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Safety of PEGylated recombinant human full-length coagulation factor VIII (BAX 855) in the overall context of PEG and PEG conjugates

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Introduction: BAX 855 is a PEGylated human full-length recombinant factor VIII (rFVIII) based on licensed rFVIII (ADVATE). The applied PEGylation technology has been optimized to retain functionality of the FVIII molecule, improve its pharmacokinetic properties and allow less frequent injections while maintaining efficacy. **Aim:** The aim of this study was to confirm that the excellent safety profile of ADVATE remains unchanged after PEGylation. **Methods:** Non-clinical safety studies with BAX 855 and its respective unbound polyethylene glycol (PEG) were conducted in several species. The distribution of a single dose of radiolabelled BAX 855 was further investigated in rats. Publically available safety data on PEG alone and PEGylated biomolecules were summarized and reviewed for specific safety findings attributable to PEG or PEGylated biopharmaceuticals. **Results:** Safety pharmacology studies in rabbits and macaques and repeated dose toxicity studies in rats and macaques identified no safety issues. Results of a distribution study in rats administered radiolabelled BAX 855 showed that radioactivity was completely excreted; urine was the major elimination route. A 28-day study in rats dosed with the unbound PEG constituent (PEG2ru20KCOOH) of BAX 855 showed no adverse or non-adverse effects. Safety data for PEG and PEG-protein conjugates indicate no safety concerns associated with PEG at clinically relevant dose levels. Although vacuolation of certain cell types has been reported in mammals, no such vacuolation was observed with BAX 855 or with the unbound PEG constituent. **Conclusion:** Non-clinical safety evaluation of PEG and BAX 855 identified no safety signals; the compound is now in clinical development for the treatment of patients with haemophilia A.

Keywords: BAX 855, conjugation, factor VIII, haemophilia, PEG, safety

Introduction

Polypeptide biopharmaceuticals are an emerging branch of therapeutics. The biotechnology revolution has made it possible to produce highly purified polypeptides in large quantities. Development of polypeptide therapeutics, however, may bring challenges, due to the susceptibility to proteolysis, their short shelf- or half-life, low solubility, rapid elimination and their potential to cause specific immune responses [1,2]. One strategy to minimize such properties is conjuga-

tion with an inert and hydrophilic macromolecule that does not alter the safety or efficacy profile of the parent molecule. Clinical experience over the last decades has shown that polyethylene glycol (PEG) is a suitable polymer for this purpose; chemical conjugation with PEG to improve the pharmacokinetic (PK) profile of a biopharmaceutical is generally known as PEGylation [2,3]. PEGylation has been shown to improve the properties of native proteins by reducing renal elimination, providing steric shielding against immunological recognition, and decreasing receptor-mediated clearance or enzymatic attack. Today, PEGylation is the dominant technique to improve properties of a biopharmaceutical by polymer conjugation [4,5]. Numerous PEGylated biopharmaceuticals for parenteral administration are currently approved for use in humans or are in clinical development. The first approved PEGylated biotherapeutic was Adagen (Enzon, 1990); the most recently approved PEGylated biopharmaceutical is Plegridy (Biogen Idec, 2014)

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[2,6]. PEGylated biopharmaceuticals, either commercially available or in clinical development, are modified with PEG polymers of 5–60 kDa in size. Many PEGylated drug candidates are currently in various phases of development, including several indicated for the treatment of haemophilia A. Bayer and Novo Nordisk have developed PEGylated variants of recombinant B-domain deleted rFVIII (BDDrFVIII), which are currently under evaluation in clinical phase III trials. Bayer's BAY 94-9027 is a BDDrFVIII that carries an engineered amino acid sequence to allow cysteine-directed PEGylation with a 60 kDa branched PEG [7]. GlycoPEGylated FVIII (N8-GP) developed by Novo Nordisk is a BDDrFVIII with a truncated B-domain that is modified with a branched 40 kDa PEG on O-linked glycans using enzymatic glycoconjugation technology [8]. In contrast, Baxalta's BAX 855 is a full-length rFVIII conjugated with 20 kDa branched PEG molecules that consist of two chains, each 10 kDa in size. BAX 855 is synthesized using a proprietary PEGylation methodology developed by Nektar Therapeutics (Huntsville, AL, USA) that targets the epsilon-amino groups of lysines [9]. BAX 855 contains the same original, native full-length rFVIII protein used in ADVATE (rFVIII, Recombinant Antihemophilic Factor, Plasma/Albumin-Free Method).

The aim of PEGylated rFVIII variants was to prolong FVIII activity (i.e. increase the half-life), which would allow for less frequent infusion (or higher trough levels) while maintaining efficacy of the parent rFVIII product. The benefits of longer half-life, however, must not be compromised by introducing an increased safety risk attributable to combination with a chemical polymer. The safety of PEG and PEGylated products has been demonstrated in numerous studies [10–19].

In developing BAX 855, the applied PEGylation technology was optimized to retain functionality of the FVIII molecule, improve its PK properties and provide additional therapeutic options in the personalization of haemophilic care. In addition, non-clinical studies conducted by Baxalta, have demonstrated that the excellent safety profile established for ADVATE remains unchanged for the PEGylated rFVIII product, BAX 855. The safety evaluation of BAX 855 was based on data from animal model studies with BAX 855, and published data on PEGylated biopharmaceuticals extracted to determine whether chemical modification with PEG impaired the safety profile of parent drug molecules. Data from a repeated dose toxicity study with an unbound PEG-acid (PEG2ru20KCOOH) most relevant to BAX 855 as it would be liberated from the conjugate after 100% catabolism of the protein were also evaluated. The hypothetical elimination pathways of BAX 855 and PEG are shown in Fig. 1.

