

# **Neuropathology Consultative Review: Evaluation of Nonclinical Studies for Rebinyn**

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**Application #:** BLA 125611

**Product Name:** Rebinyn

**Drug:** A recombinant Factor IX molecule, pegylated with a 40K-PEG (referred to as 40K PEG-rFIX or PEG N9-GP)

**Indications:** Treatment and prophylaxis of bleeding in patients with hemophilia B and perioperative management

**Patient population:** At birth in patients with Hemophilia B

**Proposed Clinical Dosing Regimen:**

- Control and prevention of bleeding episodes: 40 IU/kg body weight for minor and moderate bleeds, and 80 IU/kg body weight for major bleeds. Additional doses of 40 IU/kg can be given.
- Perioperative management: Pre-operative dose of 40 IU/kg body weight for minor surgery, and 80 IU/kg body weight for major surgery. Consider two repeated doses of 40 IU/kg (in 1-3 day intervals) within the first week after major surgery. Frequency may be extended to once weekly after the first week until bleeding stops and healing is achieved
- Routine prophylaxis: 40 IU/kg once-weekly.

**Background and Focus of Review:**

Twelve nonclinical study reports were received. They include studies in mice (2), rats (6), and monkeys (4). Studies were reviewed in the order of species (mice, rats, monkeys). Each nonclinical study was reviewed with a focus on the evaluation of pathology in the central nervous system, and the effects of the vehicle (40K-Polyethylene Glycol or PEG) in the brain. My thoughts impacting interpretation/significance within each study are noted as "Reviewer Comments" in the section titled *Review of Individual Nonclinical Studies (Pages 6-20) in this report*. Most tables were excerpted from the sponsor's submission. Where appropriate, tables were generated to highlight or integrate findings.

### **Overall Results:**

- Nonclinical studies clearly reveal **PEG accumulation** (within the blood vessels in **the brain**, within the subepithelial connective tissue of the choroid plexus, and within the lining epithelial cells in the choroid plexus - where it **persists following a non-dosing recovery period**).
- Nonclinical studies suggest a **slight increased susceptibility of males over females**.
- All nonclinical studies were **limited to adult animals** (no juvenile animal studies were available for review).

### **Reviewer's Conclusion, Discussion, and Recommendations:**

The primary method of histopathological evaluation was routine screening under light microscopy (LM) with hematoxylin and eosin (H&E) stained paraffin-embedded sections. Some studies included immunohistochemistry (IHC) that localized PEG within brain tissues, and one electron microscopy (EM) study was conducted to identify cellular localization of PEG within the choroid epithelial cells. It appears that brain section sampling was limited to approximately 3-5 sections. Additional sections of the choroid plexus were included. Spinal cord (usually three sections) was also evaluated.

No dedicated neuropathology evaluation was conducted which would include processing of the brain by perfusion fixation, and staining with multiple stains to evaluate all components of the nervous system. Given the nature of PEG to navigate past fenestrated endothelium of blood vessels (such as in this case from the subepithelial connective tissue into choroid plexus epithelial cells), it remains unknown if PEG accumulation also increased within adjacent ependymal cells lining the ventricles, and/or around the circumventricular organs (which are multiple neuroanatomical areas within the brain that do not have a blood brain barrier such as the median eminence, subfornical organ, area postrema, pineal gland etc). Similarly, it is not known whether PEG can accumulate within the neurons of the dorsal root ganglion within the peripheral nervous system and potentially impact sensory nerve functions (since these are not routinely tested in nonclinical settings, it could be missed).

Given the end-points utilized within the nonclinical studies conducted, PEG was found within the epithelial cells lining the choroid plexus, in the subepithelial connective tissue, and within blood vessels in the brain parenchyma. No PEG was noted within the brain parenchyma indicating an intact and functional blood brain barrier. The presence of PEG within the choroid plexus (considered to be the first line of defense into the brain) reflects the need to assess the integrity of the blood-CSF barrier. Immunohistochemistry with anti-PEG was also included in some rat studies which clearly demonstrated that IHC is a more sensitive end-point (compared to light microscopy- routine staining with

H&E) in localizing PEG within tissues. However, IHC evaluations were limited to the presence/absence of PEG within the tissues. Estimation on the distribution and/or severity of PEG accumulation was limited/lacking. Electron microscopy (EM) was also conducted in a rat study clearly localizing the PEG to being within intracytoplasmic Type III vesicles (based on the pathologists' classification of four types of "vesicles"). It is speculated by the reviewer that the content in intracytoplasmic vesicles were derived by pinocytosis from the PEG in the underlying connective tissue. However, given that the EM was evaluated at a single time-point (26 weeks post-dosing), the subsequent life-cycle of PEG within the vesicle remains unclear. Specifically, it is unknown if there is seepage and accumulation into the CSF over time with repeated and chronic dosing regimens, especially if there is potential to exceed the clearance rate out of the CSF. Evaluation of other sites (subepithelial choroid plexus, blood vessels, and lining microvillar areas) was absent/limited and likely beyond the scope of the study conducted. In conclusion, an evaluation of the distribution of PEG accumulation (with morphological criteria and severity grades) within the tissue would be best examined with IHC (in conjunction with routine H&E).

The toxicological and clinical significance of PEG accumulation within the choroid plexus and integrity of the blood-CSF barrier remains unclear. Since evaluations under various studies were limited to one (terminal sacrifice) or two (recovery sacrifice) time-points, it is not possible to glean the significance of PEG accumulation within vesicles over time. Does the PEG move into and accumulate within the cerebrospinal fluid (CSF)? What is the rate of clearance from the CSF? A time-course study and/or PEG kinetics within the brain tissues and/or CSF would be most useful. One rat study conducted by the sponsor indicates that cerebrospinal fluid (CSF) was collected, but no data/results were included. If present within the CSF, clinical doses can potentially be based on safety margins derived from steady state levels within the CSF conducted in nonclinical studies where no adverse toxicity is noted.

Given that the indication includes a pediatric population (as early as at birth) and the knowledge that the developing brain in children are more susceptible to toxicants (Saunders et al, 2012), it is recommended that PEG accumulation in the CSF/brain tissue is evaluated in juvenile animals with dedicated neuropathology end-points, and in an experimental design that includes pharmacokinetic sampling for PEG within the brain tissue (including choroid plexus) and/or CSF. A dedicated neuropathology evaluation would include a palette of stains to evaluate the various cell types and structures [H&E, FJB, LFB, silver stain, IHC (GFAP< Iba1, Olig 1)], additional brain sectioning (including circumventricular organs) and a screening of peripheral and autonomic nervous systems. Such a study would lend confidence to the lack of any adverse findings.

This review is focused on neuropathology evaluation in the nonclinical studies submitted. I have not reviewed whether any specific neurobehavioral and/or neurochemical end-points were assessed. Such complementary end-points should also be included in a well-conducted study evaluating potential neurotoxicity.

