitivity to infections, this model can only be utilized on a case-by-case basis.

No signs of toxicity were seen with BAY 94-9027 in the chronically treated IDF rats with twice-weekly dosing of up to 1200 IU/kg for 26 weeks. The number of animals that died in the highest dose group of 1200 IU/kg (4 out of 45 over the course of 52 weeks on study) was slightly higher than in the other dose groups or control (1 or 2 animals out of 45). These deaths are not attributed to treatment with BAY 94-9027. All animals that died in the highest dose group showed pathologies that can be attributed to background changes. It is concluded that there was no BAY 94-9027-related mortality, adverse clinical observations, body weight or food consumption changes, or ophthalmology observations. No toxicologically important differences were noted in hematology, clinical chemistry, or urinalysis parameters. There were no BAY 94-9027-related macroscopic or microscopic findings or organ weight changes at weeks 13 and 26 and through the recovery portion of the study. No PEG-related cellular vacuolation was seen in any of the > 40 organs and tissues (including macrophages) evaluated histologically. Toxicokinetic assessment revealed good exposure in all animals except 1 animal dosed with 400 IU/kg that had also a positive ADA titer.

There was no accumulation of PEG in pivotal organs (brain including choroid plexus, kidney, or spleen) as demonstrated by the lack of a positive immunoreaction to anti-PEG monoclonal antibody by IHC. Cerebrospinal fluid samples analyzed from all time points of all dose groups with a sensitive assay were negative for PEG supporting the IHC results. The concentrations of PEG in plasma were not measurable in the low-dose group (40 IU/kg) and increased with dose in the group 3 (400 IU/kg) and group 4 (1200 IU/kg) rats. In the highest dose group, the mean PEG concentration measured in plasma 72 hours after last dose on week 26 was 473 ± 57 ng/mL in the rats. No PEG was measured in plasma after the 26-week treatment-free period.

Toxicokinetics of BAY 94-9027 and PEG in plasma in the IDF rats were comparable to the results from the experiments with euthymic rats (data not shown), indicating no relevant differences in exposure to BAY 94-9027 between the different strains. It is reported that the number of granulocytes, monocytes/macrophages, erythrocytes, B cells, and natural killer (NK) cells in the blood of IDF rats were usually within the normal range; the innate immune system in IDF rats is well developed. It can be concluded that macrophage function and the role of the RES in PEG clearance are likely comparable between IDF rats and immunocompetent animals and that the chronic study with BAY 94-9027 is an adequate model for long-term treatment and risk assessment for humans.

In combining sensitive methods evaluating potential PEG presence in organs and tissues (IHC), and evaluating the plasma and CSF concentrations in combination with standard H&E histopathology technique, the chronic study showed that BAY 94-9027 did not induce PEG-related changes at multiples of the human dose after chronic treatment.

Conclusions
The nonclinical toxicology program supports that BAY 94-9027 is safe when given on demand and prophylactically as
chronic treatment. There were no toxicities seen related to the rFVIII protein or the PEG moiety in any of the studies. Specifically, no cellular vacuolation or PEG accumulation was detectable up to high multiples of the clinical dose and after chronic dosing. The NOAEL in the chronic rat toxicity study was the highest dose of 1200 IU/kg or 0.04 mg PEG/kg given twice per week.

Acknowledgments
We are grateful to Dr. Claudia Stark for her toxicology expertise, critical review and helpful comments to the manuscript. Further, we thank the many people who contributing to the experimental data.

Declaration of Conflicting Interests
The author(s) declared no potential, real, or perceived conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD
Inge A. Ivens https://orcid.org/0000-0001-5506-363X

References

1. Mei B, Pan C, Jiang H, et al. Rational design of a fully active, long-acting PEGylated factor VIII for hemophilia a treatment. Blood. 2010;116(2): 270–279.

2. Reding MT, Ng HJ, Poulsen LH, et al. Safety and efficacy of BAY 94-9027, a prolonged-half-life factor VIII. J Thromb Haemost. 2017;15(3): 411–419.