The toxicity study with PEG2ru20KCOOH performed in the context of preclinical development of BAX 855 contributed to the publically available safety

reports on PEG. Published information on distribution, metabolism and excretion, which are highly relevant to the safety of PEG and PEGylated biopharmaceuticals, are also described. As a whole, these comprehensive data aim to demonstrate the safety of BAX 855.

Distribution, metabolism and excretion of unbound PEG, PEGylated biopharmaceuticals and BAX 855

Distribution, metabolism and excretion from the organism are important determinants for the safety of a compound. PEG is the acronym for a chemical family of uncharged polyethers consisting of repeated units of ethylene oxide and having amphiphilic properties and very low chemical reactivity; only the terminal hydroxyl groups of the molecule may be accessible for covalent bonding. As with the PEG used for conjugation to rFVIII in BAX 855, PEGs are frequently modified at the chain terminus, rendering them less reactive (methoxy-PEG, mPEG; endcapped PEGs). The pharmacokinetics, metabolism and distribution of PEG after parenteral application were recently discussed in detail by Baumann *et al.* [20]. Briefly, PEG was shown to be readily distributed and excreted in several animal species. PEG size is an important determining factor for circulation time and excretion pathway [21]. Some reports referring to enzymatic metabolism of PEG molecules via alcohol (ADH) and aldehyde dehydrogenase might be misinterpreted [22,23]. Because PEG represents a family of compounds with large variations in terminal groups and size, it is important to distinguish literature covering PEGs with free hydroxy groups from references discussing endcapped PEGs used to modify pharmaceuticals. Still, PEG may be metabolized at its terminal ends, but the ratio is rendered negligible due to modification of the polyethylene glycol chain ends with, e.g. methoxy-capping [20]. Ether linkages connecting ethylene glycol subunits in the PEG chain are highly stable *in vivo* due to the absence of etherases in eukaryotes. Nevertheless, the formation of PEG peroxides *in vitro* is known to occur when PEG solutions are stored in the presence of oxygen [24]. PEG chains may be cleaved by free radicals *in vivo* after phagocytosis of PEG or PEG-containing particles when reactive oxygen species are present in the (phago)lysosome, but at a minor rate, with no detectable toxicological relevance [24].

For non-glycosylated globular proteins, the threshold for glomerular filtration is approximately 70 kDa [24]. PEG molecules differ from globular proteins as PEG chains are highly hydrated, which amplifies the hydrodynamic radius of PEG in aqueous solutions. Furthermore, the flexible and linear PEG molecules are able to migrate through glomerular pores despite their