In general, given the generally inert nature of PEG, the lack of PEG within the brain parenchyma (limited to within blood vessels reflecting an intact blood brain barrier) in adult animals, and limited recovery following non-dosing periods in some nonclinical studies, risk assessment may be based on the lack of adverse findings on histopathology evaluation. However, considering the chronic dosing regimen and the patient population for this proposed drug, clinical dosing may potentially be based on safety margins derived from a low observed effect level (LOEL) in a well-conducted dedicated neuropathology evaluation within a chronic nonclinical toxicology study (including juvenile animals).

### **Terminology of Pathology Diagnoses:**

#### **Epithelial cells and ependymal cells:**

There are two types of epithelial cells that line the ventricles containing cerebrospinal fluid (CSF). These include the choroidal epithelial cells and the ependymal cells. The ependymal cells exist as a single layer of cells lining the ventricular surface adjacent to the neural tissue (brain parenchyma). On the other hand, the choroid plexus epithelial cells is a tuft of tissue that extends and projects into the ventricular CSF, and, unlike the ependymal cells, is not in intimate contact with the brain parenchyma. The tissue tuft includes the lining choroidal epithelial cells, subepithelial stromal connective tissue and blood vessels containing fenestrated endothelial cells. The nonclinical studies in this report refer to evaluation of the epithelial cells of the choroid plexus.

#### **Vacuoles/Vacuolation and Vesicles:**

The terms vacuoles and vesicles are often used interchangeably among pathologists and refer to membrane bound cellular organelles containing fluid, substrate, or pigment etc. For example, vacuolated macrophages refer to macrophages scarfing up material from its immediate environment (phagocytosis) to within its cytoplasm (routine function for macrophages). The resulting intracytoplasmic organelle containing the scarfed extracellular material is referred to as a vacuole. In the nonclinical studies reviewed in this report, the use of the term “vesicle” was limited to EM studies whereas the term “vacuoles” were used with LM evaluation. Both organelles are intracellular and membrane-bound. The vesicles noted under EM in the epithelial cells of the choroid plexus are speculated by the reviewer to be derived via pinocytosis of PEG into the cell from the surrounding connective tissue. However, the subsequent fate of these vesicles containing PEG remains unclear. It is not known if the vesicles transport PEG into the

CSF and whether PEG is cleared from the CSF, and/or whether the rate of clearance exceeds potential accumulation with a chronic dosing regimen in pediatric patients.

**Blood brain barrier (BBB) and Blood-CSF barrier (BCSFB):**

Blood brain barrier refers to the impermeable endothelial cells lining blood capillaries within the brain. Blood CSF barrier refers to the choroid plexus epithelium with an underlying stroma of permeable capillaries. Hence, the BBB is endothelial, while the blood CSF barrier is epithelial. The barrier is thought to be due to tight junctions present between the cells. Both the BBB and BCSFB mediate homeostatic mechanisms to protect the neural microenvironment.

**Studies Submitted:**

**Mouse:**

- Study 210169: [<sup>3</sup>H]Sia-rFIX-40K-PEG: A study of distribution, by quantitative whole-body autoradiography, following intravenous administration to the haemophilic mouse
- Study 212166: [<sup>3</sup>H]PEG-N9-GP Polyethyleneglycol (PEG): Tissue Distribution of Radioactivity in the Haemophilic Mouse by Quantitative Whole Body Autoradiography and Qualitative Micro-Autoradiography

**Rats:**

- Study 212213: [<sup>3</sup>H]NNC 0126-0000-0116, 40 kDA Polyethylene glycol (PEG): Tissue Distribution of Radioactivity in the Rat by Quantitative Whole-Body Autoradiography and Qualitative Microautoradiography
- Study 209294: 40 K Polyethyleneglycol (PEG) Exploratory Toxicity Study by Intravenous (bolus) Administration on Alternate Days to (b) (4) Wistar Rats for 2 or 6 Weeks
- Study 210259: N9-GP Single dose Intravenous (Bolus) Administration Comparison Study in the Rat
- Study 212143: Pharmacokinetic and Immunogenicity Study in Rowett Nude (b) (4) Rats Following Twice Weekly Intravenous Administration for 6 Weeks
- Study 212513: 26 Week Toxicity Study by Intravenous Administration to Rowett Nude Rats (b) (4) Followed by a 26 Week Treatment Free Period (GLP)
- Study 214495: Transmission electron microscopic investigation of epithelial choroid plexus cells from Rowett Nude Rats dosed NNC0156- 0000-0009 for 26 weeks (JLY0426)

**Monkeys:**

- Study 209215: 40 K Polyethyleneglycol (PEG) Toxicity Study by Intravenous (Bolus) Administration to Cynomolgus Monkeys for 2, 6 or 13 Weeks

- Study 208260: 40K PEG-rFIX - 4 Week Intravenous Administration Toxicity and Safety Pharmacology Study in the Male Cynomolgus Monkey Followed by a 5 Week Treatment-free Period
- Study 208405: 40K PEG-rFIX 13 Week Intravenous Administration Immunogenicity Study in the Male Cynomolgus Monkey Followed by a 5 Week Treatment-free Period
- Study 209200: An immunohistochemical investigation of cynomolgus monkey brain tissues from two intravenous studies with 40K PEG-rFIX

### **Summary of the Nonclinical Studies:**

Two mouse studies with radiolabeled test article revealed significantly high levels in the choroid plexus 1 hour post-dosing in male pigmented Factor IX knock-out mice (Study 210169). However, this was not seen in another mouse model (Factor IX deficient albino mice – (b) (4) strain) – Study 212166. Clearly, the pharmacokinetics is different between the two mouse models at 1 hour post-dosing.

A rat study with radiolabeled test article reveals low distribution across central nervous system tissues. The pharmacokinetics at the 1 hour time-point is unremarkable compared to the pigmented male mouse (Study 210169). This study was done in male albino rats.

**Reviewer's Comment:** *Based on similar differences in mice (pigmented vs. albino), would the initial pharmacokinetics vary between (b) (4) hooded rat strain (pigmented) versus the albino rats used in the above study ?). Could pegylation be affected by pigmentation ? (Lu et al, 2011; Gurguta et al, 2006).*

Intravenous administration of PEG alone for a period of 6 weeks (dosing on alternate days) in rats (Study 209294) revealed vacuolated macrophages in the interstitium of the choroid plexus in animals treated with 33.4 mg/kg/2 days (or 117 mg/kg/week) and in males treated with 12.8 mg/kg/2 days (or 45 mg/kg/week). A review of the pathology tables includes an additional diagnosis of “inflammatory cell infiltrate choroid plexus” at 2 and 6 weeks of PEG alone. Although the incidence is sporadic, it remains unclear as to *which* additional inflammatory cell types (lymphocytes, plasma cells, neutrophils, eosinophils, etc) were present.