3. Konkle BA, Staslyshyn O, Chowdary P, et al. Pegylated, full-length, recombinant factor VIII for prophylactic and on-demand treatment of severe hemophilia A. Blood. 2015;126(9):1078–1085.

4. Mullins ES, Staslyshyn O, Alvarez-Roman MT, et al. Extended half-life pegylated, full-length recombinant factor VIII for prophylaxis in children with severe haemophilia A. Haemophilia. 2017;23(2):238–246.

5. Mahlangu J, Powell JS, Ragni MV, et al. Phase 3 study of recombinant factor VIII Fc fusion protein in severe hemophilia A. Blood. 2014;123(3): 317–325.

6. Young G, Mahlangu J, Kulkarni R, et al. Recombinant factor VIII Fc fusion protein for the prevention and treatment of bleeding in children with severe hemophilia A. J Thromb Haemost. 2015;13(6):967–977.

7. Coyle TE, Reding MT, Lin JC, Michaels LA, Shah A, Powell J. Phase I study of BAY 94-9027, a PEGylated B-domain-deleted recombinant factor VIII with an extended half-life, in subjects with hemophilia A. J Thromb Haemost. 2014;12(4):488–496.

8. Shah A, Solms A, Wiegmann S, et al. BAY 94-9027 and recombinant factor VIII Fc fusion protein: a head-to-head, randomized, crossover, pharmacokinetic study in patients with severe haemophilia A. Haemophilia. 2019; in review.

9. Shah A, Coyle T, Lalezari S, Fisher K, et al. BAY 94-9027, a PEGylated recombinant factor VIII, exhibits a prolonged half-life and higher area under the curve in patients with severe haemophilia A: Comprehensive pharmacokinetic assessment from clinical studies. Hemophilia. 2018; 24(5):733–740.

10. Reding MT, Ng HJ, Tsenekidou-Stoefer D, Linardi C, Lalezari S. Safety of long-term prophylaxis with BAY 94-9027: Interim results of >5 years of treatment in the PROTECT VIII extension trial. Haemophilia. 2018; 24(Suppl 5):W-P-001 (404).

11. US Food and Drug Administration. 2016. ADYNOVATE Prescribing Information. Accessed February 2019. https://www.fda.gov/downloads/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/UCM472594.pdf.

12. US Food and Drug Administration. 2018. JIVI Antithemophilic Factor (Recombinant) PEGylated-aucl. Prescribing Information. Accessed February 2019. https://www.fda.gov/downloads/BiologicsBloodVaccines/UCM618979.pdf.

13. Ivens IA, Achanzar W, Baumann A, et al. PEGylated biopharmaceuticals: current experience and considerations for nonclinical development. Toxicol Pathol. 2015;43(7):959–983.

14. Ivens IA, Baumann A, McDonald TA, Humphries TJ, Michaels LA, Mathew P. PEGylated therapeutic proteins for haemophilia treatment: a review for haemophilia caregivers. Haemophilia. 2013;19(1):11–20.

15. Turecek PL, Bossard MJ, Schoetens F, Ivens IA. PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. J Pharm Sci. 2016;105(2):460–475.

16. Stid R, Fuchs S, Bossard M, Siekmann J, Turecek PL, Putz M. Safety of PEGylated recombinant human full-length coagulation factor VIII (BA 855) in the overall context of PEG and PEG conjugates. Haemophilia. 2016;22(1):54–64.

17. Webster R, Didier E, Harris P, et al. PEGylated proteins: evaluation of their safety in the absence of definitive metabolism studies. Drug Met Dispos. 2007;35(1):9–16.

18. Rudmann DG, Alston JT, Hanson JC, Heidel S. High molecular weight polyethylene glycol cellular distribution and PEG-associated cytoplasmic vacuolation is molecular weight dependent and does not require conjugation to proteins. Toxicol Pathol. 2013;41(7):970–983.