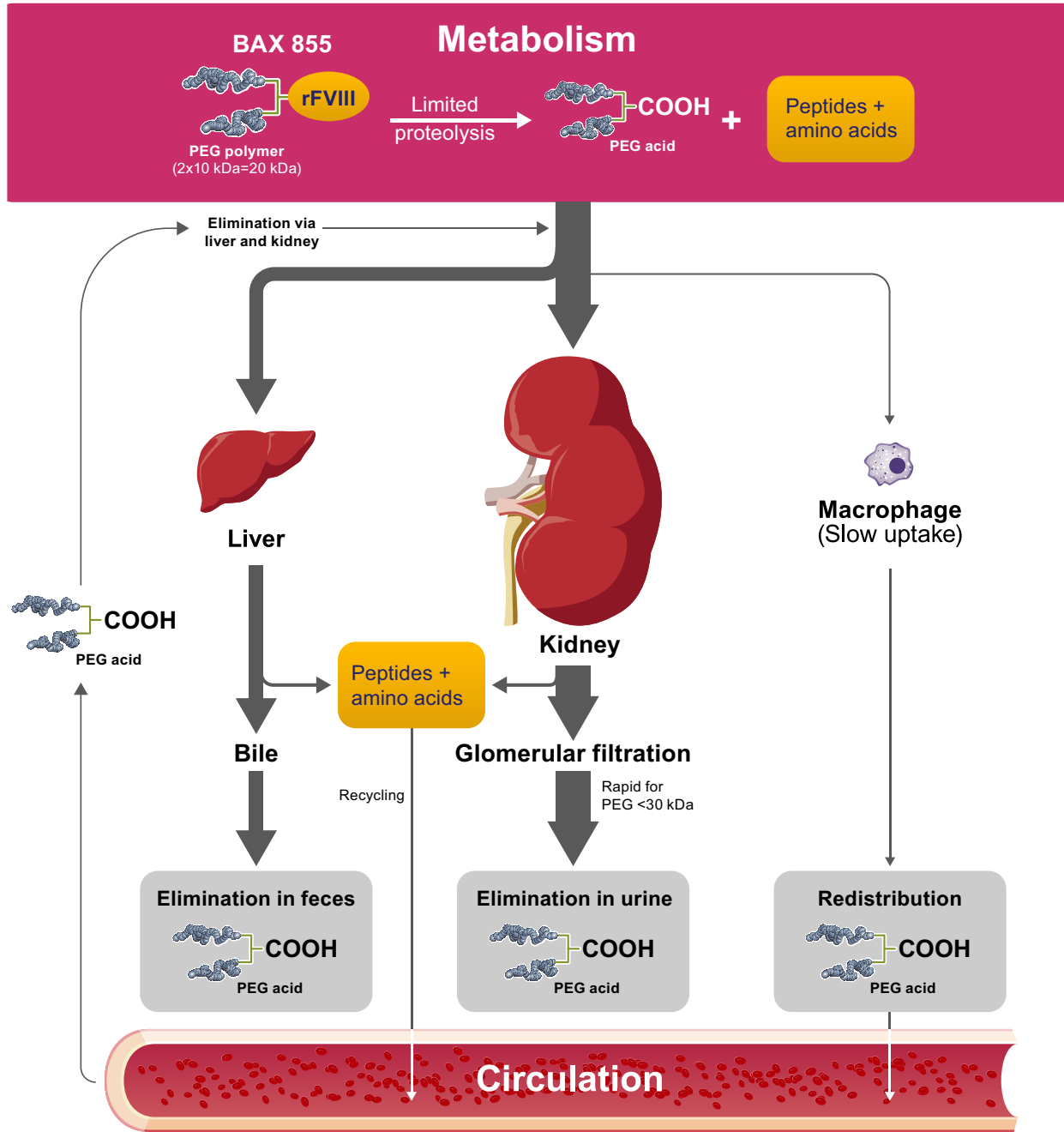


Fig. 1. Hypothetical degradation and elimination pathways of PEG-rFVIII conjugate (BAX 855); PEG-acid is the final degradation product, which is primarily eliminated via kidney and liver.

large polymer size, a process referred to as ‘reptation’ (lat. reptare – to creep) [24]. Therefore, no clear threshold for glomerular filtration can be determined for PEG. However, renal clearance is significantly slower for non-conjugated PEGs larger than 30 kDa [21]. A PEG size >30 kDa triggers increased half-life and favours other than renal clearance pathways, such as hepatic clearance or pinocytosis/phagocytosis by cells of the mononuclear phagocyte system (MPS),

also known as the reticuloendothelial system (RES) or lymphoreticular system. MPS cells are able to engulf particles (phagocytosis) to form an intracellular phagosome, which fuses with the lysosome, the compartment involved in the breakdown of cellular components, thereby generating the phagolysosome. Inside the phagolysosome, contents are subsequently degraded and released intra- or extracellularly for further processing or elimination.

PEG molecules with increasing MW tend to have higher rates of cellular clearance; similarly, macrophage uptake of the inert polymer polyvinyl alcohol tends to increase with increasing MW [25]. It has been established that the physicochemical properties of a particle's surface, such as surface charge, size, functional groups and hydrophobicity, affect the uptake of particles by phagocytic cells. *In vitro* experiments with nanoparticles coated with PEG of varying MW showed that the lower negative charge of the surface after PEGylation reduced the uptake by macrophages [25]. PEG inside of phagocytic cells may be released into the circulation by decomposition of cell membranes after apoptotic decay of the phagocytic cell or by exocytosis [20]; renal or biliar excretion follows.

The vasculature of the liver is composed of discontinuous capillary walls that allow small and large polymeric molecules such as PEG to diffuse from the blood to the extravascular region. Elimination might also occur via paracellular pathways, such as transepithelial movement across intercellular junctional complexes [26]. Liver clearance via parenchymal cells leading to excretion via bile and faeces decreases with increasing MW.

Non-clinical studies for approved PEGylated proteins have demonstrated that PEG/PEGylated proteins distribute to various compartments [20]. Variability in the distribution pattern of PEGylated biopharmaceuticals, their metabolism, and excretion complicate our ability to clearly understand the specific effects of PEGylation. However, where proteolysis and kidney clearance are the primary degradation routes for the parent protein, increasing overall size of the PEGylated conjugate will limit renal clearance. When proteins themselves are too large for renal clearance, the protein will dominate the clearance mechanism. There appear to be no explicit rules with respect to PEG size in the case of clearance by receptor-mediated processes. Thus, the variation in non-clinical data from available distribution studies in FDA and EMA databases¹² is considered to be attributable to the different biological components of the conjugates (protein or DNA of varying size, clearance mechanism and pharmacological target) and to the size and quantity/dose of the PEG molecules used for chemical modification.

Distribution, metabolism and excretion of BAX 855

An ADME study with radiolabelled BAX 855 investigated the distribution and excretion of BAX 855 after a single high dose given intravenously to male and female rats. Radiolabelling of a PEG-protein conjugate

with a tritium (T; ³H) label directly on the PEG was a pioneering chemical synthesis (to be published separately), as previous studies with radiolabelled PEGylated biotherapeutics were synthesized with the radiolabel on the protein moiety or the linker between PEG and the biologically active constituent. This was an important step, as PEG is almost inert to chemical modification except at its terminal hydroxyl group (which is blocked in PEG-protein conjugates) [27] or at linker structures that are highly reactive and have many different potential labelling sites. The disadvantage of using radiolabelled linkers is that the biological distribution of PEG can only be investigated as long as the linker is attached to the PEG [28]. Our ³H labelling method aimed to directly label the PEG polymer. The resulting test item for the ADME study in rats is shown in Fig. 2.