Intravenous administration of N9-GP in a nude rat model (Study 212143) for a period of 6 weeks (twice weekly dosing) showed sporadic incidence of lesions including vacuolation of the choroid plexus during routine histopathological evaluation after 6 weeks. *It is unclear if the routine histopathological evaluation was conducted in recovery animals (2 week period) in this study.* However, immunohistochemistry of the brain following 6 weeks of dosing with a 2 week recovery period revealed PEG in small intracytoplasmic vesicles in some of the choroid plexus epithelial cells in most rats, indicating persistent changes (for at least two weeks). This study also demonstrated that immunohistochemistry is more sensitive than routine histopathology evaluation with H&E in the evaluation of PEG.

*The pharmacokinetics of PEG within the brain remains unclear. Immunohistochemistry evaluation was limited to a single time-point (following a recovery period of 2 weeks). It is not known whether the initial PEG load in the choroid plexus was higher at the end of 6 weeks of treatment, and/or whether a longer recovery time would result in negative immunohistochemistry findings suggesting clearance of PEG from the brain tissues.*

A 26 week repeat-dose toxicology study (Study 212513) in nude rats also did not reveal any remarkable incidence of vacuolation within the choroid plexus with routine histopathology evaluation (H&E). However, immunohistochemistry showed a dose-related increase in immunohistochemistry staining for PEG in blood of blood vessels in the brain, and connective tissue and epithelial cells in the choroid plexus following 26 weeks of dosing. After a 26 week recovery period, PEG was still present in the epithelial cells (within vesicles) of most animals at the high dose. Lower doses were not examined, hence a no effect level could not be determined. Although the methods section indicates that CSF was collected in this study, no data was available for review.

An electron microscopic evaluation of the epithelial choroid plexus cells from Study 212513 showed that the PEG was present within intracytoplasmic vesicles of the epithelial cells of the choroid plexus.

Intravenous administration of PEG alone in monkeys (Study 209215) following 6 weeks of treatment at 12.8 mg/kg of PEG revealed vacuolation of the “ependymal” (interpreted by the reviewer as epithelial cells, given that epithelial cells line the choroid plexus, whereas ependymal cells line the ventricles – see Johanson et al, 2011) cells of the choroid plexus following routine histopathological evaluation. *However, no immunohistochemistry was conducted.*

Intravenous administration of the test article for 4 weeks with a 5 week recovery period in monkeys [Study 208260 with routine histopathology evaluation (H&E) and IHC in Study 209200] revealed PEG accumulation in all dose groups in blood located within the brain blood vessels. In addition, PEG was detected in the connective tissue of the choroid plexus, and the cytoplasm of few choroid plexus epithelial cells in monkeys dosed with 15.3 mg and 45 mg 40K PEG-rFIX/kg/week. Following 4 weeks of dosing and a 5 week recovery period, PEG was not detectable in the choroid plexus of monkeys dosed with 4.2 mg and 15.3 mg PEG-rFIX/kg/week After 1 week of recovery, PEG was detected in the connective tissue of the choroid plexus, in the cytoplasm of few choroid plexus epithelial cells, and in the blood located within the brain blood vessels of monkeys dosed with 45 mg PEG-rFIX/kg/week. *However, it appears that the experimental design did not include evaluation of animals dosed with 45 mg 40K PEG-rFIX/kg/week following a 5 week recovery period.*

Intravenous administration of the test article for 13 weeks with a 5 week recovery period (Study 208405 with pathology evaluation in Study 209200) did not reveal PEG in choroid plexus or in any of the brain structures investigated or in the brain blood vessels. However, immunohistochemistry was not used in these studies.

## **Review of Individual Nonclinical Studies:**

### **Study 210169: 40K-PEG-fFIX - [<sup>3</sup>H]Sia-rFIX-40K-PEG: A study of distribution, by quantitative whole-body autoradiography, following intravenous administration to the haemophilic mouse**

**Objective:** determine the tissue distribution of radioactivity in the male hemophilic mouse following a single intravenous administration of [<sup>3</sup>H]Sia-rFIX-40K-PEG using quantitative whole-body autoradiography.

Single administration at 2.2 mg/kg in a mouse hemophilia model (male pigmented FIX-KO strain). Sacrifices at 1, 12, 24, 96 hrs, and 7, 10, and 14 days post-administration. No histopathology.

**Table 1:** Concentrations of radioactivity in CNS tissues of male knock out Factor IX mice after a single intravenous administration of [<sup>3</sup>H]Sia-rFIX-40K-PEG at a dose level of 2.2 mg/kg body weight:

Tissue	µg eq. of rFIX-PEG/gram of tissue							
	Animal # Sampling Time	337M 1 hr	339M 12 hr	342M 24 hr	343M 96 hr	345M 7 days	347M 10 days	349M 14 days
Plasma		<b>6.13</b>	3.26	3.47	2.05	1.02	0.499	0.074
Brain		0.131	0.057	0.077	0.096	0.072	0.071	0.054
Choroid Plexus		<b>5.68</b>	0.376	0.326	0.33	0.276	0.233	0.129
Spinal cord		0.186	0.083	0.093	0.112	0.067	0.066	0.058
Uveal tract/retina		1.17	0.877	0.235	0.282	0.238	0.257	0.263

***Reviewer Comment:*** The distribution of rFIX-PEG in the choroid plexus is remarkably close to the amount in plasma at 1 hr post-dosing in a mouse hemophilia model.

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### **Study 212166: [<sup>3</sup>H]PEG-N9-GP Polyethyleneglycol (PEG): Tissue Distribution of Radioactivity in the Haemophilic Mouse by Quantitative Whole Body Autoradiography and Qualitative Micro-Autoradiography.**

**Objective:** determine the tissue distribution of radioactivity in the male hemophilic mouse following a single intravenous administration of [<sup>3</sup>H]PEG-N9-GP using quantitative whole-body autoradiography techniques.

Single administration at 2.8 mg/kg in a mouse hemophilia model (Factor IX deficient albino mice – (b) (4) ). Sacrifices at 1,12, 24, 96 hrs, and 7,14, 35, 63, and 84 days post-administration. No histopathology.



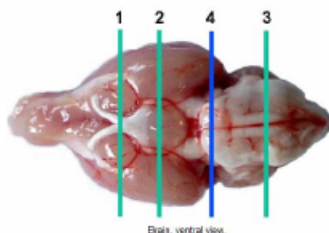
## Method:

\*Ten tissues were sampled in total as the brain was divided into 3 regions as described (below). Regions 1, 2 and 3 were prepared for sectioning.

1. Level of optic chiasm including the basal ganglia, septum, cortex, anterior hypothalamus

2. Level of hippocampus containing the cortex and brain stem at the transition of diencephalon to mesencephalon

3. Containing the cerebellum and brain stem (medulla oblongata)



**Reviewer Comment:** Brain was sampled in three regions. It is unclear if neuroanatomical areas within each region were dissected out or regions (as defined in the Method above) containing the specific neuroanatomical areas were sampled together per slice as shown in the figure above.