19. Rovira ARI, Bennet BM, Bolon B, et al. Scientific and Regulatory policy committee points to consider: histopathologic evaluation in safety assessment Studies for PEGylated Pharmaceutical Products. Toxicol Pathol. 2018;46(6):616–635.

20. Bendele A, Seeley J, Richey C, Sennello G, Shopp G. Short communication: renal tubular vacuolation in animals treated with polyethylene-glycol-conjugated proteins. Toxicol Sci. 1998;42(2):152–157.

21. EMA CHMP Safety Working Party’s response to the Paediatric Committee (PDCO) regarding the use of PEGylated drug products in the paediatric population: November 16, 2012, EMA/CHMP/SWP/647258/2012. Accessed May 15, 2019. https://www.ema.europa.eu/en/documents/scientific-guideline/ema-chmp-swp-pedo-reason-use-pediatric-drug-products_en.pdf.

22. Frazier KS. Antisense oligonucleotide therapies: the promise and the challenges from a toxicologic pathologist’s perspective. Toxicol Pathol. 2015; 43(1):78–89.

23. EMA CHMP Guideline on repeated dose toxicity. 18 March 2010. http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/default.jsp&mid=WC0b01ac058001da73.

24. Baumann A, Piel I, Hucke F, Sandmann S, Hetzel T, Schwarz T. Pharmacokinetics, excretion, distribution, and metabolism of 60-kDa polyethylene glycol used in BAY 94-9027 in rats and its value for human prediction. Eur J Pharm Sci. 2019;130:11–20.

25. US Animal Welfare Act, Public Health Service Policy on Humane Care and Use of Laboratory Animals, Guide for the Care and Use of Laboratory animals and American Veterinary Medical Association Guidelines on Euthanasia.

26. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH M3(R2) Guideline on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals September 2010. Accessed May 15, 2019. https://www.ich.org/fileadmin/Public_Web_Site/ICHSite/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2__Guideline.pdf.

27. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use ICH S6(R1) Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals June 2011. Accessed May 15, 2019. https://www.ema.europa.eu/en/documents/scien
28. German Animal Welfare Law ("Tierschutzgesetz" in line with the "European convention for the protection of vertebrate animals used for experimental and other scientific purposes" (European Treaty Series – No. 123, 1986)).

29. Rasmussen CE, Nowa J, Larsen JM, Bottomley A, Rowles A. Offenberg H. Evaluation of nonacog beta pegol long-term safety in the immune-deficient Rowett Nude Rat (Crl: NIH-Foxn1nu). *Toxicol Pathol*. 2016; 44(5):726–737.

30. Rasmussen CE, Nowak J, Larsen JM, et al. Long-term safety of PEGylated coagulation factor VIII in the immune-deficient Rowett Nude Rat. *J Toxicol*. 2017;2017. doi: 10.1155/2017/:8496246.

31. Hougen HP. The athymic nude rat Immunobiological characteristics with special reference to establishment of non-antigen-specific T-cell reactivity and induction of antigen-specific immunity. *APMIS*. 1991;99(Suppl 21): 9–39.

32. Rolstad B. The athymic nude rat: an animal experimental model to reveal novel aspects of innate immune responses? *Immunol Rev*. 2001;184(1): 136–144.

33. Schuurman HJ. The nude rat. *Hum Exp Toxicol*. 1995;14(1):122–125.

34. Lenting PJ, Van Schooten CJM, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. *J Throm Haemos*. 2007;5(7): 1353–1360.

35. Swystun LL, Notley C, Georgescu I, et al. The endothelial lectin clearance receptor CLEC4 M binds and internalizes factor VIII in a VWF-dependent and independent manner. *J Thromb Haemost*. 2019;17(4):681–694.

36. Björnsdóttir I, Sternebring O, Kappers WA, et al. Pharmacokinetics, tissue distribution and excretion of 40 kDa PEG and PEGylated rFVIII (N8-GP) in rats. *European J Pharmaceut Sci*. 2016;87:58–68.