Starting material for synthesis of tritium (T; ³H) labelled PEGylated rFVIII was PEG2ru20KCOOH (PEG-acid), which was also investigated in a repeated dose toxicity study in rats, with 10 000-fold exposure compared with PEG administered during prophylaxis with BAX 855. Coupling of (T)-PEG2ru20K-NHS reagent to full-length rFVIII was performed in a manner similar to the modification step used to generate BAX 855. The NHS ester of the tritium-labelled reagent reacts with the epsilon-amino groups of lysines in rFVIII to form stable amide bonds. After the reaction, the conjugate is purified by ion-exchange chromatography and subsequently concentrated by ultrafiltration/diafiltration (UF/DF).

Tritium exchange as a radiolabelling technique has the risk that tritium may be lost through exchange with water. Therefore integrity and stability of the labelled PEG-rFVIII conjugate was tested before, during and 1 year after the ADME study. The presence of tritium on the PEG portion of the PEG-rFVIII conjugate was measured by radio-immunoblot and western blot by staining with a monoclonal anti-PEG antibody. In addition, the agent tested in the ADME study was characterized for its FVIII-specific activity and pharmacokinetic studies were performed in FVIII-deficient mice in comparison to non-PEG tritiated BAX 855. These studies confirmed that (i) radiolabelled PEG-rFVIII was stable throughout the study,

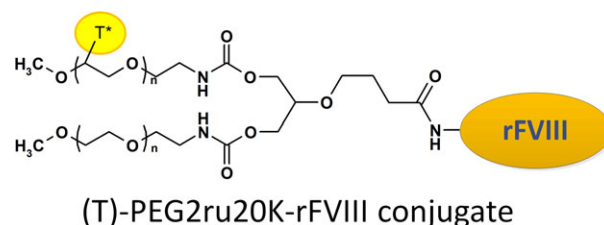


Fig. 2. (T; ³H) labelled PEGylated rFVIII, test item of the single-dose ADME study in rats.

¹<http://www.accessdata.fda.gov/scripts/cder/drugsatfda/>.

²http://www.ema.europa.eu/ema/index.jsp?curl=/pages/medicines/landing/epar_search.jsp&mid=WC0b01ac058001d124.

(ii) radiolabel was present on PEG, (iii) FVIII activity was maintained, despite introduction of the label and (iv) prolongation of the half-life of FVIII was not impeded by radiolabelling (data not shown).

Single doses of 1 and 2 mg FVIII protein kg^{-1} body weight (BW) corresponded to FVIII activities of 2088 and 4176 IU kg^{-1} BW respectively. These activity doses were accompanied by PEG doses of approximately 0.12 and 0.24 mg PEG kg^{-1} BW respectively. Urine, faeces and selected tissues were collected up to 1008 h (6 weeks) post-dose. BAX 855 was well tolerated in all rats. The distribution of drug-derived radioactivity was extensive, with the highest concentrations of radioactivity observed in plasma, blood, mesenteric lymph nodes, spleen, liver, adrenal glands and kidneys. However, after high doses of radiolabelled BAX 855 (25–50 \times the maximum clinical dose of 80 IU kg^{-1}), only marginal radioactivity levels were measured in the brain and spinal cord over 6 weeks. The average brain:plasma concentration ratio was clearly <1 (0.013–0.333) for all time points measured. These data demonstrate that even at very high doses of BAX 855, PEG did not reach the brain or related tissues at clinically relevant levels. Furthermore, radioactivity was completely excreted within 6 weeks, indicating that PEG that had been distributed into tissues was subsequently redistributed to the circulation and excreted (see Fig. 1) [29].

Cell vacuolation after administration of unconjugated PEG and PEG-protein conjugates

PEG is an inert molecule. Based on this stability, certain PEG and PEG-containing biopharmaceuticals may cause vacuolation of certain cell types after repeated treatment with high parenteral doses in animals. Based on the absence of tissue damage or any other signs of toxicity, vacuolated cells observed in some animal studies are considered to be a non-adverse consequence of an adaptive PEG removal process. Functional parameters of organs were not affected by vacuolation [30].

Tumour necrosis factor binding protein (TNF-bp) PEGylated with a 20 kDa PEG caused renal tubular vacuolation in rats at daily i.v. doses of 10, 20 or 40 mg kg^{-1} BW for 3 months. Vacuolation resulting from the high doses (20 and 40 mg kg^{-1} day $^{-1}$) was only partly reversible within the recovery period of 2 months. Tubular vacuolation did not lead to necrosis, but did cause distortion of tubular profiles and compression of nuclei [31]. The mode of action is considered to be related to specific binding of the TNF-bp to tubular epithelial cells and subsequent receptor-mediated uptake and pinocytosis [31].

Vacuole formation has also been reported for haemoglobin (Hb) and bovine serum albumin (BSA), both conjugated to a 5 kDa PEG [32]. Rats treated with a

single i.v. dose of ~ 500 mg kg^{-1} BW of both PEG-Hb and PEG-BSA were examined for vacuole formation in the heart, lung, liver, spleen and kidneys up to 30 days post infusion. Seven days after treatment, renal tubular cell vacuoles and splenic vacuolated macrophages were recorded in all animals treated with PEG-Hb. After 30 days, only 20% of animals had vacuoles in tubular cells, and no vacuolated macrophages were present in the spleen. Rats receiving PEG-BSA were free of renal tubular cell vacuoles at both time points, but all animals had splenic vacuolated macrophages, with decreasing severity at the later time point. Formation of vacuoles in the proximal tubules is also a normal process whereby proteins such as haemoglobin are recycled and supplied to metabolic pathways. Within this process, saturation of the tubular lumen with haemoglobin can result in bulbular enlargements that pinch off from cell membranes to form absorption droplets.