**Table 2:** Concentrations of radioactivity in CNS tissues of male mice after a single intravenous administration of [ $^3\text{H}$ ]PEG-N9-GP at a nominal dose level of 2.80 mg/kg body weight:

Tissue	$\mu\text{g eq. of PEG-N9-GP/gram of tissue}$									
	Sampling Time	1 hr	12 hr	24 hr	96 hr	7 days	14 days	35 days	63 days	84 days
Plasma		27	12.6	10.2	3.51	3.37	1.05	0.311	0.101	0.109
Brain		0.265	0.172	0.121	BLQ	0.057	0.042	BLQ	BLQ	BLQ
Choroid Plexus		<b>0.276</b>	0.458	0.512	0.358	0.533	0.479	0.265	0.129	0.126
Spinal cord		0.334	0.19	0.211	0.072	0.173	0.044	BLQ	BLQ	0.044
Uveal tract/retina		1.61	1.58	1.64	0.736	2.49	0.633	0.896	0.32	0.912
Meninges		1.15	0.645	0.678	0.36	1.09	0.287	0.242	0.073	BLQ

**Results:** Most of the regions of the brain, including blood in the vascular system, contained low levels of radioactivity (++) at each of the sampling times. In the cerebrum, the various regions of the cortex also contained these levels (++) throughout all sampling times, as did the corpus callosum, putamen and accumbens regions. Levels were also low across the hippocampus, thalamus and hypothalamus, at all sampling times.

In the cerebellum, background or low levels of radioactivity (+ or ++) were associated with the white and grey matter, at all sampling times. The pons region of the cerebellum contained low levels of radioactivity (++) at all sampling times. At 1 and 24 hours the pyramidal tract also contained low levels (++) but this structure was not sectioned at 96 hours.

Of specific note were higher levels, i.e., moderate (+++) at all sampling times, associated with the choroid plexus. At the 1 hour sampling time denser regions of silver grains were present which were possibly related to the vascular system. At 24 and 96 hours denser clumping was mainly associated with central vascular regions.

Micro-autoradiography work generally complemented the macroautoradiography work very well and helped to highlight a number of regions of specific uptake in certain tissues. Low but detectable levels were observed throughout the brain, indicating that the blood/brain barrier may have been penetrated to a limited degree. In addition, there was a notable presence of radioactivity at moderate levels in the choroid plexus, including vascular regions, with low levels in the cerebrospinal fluid.

**Reviewer Comment:** *It is unclear why the increased level of radioactivity 1 hr post-dosing in the choroid plexus noted in Study 210169 was not seen in this study. Clearly, the pharmacokinetics is different between the two mouse models.*

**Study 212213: [<sup>3</sup>H]NNC 0126-0000-0116, 40 kDA Polyethylene glycol (PEG): Tissue Distribution of Radioactivity in the Rat by Quantitative Whole-Body Autoradiography and Qualitative Microautoradiography**

**Objective:** to determine the tissue distribution of radioactivity using [<sup>3</sup>H]-PEG.

Single administration at 0.6 mg/kg in male albino rat. Sacs at 1, 12, 24, 96 hrs, and 7, 14, 35, 63 and 84 days post-dose

No histopathology.

Table 4: Concentrations of radioactivity in the tissues of male albino rats after a single intravenous administration of [<sup>3</sup>H]PEG at a nominal dose level of 0.6 mg/kg body weight

Tissue	µg eq. of [ <sup>3</sup> H]-PEG /gram of tissue									
	Sampling Time	1 hr	12 hr	24 hr	96 hr	7 days	14 days	35 days	63 days	84 days
Plasma		4.63	2.56	2.06	0.741	0.327	0.188	0.041	0.012	0.007
Brain		0.043	0.031	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Choroid Plexus		0.379	0.221	0.168	0.147	0.169	0.213	0.167	0.276	0.066
Spinal cord		0.036	0.024	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Uveal tract/retina		0.508	0.425	0.246	0.313	0.075	0.035	0.023	BLQ	BLQ
Meninges		0.560	0.251	0.145	0.278	0.150	0.038	0.076	BLQ	BLQ

**Reviewer Comment:** The pharmacokinetics at the 1 hour time-point is unremarkable compared to the pigmented male mouse (Study 210169). This study was done in male albino rats. Albino mice also showed unremarkable values 1 hour postdosing (Study 212166).

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**Study 210259: N9-GP: (40K PEG-rFIX) Single dose Intravenous (Bolus) Administration Comparison Study in the Rat**

Objective: compare two batches of the test article

Method: Histopathology was conducted (with peer review). The pathology data presented in this report reflects the consensus between the study pathologist and the peer reviewing pathologist.

Brain plus three sections of spinal cord (cervical, thoracic, lumbar).

Results in path report state unremarkable macroscopic findings, and that “Microscopic findings were generally infrequent, of a minor nature and consistent with the usual pattern of findings in animals of this strain and age.”

**Reviewer Comment:** *Histopathology data tables were not submitted.*

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**Study 209294: 40 K Polyethyleneglycol (PEG): Exploratory Toxicity Study by Intravenous (bolus) Administration on Alternate Days to (b) (4) Wistar Rats for 2 or 6 Weeks**

Objective: to evaluate toxicity of PEG alone.

Pathology report included (with peer review).

Method: Macroscopic observations included observation of the brain, pituitary gland and cranial nerves. Brain histology included 3 sections (cerebellum, cerebrum, midbrain to include choroid plexus).

Spinal cord was collected and “retained pending future requirement”. It is stated that “samples (wax blocks and slides) of the brain (choroid plexus) from the Main study and Satellite animals were dispatched to the Sponsors pathologist for supplementary processing. Results from any supplementary processing is reported separately and not as part of this study.”

**Reviewer Comments:**

*Brains were likely removed immediately post-euthanasia (as opposed to fixation in-situ for 24-48 hrs). Histopathology was done on immersion-fixed brains (as opposed to perfusion-fixed brains).*

*No additional reference to data from supplementary processing (as noted in the Methods section above) was noted.*

**Results:**

Macroscopic Findings:

No treatment related lesions after 2 or 6 weeks of treatment with 40K PEG.

Microscopic Findings:

No treatment related lesions were noted after 2 weeks.

After 6 weeks, lesions were noted in the brain, spleen, mesenteric and mandibular lymph nodes.

Lesions in the brain are summarized below:

Vacuolated macrophages were seen in the interstitium of the choroid plexus in animals treated with 33.4 mg/kg/2 days (or 117 mg/kg/week) and males treated with 12.8 mg/kg/2 days (or 45 mg/kg/week).

<b>Summary of treatment related findings in the brain for animals killed after 6 weeks of treatment</b>						
Group/sex	1M	3M	4M	1F	3F	4F
Dose (mg/kg/week)	0	45	117	0	45	117
Vacuolated macrophages in interstitium of chroid plexus						
Minimal	0	3	6	0	0	6
Total	0	3	6	0	0	6
Number of tissues examined	6	6	6	6	6	6

Brain/choroid plexus was examined from both the main and satellite animals.

**Reviewer Comment:** In addition to the summary table above (noted in the pathology report), additional lesions in the brain (noted in the pathology tables) include Inflammatory Cell Infiltrate Choroid Plexus at the end of 2 weeks and 6 weeks as shown in the reviewer generated table below:

Table 3:

**Histopathology - group distribution of findings for animals killed after 2 weeks of treatment**

		Group/Sex:	1M	2M	1F	2F
Tissue and Finding		Number:	3	6*	3	6*
Brain	Number Examined:		3	6	3	6
Inflammatory Cell Infiltrate Choroid Plexus			0	0	0	1

**Histopathology - group distribution of findings for animals killed after 6 weeks of treatment**

		Group/Sex:	1M	3M	4M	1F	3F	4F
Tissue and Finding		Number:	6*	6*	6*	6*	6*	6*
Brain	Number Examined:		6	6	6	6	6	6
Inflammatory Cell Infiltrate Choroid Plexus			0	2	0	0	0	0

\* Brain only evaluated in 6 animals, for remaining tissues only 3 examined

**Reviewer Comments:**

Based on administration of 40K PEG alone, repeat-dosing reveals lesions in the choroid plexus. These include findings of inflammatory cell infiltrate (at two and six weeks of treatment), and the presence of vacuolated macrophages in the interstitium of the choroid plexus at the end of 6 weeks in all animals (both sexes) administered 33.4 mg/kg every 2 days, and at the end of 2 weeks in all males administered 12.8 mg/kg/2 days.

In light of the presence of vacuolated macrophages in the choroid plexus, details on the additional finding of inflammatory cell infiltrate (noted in the pathology tables) is lacking in the pathology report. It is unclear which additional inflammatory cell types (lymphocytes, neutrophils, eosinophils, etc) were also present.

**Deficiencies:**

Morphological criteria that define the grading scale are not included in the pathology report.

Whether the choroid plexus was consistently sampled in all animals is not confirmed.

Any supplementary processing (as noted in the Methods section) is not documented.

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**Study 212143: Pharmacokinetic and Immunogenicity Study in Rowett Nude**

**(b) (4) Rats Following Twice Weekly Intravenous Administration for 6 Weeks**

Objective: to determine the pharmacokinetics and potential immunogenicity of N9-GP in nude rats.

Twice weekly administration at 0.205 mg/kg (40 U/kg) or 6.15 mg/kg (1200 U/kg),  
(followed by a 2 week recovery period ?) see Reviewer Comment below

Method: Histopathology and immunohistochemistry were both included. The aim of the immunohistochemistry end-point was to explore whether the PEG part of the test article N9-GP, a PEGylated recombinant human coagulation factor IX (rFIX), could be detected in the brain choroid plexus in the nude rat.

Macroscopic observations included observation of the brain, pituitary gland and cranial nerves. Brain histology included 3 sections (cerebellum, cerebrum, midbrain), spinal cord (transverse and longitudinal section at the cervical, lumbar and thoracic levels). PEG-immunohistochemistry staining included the brain.

**Reviewer Comment:** *The experimental design is unclear. For histopathology, it appears that the animals were sacrificed at the end of 6 weeks. For immunohistochemistry, it appears that the evaluation was conducted in all 13 rats/group following 6 weeks of dosing and 2 weeks of recovery.*

**Results:**

Histopathology - Brain:

**Histopathology - group distribution of findings for animals killed after 6 weeks**

Tissue and Finding	Group/Sex:	1M	2M	3M	1F	2F	3F
	Number:	5	13	12	5	11	13

Brain	Number Examined:	5	13	12	5	11	13
Perivascular Cuffing		0	1	0	0	0	0
Vacuolation of Choroid Plexus		0	1	0	0	0	0
Vasculitis with Associated Pigment, Focal - Subependymal		0	0	1	0	0	0
Spinal C. Cerv.	Number Examined:	5	13	12	5	11	13
Spinal C. Thor.	Number Examined:	5	13	12	5	11	13
Spinal C. Lumb.	Number Examined:	5	13	12	5	11	13

#### Immunohistochemistry - Brain:

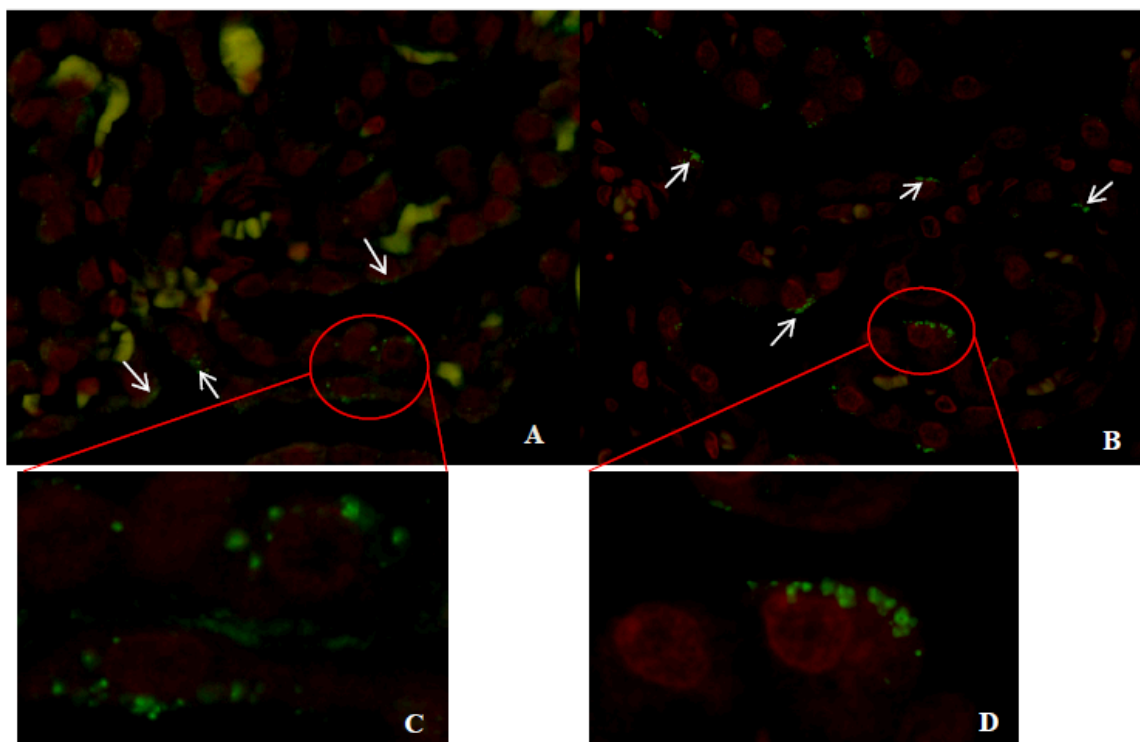
In the group administered 6.15 mg/kg of N9-GP with a 2 week recovery period, “PEG was detected in small vesicles the cytoplasm in some of the choroid plexus epithelial cells in all male rats and in 11 out of 13 female rats. PEG was weakly detected in the choroid plexus connective tissue in 4 out of 12 male rats. No PEG was detected in any other brain structure.”