Vacuolated cells have also been observed in non-clinical studies conducted for several approved PEGylated biopharmaceuticals (Somavert, Macugen, Cimzia, Krystexxa and Omontys) [33–37]. Rudmann *et al.* [38] recently reported the influence of molecular size of unconjugated PEG on cytoplasmic vacuolation. The distribution and toxicity of three PEG molecules (100 mg kg^{-1} of linear 10, 20 and 40 kDa PEG) was investigated after repeated i.v. injection in rats for 3 months; 10 kDa PEG was injected daily, 20 kDa PEG every other day and 40 kDa once weekly. It was demonstrated that increasing PEG MW strongly reduced PEG immune-historeactivity in renal tubules of rats. The PEG immune-historeactivity increased, however, in other cell populations with increasing MW of the PEG, especially splenic macrophages and choroid plexus epithelial cells. PEG-related histological changes were generally limited to rats dosed with 40 kDa PEG, where PEG immune-historeactivity was associated with macrophage and choroid plexus epithelial cell vacuolation, as well as epithelial vacuolation and degeneration of renal tubules. Based on these data, the effect of a PEG size of 10–40 kDa on cellular distribution of immune-reactive PEG and PEG-related histological changes was clearly demonstrated. Chronic dosing of biologics conjugated to PEG ≥ 40 kDa may have greater potential for vacuolation in macrophages, choroid plexus epithelial cells and renal tubular epithelial cells [38].

The occurrence of vacuolated cells in choroid plexus epithelial cells (ependymal cells) has been widely discussed in relation to the safe use of PEGylated biopharmaceuticals. The EMA's CHMP safety working party reviewed repeated dose toxicity studies for PEGylated biologics that are approved or under development for use in children (including study results outside the public domain) and concluded that PEG vacuolation of ependymal cells in animals was only

observed if several conditions were met [39], i.e. the study was conducted in macaques, a PEG moiety size of at least 40 kDa was applied, the study lasted ≥ 6 weeks and the administered dose comprised at least a cumulative monthly PEG exposure of $0.4 \mu\text{mol kg}^{-1} \text{ month}^{-1}$.

In 2013, an article discussing PEG-associated vacuolation in macrophages was published [30]. Vacuolation was predominantly reported for tissues comprising the RES and was without apparent toxicological significance. Following high exposure to PEGylated biopharmaceuticals, vacuolated macrophages were observed in several tissues, including the liver, kidney, bladder and choroid plexus. These findings were considered to reflect normal physiological processing of foreign material by scavenger phagocytic cells, producing no apparent effect on cell function or cell viability. Other cell types, such as hepatocytes, cells of urinary bladder, epididymis, adrenal cortex, synoviocytes, ciliary bodies of the eyes and choroid plexus of the brain previously showed vacuolation after high PEG exposure. For PEG therapeutic proteins, dose- and duration-dependent PEG accumulation and cytoplasmic vacuolation was non-specific or frequently target-associated, as described for renal tubular epithelial cells [31] and neurons without cellular damage or apparent effect on neuronal function (e.g. nerve conduction velocity) [30].

In conclusion, vacuolation is considered an effect of lysosomal processing after injection of high doses of vascular persistent but highly soluble test article such as PEG.

PEG exposure with PEGylated biopharmaceuticals and BAX 855

The estimated PEG dose for a single dose of approved PEGylated biopharmaceuticals ranges from 0.026 to 176 mg PEG per person per application (Table 1). For BAX 855, the dose of one FVIII activity unit is associated with an exposure to $0.095 \mu\text{g PEG}$. Dosing of BAX 855 depends on indication and use. Prophylactic dosing in a completed Phase 2/3 pivotal study with BAX 855 (ClinicalTrials.gov Identifier: NCT01736475) was up to 60 IU kg^{-1} twice weekly. The maximum anticipated prophylactic dose is 80 IU kg^{-1} , given twice weekly. Doses of up to 100 IU kg^{-1} may be used preoperatively (over a limited number of days) in an ongoing Phase 3 surgery study. Table 2 lists the PEG doses associated with these clinical FVIII dosing regimens. The maximum anticipated prophylactic dose of 80 IU kg^{-1} BAX 855 would result in a PEG application of $7.6 \mu\text{g kg}^{-1} \text{ BW}$ or 0.46 mg (for a 60 kg individual, the standard BW used in Table 1). Higher, short-term exposure with BAX 855 before or after surgery is not taken into