“PEG was detected in the connective tissue of the choroid plexus and in the blood in the blood vessels of the brain from the early decedent male rat No. 27. This animal received only 2 administrations of 1200 Units/kg prior to death and harvesting of tissues without a 2 week recovery period.”

PEG positive staining summarized per group and sex, 6 weeks dosing and 2 weeks recovery:

Group	Sex/N	NNC0156-0009/kg twice weekly		Total PEG	Brain tissue		Blood in brain blood vessels
		Units	Mg PEG/dose	Mg	Choroid plexus:		
					Connective tissue	Epithelial cells	
1	M/2	0	0	0	0	0	0
	F/2	0	0	0	0	0	0
2	M/13	40	0.2	2.4	0	0	0
	F/13				0	0	0
3	M/12	1200	6.4	77	4	12	0
	F/13				0	11	0

M = male, F = female



**Figure 1 Detection of PEG in nude rat choroid plexus epithelial cells, 6 weeks dosing and 2 weeks recovery**

Immunohistochemical staining of PEG (white arrows) in small vesicles in the cytoplasm of the epithelial cells in choroid plexus (A, B) following 6 weeks dosing with 1200 U NNC0156-0009/kg twice weekly and 2 weeks recovery. C and D are digital enlargements of the areas of interest in figs A and B. (Images A and B were taken with a 60x objective) (green = PEG and red = nuclei staining).

**Reviewer Comment:**

*Clearly, PEG is seen after a 2 week non-dosing/recovery period. The life cycle of PEG remains unclear.*

*It is not known whether the initial PEG load in the choroid plexus was higher at the end of 6 weeks, and/or whether a longer recovery time would result in negative immunohistochemistry findings.*

**Deficiencies:**

*It is unclear if the routine histopathological evaluation was conducted in recovery animals.*

*Morphological criteria for the above lesions are not noted in the pathology report.*

*Whether the choroid plexus was consistently sampled in all animals is not confirmed.*

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**Study 212513: 26 Week Toxicity Study by Intravenous Administration to Rowett Nude Rats (b) (4) Followed by a 26 Week Treatment Free Period (GLP)**

**Objective:** Repeat-dose chronic toxicology study.

Repeat administration in nude rats at doses of 0.205 mg/kg (40U/kg), 150 U/kg, 600 U/kg, and 6.15 mg/kg (1200 U/kg), every 5 days for 26 weeks + 26 week recovery period

**Reviewer Comment:** *mg/kg conversions for the mid doses (150 and 600 U/kg) were not included*

**Method:** Histopathology and immunohistochemistry were included. The aim of the immunohistochemistry end-point was to explore whether the PEG and/or the protein part of the test article N9-GP, could be detected in the brain choroid plexus in the nude rat.

**CSF samples taken from control and high dose group.** It is stated that “The cerebrospinal fluid samples were stored frozen (approximately -70°C or below) pending shipment to the Sponsor’s representative. Any assay procedures and results were not part of this study and were reported separately.”

**Reviewer Comment:** *No additional reference to data from CSF evaluation was noted.*

**Method:** Brain histology included:

- 3 paraffin-embedded sections (cerebellum, cerebrum, midbrain and choroid plexus of the brain), spinal cord (transverse and longitudinal section at the cervical, lumbar and thoracic levels). It is stated that two serial sections of the brain were prepared – one section was dispatched to the sponsor along with the tissue block.
- Brain (the choroid plexus) from the left and right lateral ventricle was epon-embedded from the first 2 surviving animals from all groups (male and female) at the terminal kill of the main and recovery phases. *(Reviewer notes that these sections were used for the Electron Microscopy Study 214495 reviewed below)*

## **Results:**

Histopathology: Routine histopathology did not reveal a notable increase in the incidence of cytoplasmic vacuolation in the choroid plexus compared to the controls.

## **Immunohistochemistry:**

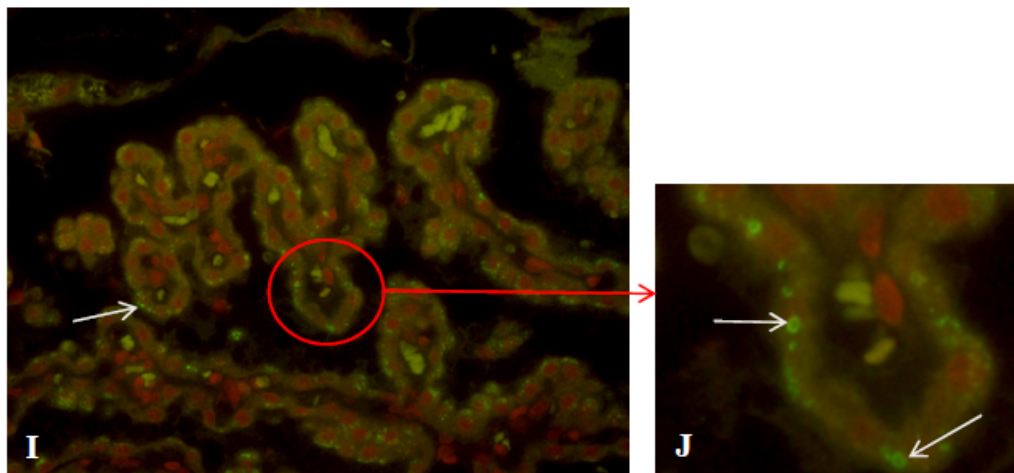
The protein part of the test article was not detected in any animal.

PEG IHC staining in the brain choroid plexus is summarized in the table below:

Dose – NNC0156-0000-0009 (IU/kg)	0		40		150		600		1200	
Number of animals	Main		Main		Main		Main		Main	
	M: 18	F: 18	M: 18	F: 18	M: 18	F: 18	M: 18	F: 18	M: 18	F: 18
	Recovery		Recovery		Recovery		Recovery		Recovery	
	M: 4	F: 4	M: 0	F: 0	M: 0	F: 0	M: 0	F: 0	M: 9	F: 8
IHC staining (no. PEG positive)										
Blood in blood vessels	0	0	9	8	17	17	18	17	18	18
Connective tissue	0	0	9	0	17	15	18	18	18	18
Epithelial cells (detected in small vesicles)	0	0	9	8	17	11	18	18	18	18
Recovery animals										
Blood in blood vessels	0	0	-	-	-	-	-	-	0	0
Connective tissue	0	0	-	-	-	-	-	-	0	0
Epithelial cells (detected in small vesicles)	0	0	-	-	-	-	-	-	9	8

- not examined





**Figure 3** Detection of PEG in nude rat choroid plexus epithelial cells, 26 weeks dosing of NNC156-0009/kg/5th day and 26 weeks off treatment

Immunohistochemical staining of PEG (white arrow) in the cytoplasm of the epithelial cells in choroid plexus, 1200 IU (I) NNC0156-0009/kg/5th day followed by 26 week off treatment. J is digital enlargements of the area of interest in figs I (Images I was taken with a 60x objective) (green = PEG and red = nuclei staining)

**Reviewer Comment:** *Given the presence of PEG after 26 weeks of recovery in the high dose group, it is critical that a NOAEL/LOAEL be determined through review and examination of the lower dose groups by the reviewing pathologist.*

**Study 214495: Transmission electron microscopic investigation of epithelial choroid plexus cells from Rowett Nude Rats dosed NNC0156- 0000-0009 for 26 weeks (JLY0426)**

**Reviewer Comment:** *Samples are from Study 212513*

**Objective:** Stated as an investigation to determine “if the PEG containing vesicles identified by immunohistochemistry in the choroid plexus epithelial cells had affected the organelles or other cell structures normally present in the cells.”

**Reviewer Note:** *Not so much whether it affects other organelles but more to identify the organelle containing PEG (lysosomes ?) and whether there is seepage into the CSF with repeat-dosing over time.*

**Method:** Epoxy embedded tissue blocks (from Study 212513), 1μ sections, and contrast stained.

**Results:** The different vesicles types in the cytoplasm were classified by the study pathologist as:

Type 1 = rough endoplasmic reticulum (rER)/Golgi vesicles, mucinous content  
Type 2 = undefined. Probably mucin droplets

Type 3 = PEG vesicles (verified by immunohistochemistry at paraffin embedded tissue for light microscopy evaluation in study 212513)

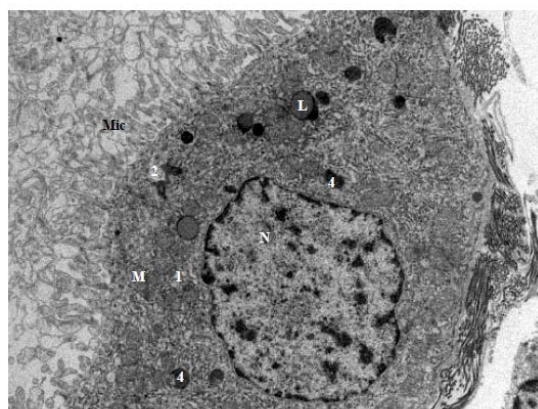
Type 4 = “dense body” lysosome and lysosome related/lipofuscin

The vesicle Type 3 was recognized containing PEG droplets. The vesicles were described as smaller “gray” electron dense droplets/vesicles.

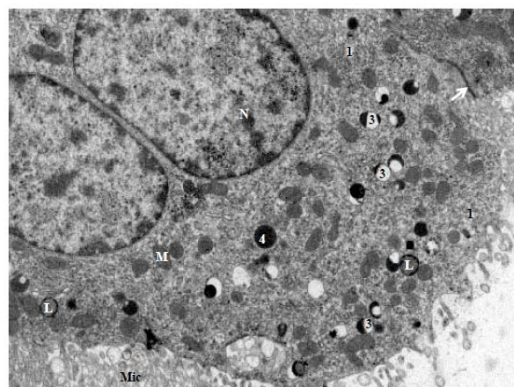
Distribution of vesicle types between controls and treated rats is shown below:

Treatment	Vesicle type:			
	1 rER/Golgi vesicles	2 large mucin droplets	3 PEG vesicles	4 lysosome related/lipofuscin
Vehicle control	+	+	0	+
1200IU nonacog beta pegol/kg/5 <sup>th</sup> day	+	+	+	+

Illustrations of epoxy-embedded sections from a control and treated rat are depicted below:



**Figure 3** CP epithelial cell from a nude control rat treated with vehicle for 26 weeks  
A choroid plexus epithelial cell from a Rowett Nude rat in the vehicle control group.  
N = nucleus, M = mitochondria, L = lipid droplet, Mic = microvilli  
Type 1 = rER/Golgi vesicles; mucinous content  
Type 2 = undefined. Probably mucin droplets  
Type 4 = “dense body” lysosome and lysosome related/lipofuscin



**Figure 4** Choroid plexus epithelial cell from a nude rat treated with 1200IU nonacog beta pegol for 26 weeks  
A choroid plexus epithelial cell from a Rowett nude rat treated with 1200IU nonacog beta pegol/kg/fifth day.  
N = nucleus, M = mitochondria, L = lipid droplet, Mic = microvilli  
Type 1 = rER/Golgi vesicles; mucinous content  
Type 3 = PEG vesicles (verified by immunohistochemistry)  
Type 4 = “dense body” lysosome and lysosome related/lipofuscin

**Reviewer Conclusions:** The earlier immunohistochemistry findings in Study 212513 reveal PEG not only in cells but also in the subepithelial areas (connective tissue) within the choroid plexus and within blood vessels in the brain parenchyma at 26 weeks post-dosing and only in the epithelial cells following a 26 week recovery period. This EM study (samples taken from Study 212513) was limited to evaluation of PEG in the epithelial cells alone. No comment on the presence of PEG in the connective tissue is noted in this EM study. It is speculated that the content in intracytoplasmic vesicles were derived by pinocytosis from the PEG in the underlying connective tissue. Given that the EM was evaluated at a single time-point (26 weeks post-dosing), the subsequent life-cycle of PEG within the vesicle remains unclear. It is unknown if there is seepage and accumulation into the CSF over time with repeat-dosing, especially if there is potential to exceed the clearance rate out of the CSF. It would be most useful to know whether PEG is eventually seen within the CSF. Although

*the protocol for 212513 states CSF was collected, no further information/data was provided in the report for that study.*

*As an aside, it is noted that the classification for vesicles appears to be based on morphological criteria of content within the membrane limited vesicles. No special stains for organelles (lysosomes) or ultrastructural material (lipofuscin) on comparable sections were completed to confirm stated cellular features. The terminology for “vacuoles” and “vesicles” can often be used interchangeably by pathologists. Intracellular vacuoles may develop by multiple pathways. These include absorbing substances into the cytoplasm via pinocytosis, or swelling of intracellular organelles (mitochondria), from lysosomes, by (auto)phagocytosis etc.*

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**Study 209215: 40 K Polyethyleneglycol (PEG) Toxicity Study by Intravenous (Bolus) Administration to Cynomolgus Monkeys for 2, 6 or 13 Weeks**

Objective: to assess the systemic toxic potential of 40 K Polyethyleneglycol (PEG)

Males, 12.8 mg/kg/every 2 days for 2 or 6 weeks; and 2 mg/kg/every 2 days for 13 weeks.

Histopathology was included (with peer review). No immunohistochemistry.

Method: Brains likely removed post-euthanasia. Macroscopic observations included observation of the brain, pituitary gland and cranial nerves. Brain histology included 3 sections (cerebellum, cerebrum, midbrain and medulla), spinal cord (transverse and longitudinal section at the cervical, lumbar and thoracic levels).