Table 1. PEG exposure with approved PEGylated biopharmaceuticals

Product (Approval date)	PEG size	Typical dose	Frequency/route	PEG exposure/dose	Indication	References
Adagen (1990) (PEG-adenosine deaminase bovine)	5 kDa multiple‡	1200 IU *	Once weekly/i.m.	n.a.	Severe combined immuno deficiency	[49]
Oncaspar (1994) (Pegaspargase)	5 kDa multiple‡	4000 IU* $\approx <47 \text{ mg}^\dagger$	Once every 2 weeks or less/i.m. or i.v.	127 mg	Acute lymphoblastic leukaemia, hypersensitivity to asparaginase	[50]
Peg-Intron (2000) (Peginterferon- α -2b)	12 kDa	0.064 mg^*	Once weekly/s.c.	ca 0.026 mg	Hepatitis C	[51]
Pegasys (2001) (Peginterferon- α -2a)	40 kDa	0.18 mg	Once weekly/s.c.	0.12 mg	Hepatitis B and C	[52]
Neulasta (2002) (Pegfilgrastim)	20 kDa	6 mg	Once every 3 weeks or less/s.c.	6 mg	Neutropenia	[53]
Somavert (2003) (Pegvisomant)	5 kDa multiple‡	10 mg	Once daily/s.c.	n.a.	Acromegaly	[37]
Macugen (2004) (PEG-aaptanib)	40 kDa	0.3 mg	Once every 6 weeks/ intravitreal	0.24 mg	Age related macular degeneration	[33]
Mircera (2007) (mPEG-Epoetin beta)	30 kDa	0.036 mg^*	Once every 2 or 4 weeks/s.c. or i.v.	0.018 mg	Renal anaemia	[54]
Cimzia (2008) (Certolizumab pegol)	40 kDa	400 mg	Once every 2 or 4 weeks/s.c.	176 mg	Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis	[36]
Krystexxa (2010) (Pegloticase)	10 kDa multiple‡	8 mg	Once every 2 weeks/i.v.	24 mg	Chronic gout	[35]
Omontys (2012) (Peginesatide)	40 kDa	4.2 mg^*	Once monthly i.v. or s.c.	4.36 mg	Anaemia in chronic kidney disease	[34]
Plegridy (2014) (Peginterferon beta-1a)	20 kDa	125 μg	Every 2 weeks/s.c.	0.1 mg	Multiple sclerosis	[6]

Data from specified reference and/or <http://www.rxlist.com>; n.a., information not available.

*Based on an adult with 60 kg BW and 1.6 m^2 body surface according to Auletta [55].

†Specific activity at least 85 IU/mg.

‡Multiple PEG polymer chains attached.

Table 2. PEG dose with expected doses of BAX 855 in clinical studies

Study	Dosing regimen	FVIII dose (IU/kg)	PEG exposure per dose (mg)*
Prophylactic dosing Ph 2/3 pivotal study	Twice weekly	60	0.34
Continuation study	Twice weekly	80	0.46
Paediatric study	Twice weekly	80	0.46
Ph 3 Surgery study	Daily (limited duration)	100	0.57

*Based on 60 kg body weight and a PEG dose of 0.095 µg per IU FVIII.

account as the contribution to an overall increase in cumulative monthly or lifelong PEG exposure would be minimal. This absolute dose of <1 mg results in a lower PEG exposure than that of several approved PEGylated proteins such as Pegaspargase (Oncaspar), Pegfilgrastim (Neulasta), Pegvisomant (Somavert), Certolizumab pegol (Cimzia), Pegloticase (Krystexxa) and Peginesatide (Omontys) (see Table 1). Compared with once-a-month treatment with Certolizumab (176 mg of 40 kDa PEG for 60 kg individual) in particular, the monthly PEG exposure with BAX 855 dosed 10 times (twice weekly application of 80 IU kg⁻¹ BW resulting in a PEG dose of 76 µg kg⁻¹ BW or 4.6 mg per person) would be 38 times lower.

The cumulative PEG dose is important in considering possible accumulation and vacuolation of ependymal cells *in vivo*. As described above, the CHMP Safety Working Party recently discussed the risk of ependymal cell vacuolation caused by PEGs (mainly >40 kDa) at a monthly PEG exposure of ≥0.4 µmol kg⁻¹ month⁻¹ and recommended addressing this risk before conducting longer term clinical trials in children. In applying this recommendation to the BAX 855 programme, the dose of 7.6 µg PEG kg⁻¹ per 80 IU kg⁻¹ BW dose can be converted to µmol. The MW of the PEG attached to rFVIII in BAX 855 is 20 kDa, hence the clinical PEG exposure of 7.6 µg PEG kg⁻¹ BW per day equals 7.6 µg/20 000 µg/µmol × 10 doses per month = 0.0038 µmol/kg BW/month, more than 100 times below the threshold of 0.4 µmol kg⁻¹ BW month⁻¹.

It can be concluded that for BAX 855, (i) the monthly PEG exposure is substantially lower than for other approved PEGylated products, (ii) the PEG exposure is substantially lower than the threshold for ependymal cell vacuolation observed in animal studies (≥0.4 µmol kg⁻¹ BW month⁻¹) and (iii) the size of the PEG (20 kDa) allows complete elimination from the body without any risk of accumulation. Thus, the PEG exposure derived from BAX 855 over a chronic treatment period is considered to be low and to pose no safety concern.

Safety data on unbound PEG

PEG is found in pharmaceuticals for topical (ointments), oral (excipients, laxatives), ophthalmic (eye

drops and creams), rectal (suppositories) and parenteral (excipients) administration, providing evidence for its clinical safety. The wide use of PEG is further demonstrated by numerous entries in the FDA Inactive Ingredient List³. Several other frequently used pharmaceutical excipients, such as Tween 20/80 (Polysorbate 20/80) or Poloxamer 188 (Pluronic® F-68; Sigma-Aldrich, St. Louis, MO, USA), also contain repeated ethoxy-groups and release small PEG molecules when metabolized.