It is stated that “....samples (wax blocks and slides) of the brain, including the choroid plexus, were dispatched to the sponsor’s pathologist for supplementary processing. The findings from any supplementary processing will be reported separately and will not be a part of this study. The wax block generated for the brain from animal Nos. 183, 191 and 193, which were initially retained at HLS, were dispatched to the Sponsor. Any information generated from an assessment of these samples will be reported separately by the Sponsor. These blocks were returned to HLS for retention in the archive.”

Results:

Minimal vacuolation of ependymal cells of the choroid plexus following 6 weeks of treatment at 12.8 mg/kg. No cellular vacuolation was seen in other organs examined.

**Reviewer Comment:** *What is stated as ‘ependymal cells’ above more likely refers to the choroid epithelial lining cells*

Group/sex	1M	2M	3M
Weekly Dose (mg/kg)	45	45	7
Vacuolation of ependymal cells of choroid plexus			
Minimal	0	2	0
Total	0	2	0
Number of tissues examined	3	3	3

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**Study 208260: 40K PEG-fFIX - 4 Week Intravenous Administration Toxicity and Safety Pharmacology Study in the Male Cynomolgus Monkey Followed by a 5 Week Treatment-free Period**

Objective: 28 day tox/TK study and safety pharmacology

Five doses over a 4 week period (~ once a week). Doses included, 0, 1.9 mg/kg (350 U/kg), 15.3 mg/kg (1300 U/kg), 45 mg/kg (3750 U/kg).

Histopathology included with peer review.

Method: Brain (5 slides per animal), spinal cord (cervical, lumbar, thoracic).

Fast-track histopathology for Group 4 dose determination was based on the histopathology of the lungs, heart, kidney and brain (focus on thrombus formation) from animals in Groups 1, 2, and 3.

It is stated that “....all histological brain slides (5 slides per animal from all 29 animals) were dispatched directly to the Study Monitor. Review of these slides is for research purposes only at the discretion of the Sponsor and, therefore, form no part of this study”.

**Reviewer Comment:** *It appears that Group 4 animals were dosed separately in time compared to Groups 1, 2, and 3 in this experimental design. The results of the Fast-track histopathology for Group 4 were not available for review in this report.*

The pathology report for this study is not included.

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**Study 208405: 40K PEG-rFIX 13 Week Intravenous Administration Immunogenicity Study in the Male Cynomolgus Monkey Followed by a 5 Week Treatment-free Period**

Objective: To investigate time course of antibody development

Once weekly dosing at 1.3 mg/kg (200 U/kg) for 13 weeks with a 5 week recovery period.

Method: Brain (5 slides per animal), spinal cord (cervical, lumbar, thoracic). It is stated that “Following completion of the processing/evaluation, any tissue blocks containing the choroid plexus were returned to the Study Monitor/Novo Pathologist. These blocks are for investigations unrelated to this study.”

**Reviewer Comments:**

No further information/data on the aforementioned tissue blocks containing choroid plexus was provided in this report.

There is no pathology report available.

**Study 209200: An immunohistochemical investigation of cynomolgus monkey brain tissues from two intravenous studies with 40K PEG-rFIX**

Objective: To examine the extent of polyethyleneglycol (PEG) present in the brain tissues included the choroid plexus and tissues from studies 208260 and 208405

Method: Brain tissue sections including choroid plexus (CP) were immunohistochemically stained for PEG using a commercial anti-PEG antibody and the slides were examined by microscopy

**Results:**

Study 208260:

Immunohistochemical detection of PEG (from tissues collected in studies 208260 and 208405) in the choroid plexus and brain blood vessels is summarized in the table below:

Cynomolgus monkey			Main animals			Recovery animals		
Treatment:			Brain blood vessels	Choroid plexus		Brain blood vessels	Choroid plexus	
Duration	Dose of 40K PEG-rFIX							
No of weeks	mg/kg/w	Total	blood	Connective tissue	Epithelial cells	Blood	Connective tissue	Epithelial cells
13 w, 5 w rec	2.3	30	0	0	0	0	0	0
4 w and	0	0	0	0	0	0	0	0
5 w	4.2	16.8	weak	0	0	0	0	0
recovery	15.3	61.2	yes	yes	few pos	0	0	0
	45*	180	yes	yes	Few pos	yes	yes	Few pos

\* = only 1 week of recovery

Study 208260:

After 4 weeks dosing, PEG was detected in all dose groups (4.2, 15.3 mg and 45 mg 40K PEG-rFIX/kg/week) in blood located within the brain blood vessels. In addition,

PEG was detected in the connective tissue of the CP, and the cytoplasm of few CP epithelial cells in monkeys dosed with 15.3 mg and 45 mg 40K PEG-rFIX/kg/week. Following 4 weeks of dosing and a 5 week recovery period, PEG was not detectable in the CP of monkeys dosed with 4.2 mg and 15.3 mg PEG-rFIX/kg/week.

After 1 week of recovery, PEG was detected in the connective tissue of the CP, in the cytoplasm of few CP epithelial cells, and in the blood located within the brain blood vessels of monkeys dosed with 45 mg PEG-rFIX/kg/week.

**Reviewer Comment:** *It appears that the experimental design did not include evaluation of animals dosed with 45 mg 40K PEG-rFIX/kg/week following a 5 week recovery period.*

Study 208405:

PEG could not be detected in CP or in any of the brain structures investigated or in the brain blood vessels after 13 weeks dosing of 2.3 mg 40K PEG-rFIX/kg/week followed by 5 weeks of recovery.

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### **Missing Pathology Data and Deficiencies:**

- Study 210259 (histopathology data tables were not available)
- Study 209294: It is not clear if the choroid plexus was consistently sampled in all animals, and/or whether any supplementary processing (noted in the Methods section) was completed.
- Study 212513: CSF was collected but data is unavailable for review.
- Studies 209215, 208260 and 209200 – data is limited to routine histopathology evaluation with H&E. No immunohistochemistry was done.

### **References:**

- Lu C, Kim BM, and Chai KY, 2011: Design, synthesis and evaluation of PEGylated lipoic acid derivatives with functionality as potent anti-melanogenic agents. Eur J Med Chem. 2011 Oct;46(10):5184-8. doi: 10.1016/j.ejmech.2011.07.056. Epub 2011 Aug 5
- Gurguta C, Kauer C, Bergholz U, Formann E, Steindl-Munda P, Ferenci P. Tongue and skin hyperpigmentation during PEG-interferon-alpha/ribavirin therapy in dark-skinned non-Caucasian patients with chronic hepatitis C. Am J Gastroenterol. 2006 Jan;101(1):197-8.
- Saunders NR, Liddelow SA, and Dziegielewska, KM. Barrier mechanisms in the developing brain. Front in Pharmacol. 2012. Vol 3, Article 46, 1-18.

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