As early as 1950, Smyth comprehensively summarized the toxicity of PEGs of 62 Da (ethylene glycol) to 10 kDa [40]. However, these results are of questionable relevance as PEG materials were less pure at that time and contained a broad distribution of PEG moieties. More recent reviews were published in 2005 and 2007 [19,27]. Even for the most toxic ethylene glycol, the LD₅₀ value (for a single oral dose in guinea pigs) was as high as 6.6 g kg⁻¹ BW, due to the toxic metabolite, oxalic acid. Studies of chronic oral toxicity in dogs revealed no adverse effects for PEGs of 200 Da to 6 kDa at doses of 2% of the diet for 1 year. With increasing PEG size, oral toxicity (LD₅₀) decreased to > 50 g kg⁻¹ BW in rats for 10 kDa PEG. No absorption via the gastrointestinal tract was reported for PEGs >6 kDa [41]. Intravenous application of PEG of low MW may cause kidney lesions at high dosage. Table 3 summarizes the results for PEG toxicity after i.v. administration. Even repeated i.v. dosing with amounts matching the g kg⁻¹ BW in several species caused no adverse events, adverse reproductive or teratogenic effects. PEGs were neither mutagenic nor carcinogenic [27].

Extensive safety evaluation on PEG alone was conducted by Schering-Plough for submission of Peg-Intron. The 12 kDa mPEG was not mutagenic in the standard battery of *Salmonella* and *Escherichia* strains, and a chromosomal aberration test in human lymphocytes was negative. A mouse micronucleus study showed that mPEG did not cause micronucleus formation after intraperitoneal (i.p.) injection. A 13-week study in rats, in which mPEG was administered s.c. twice weekly at doses up to 2276 µg m⁻² week⁻¹ (379 µg kg⁻¹ week⁻¹), revealed no mPEG-related findings under macroscopic or microscopic examination. In a 13-week (4-week recovery) study in macaques (s.c. twice weekly at doses up to 2276 µg m⁻² week⁻¹; 190 µg kg⁻¹ week⁻¹), no mPEG-related findings were observed under macroscopic or microscopic examination. An embryo-foetal development study in rats with mPEG administered daily at doses up to 800 µg m⁻² per day (133 µg kg⁻¹) from day 6 through 17 after mating showed no signs of toxicity, no maternal or *in utero* effects. No signs of toxicity, maternal or *in utero* effects were noted in an embryo-foetal development

³<http://www.accessdata.fda.gov/scripts/cder/IIG/index.cfm>.

Table 3. PEG toxicity studies using intravenous application

PEG	Dose	Species	Results	References
PEG-4, -8, -20, -32, -75, -150 (200–7500 Da)	i.v. 350 mg kg ⁻¹ day ⁻¹ for 5 weeks	Rabbit	Cloudy swelling of renal tubular epithelium and hepatic parenchyma across all treatment groups	[56]
PEG-6 (300 Da)	i.v. <500 mg kg ⁻¹ day ⁻¹ for 5 weeks	Rabbit	No adverse effects	
PEG-6 (300 Da)	i.v. 1250 mg kg ⁻¹ day ⁻¹ for 4 weeks	Rat	No adverse effects	[57]
	i.v. 350 mg kg ⁻¹ day ⁻¹ for 29 days	Macaque	No adverse effects	
PEG-75 (4 kDa)	i.v. 90 mg kg ⁻¹ day ⁻¹ for 12 months	Dog	No adverse effects	[58]

study in rabbits with daily doses up to 800 µg m² (67 µg kg⁻¹) from day 7 through 19 after mating [42].

The PEG size used for BAX 855 (20 kDa) is within the range currently used in approved PEGylated biotherapeutics, i.e. 5–40 kDa (Table 1). According to Yamaoka *et al.*, the half-life of a 20 kDa PEG in mice after i.v. administration is approximately 3 h (169 ± 20 min), indicating that exposure is limited and PEG of this size is quickly eliminated from the body [21]. While the 20 kDa PEG is small enough to assure rapid elimination (primarily via the kidneys and in bile after liberation from rFVIII), it is still able to sterically shield rFVIII from clearance receptors, thereby prolonging its half-life. Based on the stability of PEG *in vivo* and stable covalent bonds, metabolism of PEG-rFVIII is expected to liberate PEG2ru20K-COOH (PEG-acid) after catabolism of the protein part (Fig. 1, for structure see Fig. 3). The non-clinical safety of PEG-acid was also investigated in a 28-day study conducted by Baxalta. Rats received i.v. doses of 0.65, 6.5 and 65 mg kg⁻¹ twice weekly for a total of 8 doses (for experimental details see Data S1). The dose levels selected were based on an estimation of the cumulative lifetime exposure of ~6.5 mg PEG/kg BW considering a BAX 855 dose of 80 IU kg⁻¹ BW twice weekly over 70 years. Furthermore, the high dose of 65 mg PEG kg⁻¹ BW is ~10 000 times the PEG exposure of a single clinical dose of BAX 855 and 10 times the estimation of the cumulative lifetime exposure. Delayed onset or reversibility of toxicity

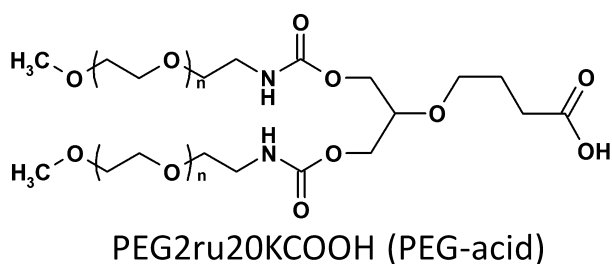


Fig. 3. PEG2ru20KCOOH (PEG-acid), expected final degradation product of BAX 855 that was also investigated in a repeated dose toxicity study in rats with 10 000-fold exposure compared with PEG administered during prophylaxis with BAX 855.

was assessed during a 4- and 13-week treatment-free period. All doses were well tolerated with no negative clinical observations, effects on BW or food consumption, ophthalmoscopy or clinical or anatomical pathology. There were no macroscopic or microscopic findings (including no signs of cell vacuolation) due to administration of PEG-acid.

Overall, acute and chronic administration of various PEGs by relevant routes of application was evaluated in several animal species. Signs of toxicity only appeared at very high doses – in the g kg⁻¹ BW range (Table 3). These data indicate that PEG has very low toxicity in animals.

Safety data for PEGylated proteins

Preclinical evaluation of approved PEGylated biopharmaceuticals (Adagen, Oncaspar, Peg-Intron, Pegasys, Neulasta, Somavert, Macugen, Mircera, Cimzia, Krytexxa, Omontys and Plegridy) included comprehensive toxicology studies with the PEG-conjugate and partly with the PEG alone. No toxicity unique to PEGylation has been reported. Several available publications also deal with adverse effects of PEG-protein conjugates, however, without relation to PEG [42–46]. If toxic effects were observed in non-clinical studies with the currently available PEGylated products, they were caused by the protein portion of the conjugate and exaggerated pharmacological effects, not by the PEG. The non-adverse formation of PEG-associated vacuoles was observed in several animal studies, but was considered to reflect normal processing of foreign material [38].

Summary of non-clinical safety of BAX 855

Safety pharmacology and single- and repeated dose toxicity studies were conducted in mice, rats, rabbits and macaques. Table 4 provides an overview of these studies and their outcomes. In summary, these study results demonstrated the safety of BAX 855. Histopathological evaluation in rats and macaques repeatedly treated with BAX 855 revealed no adverse findings in tissues. Importantly, specific histopatholog-

Table 4. Summary of non-clinical *in vivo* safety studies with BAX 855

Study	Species	Maximum dose per kg BW	Results	References
Safety pharmacology				
Thrombogenicity, Wessler test	Rabbit	900 IU kg ⁻¹	Not thrombogenic, comparable to ADVATE	[59]
Cardiovascular and respiratory safety	Macaque	600 IU kg ⁻¹	Well tolerated; no adverse test-item related findings	[59]
Toxicology				
4-week repeated dose toxicity study after intravenous application	Rat	700 IU kg ⁻¹ dosed every other day; 15 doses	No signs of toxicity or test-item related adverse effects at any dose	[47]
	Macaque	700 IU kg ⁻¹ dosed every 5 days; 6 doses		[47]
Comparative immunogenicity study of BAX 855 and ADVATE	Mouse; macaque	8, 40 µg protein kg ⁻¹ once weekly; 8 doses	Similar immunogenicity profile to ADVATE	[48]

ical examination of the brain and spinal cord of these species confirmed the absence of any vacuolation in organs of all animals that received BAX 855 [47]. Results of comparative immunogenicity studies in mice and macaques indicate that BAX 855 has a similar immunogenicity profile to ADVATE [48].

Summary and conclusion

BAX 855 is a human, full-length recombinant factor VIII (rFVIII, used in ADVATE) modified with polyethylene glycol (PEG) to extend its circulating half-life. As the safety profile of ADVATE is well established, assessment of BAX 855 focused on possible safety issues that could arise from conjugation with PEG.

PEG itself is a highly stable and inert molecule, generally considered as safe and has been used for decades in personal care products and as an excipient or drug component in various parenteral drug products and devices. The first PEGylated biopharmaceuticals produced using PEGylation technology similar to that of BAX 855 were marketed in 1990. Today, 12 PEGylated biopharmaceuticals are commercially available for use in humans. Safety data for similar PEG molecules alone or in PEG-protein conjugates with PEG of comparable or larger size and much higher PEG doses than is intended for BAX 855 indicate no safety concerns caused by or related to PEG at clinically relevant exposure levels. The only PEG-related finding reported in the literature and observed in some animal studies at extreme PEG doses after repeated administration was vacuolation of certain cell types, including ependymal cells in the choroid plexus. The probability, nature and functional consequences of such findings are not completely understood. However, it is important to note that no such vacuolation occurred in animal studies with BAX 855 or with the unconjugated 20 kDa PEG. The PEG size and clinical PEG doses

applied with BAX 855 are within a range in which vacuolation is not expected and did not occur during non-clinical testing. Non-clinical studies conducted with BAX 855 in mice, rats, rabbits and macaques revealed no adverse effects related to PEGylation of rFVIII. An ADME study showed that even a very high single i.v. dose of radiolabelled BAX 855 in rats was completely excreted within 6 weeks. A 28-day repeated dose toxicity study in rats, using PEG2ru20KCOOH (the final and significant *in vivo* degradation product of BAX 855 derived from PEG in animals and humans), was conducted at dose levels representing multiples of a cumulative lifetime dose of PEG related to BAX 855 therapy. PEG2ru20KCOOH did not cause any adverse or non-adverse effects (including vacuolation of macrophages or other cells).

In conclusion, safety evaluation of PEG and Baxalta's PEGylated rFVIII, BAX 855, based on extensive literature analysis and data from non-clinical studies identified no PEG-related safety concerns. Now undergoing clinical trials, BAX 855 may in the future offer an important therapeutic option for patients with haemophilia A.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. 28-day repeated i.v. dose rat study with PEG2ru20KCOOH.